FemHab: The effects of bed rest and hypoxia on oxidative stress in healthy women

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NEW & NOTEWORTHY

Disturbed redox balance is consistently reported following inactivity and/or hypoxia exposures. The present study provides first insight into the independent and combined effects of bed rest-induced inactivity and normobaric hypoxia on oxidative stress and the antioxidant system in healthy women. We show that hypoxia during inactivity additively impairs redox balance. Furthermore, we demonstrate that even low-intensity activity during hypoxic exposure might upregulate the antioxidant system and consequently blunt hypoxia-induced oxidative stress.

PHYSICAL ACTIVITY PROFUNDLY impacts redox balance in humans (27). Both acute exercise (37) and physical inactivity (18) are known to elicit excessive reactive oxygen species (ROS) production, leading to increased oxidative stress and blunted antioxidant status. Numerous studies have demonstrated that minimizing physical activity levels via experimental bed rest (1, 6, 41) or limb unloading (42) results in disturbed redox balance. Although the underlying mechanisms are multifactorial (38), the changes in muscle protein synthesis and proteolysis seem to be the key modulators of inactivity-related local and systemic oxidative stress (36).

Acute (20, 35) and prolonged (10, 25, 46) exposures to hypoxia are also known to independently increase oxidative stress. The mechanisms underlying this hypoxia-related ROS overproduction include increased catecholamine production (23), decreased mitochondria redox potential (16), and xanthine oxidase pathway activation (54). Recently, a number of studies investigated the redox balance responses to combined hypoxia and exercise training (39). Whereas acutely the combination of both clearly augments oxidative stress (24, 33, 53), chronic exposures to combined stimuli lead to diverse outcomes. In particular, high-intensity training has been shown to impair (34), whereas moderate-intensity training does not seem to modify (32), antioxidant status during hypoxic exposure. Furthermore, recent findings (8) suggest that moderate training performed during hypoxic confinement blunts oxidative stress secondary to antioxidant system stimulation. Collectively, these data suggest that, under prolonged hypoxia, moderate to low activity
levels may act as antioxidant-conditioning stimuli and result in augmented ROS cellular defense (8, 32).

In contrast to exercise and hypoxia, the interactive effects of unloading/inactivity and hypoxic exposure on redox balance have not been studied. Besides future space exploration in which concomitant exposures to microgravity-related unloading and hypoxia are planned within the envisaged planetary habitats (4), the combination of unloading and hypoxia is also a common feature in numerous clinical populations, such as patients with chronic obstructive pulmonary disease (COPD) and heart failure (HF) rendered inactive and hypoxic by their disease. This aspect is especially important given that an association between excess ROS production and COPD (49) and HF (48) pathogenesis is clearly established. Taken together, the well-known pathophysiological as well as beneficial effects of ROS (55) warrant further investigations of the inactivity and hypoxia-induced redox balance modulation.

In addition, the majority of studies investigating independent oxidative stress responses to inactivity (1, 6, 41, 42) and hypoxia (10, 20, 25, 35, 46) were performed in male-only cohorts. To-date studies investigating potential sex-related differences in resting (5, 47) and postinventional (3, 31) oxidative stress provided conflicting results. Although several studies reported comparable (5, 31) or higher oxidative stress levels in males (3), recent findings suggest that these may be marker specific, as higher levels of select oxidative markers have been observed in males, whereas other markers may be higher in females (47). On the other hand, higher antioxidant enzymes activity in females compared with males is consistently reported (47). Interestingly, studies failed to demonstrate a significant correlation between the levels of estrogens and levels of oxidative stress (5, 47). It therefore seems unlikely that the purported protective effect of estrogen against oxidative damage (44) could explain the observed sex-related differences in redox balance.

This study aimed to investigate the combined effects of bed rest-induced unloading and hypoxia exposure on oxidative stress and antioxidant status in healthy women. Hypoxic ambulatory confinement was also investigated to assess the effect of habitual activity during continuous hypoxic exposure. A bulatory confinement was also investigated to assess the effect of rest-induced unloading and hypoxia exposure on oxidative stress. We tested the following two hypotheses: 1) superimposing hypoxia on prolonged inactivity augments oxidative stress and blunts antioxidant status, and 2) habitual physical activity counterbalances hypoxia-induced redox balance changes.

METHOD

Participants. A total of 40 healthy, nonsmoking eumenorrheic women were screened for participation in the present study. The inclusion/exclusion criteria were based on the European Space Agency recommendations for bed rest protocols (Standardization of Bed Rest Study Conditions, August 2009). In addition, individuals exposed to altitudes above 2,000 m within 2 mo before the start of the study were ineligible to participate. All participants provided informed consent and were given detailed information regarding the research protocol and all experimental procedures. The baseline characteristics of the participants in each of the three interventions are outlined in Table 1.

### Table 1. Baseline characteristics of the participants

<table>
<thead>
<tr>
<th></th>
<th>NBR</th>
<th>HBR</th>
<th>HAMB</th>
</tr>
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<tbody>
<tr>
<td>Age, yr</td>
<td>26 ± 4</td>
<td>27 ± 4</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Height, cm</td>
<td>167 ± 5</td>
<td>167 ± 5</td>
<td>167 ± 6</td>
</tr>
<tr>
<td>Body mass, kg</td>
<td>61 ± 9</td>
<td>59 ± 8</td>
<td>60 ± 8</td>
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<tr>
<td>BMI, kg/m²</td>
<td>21 ± 2</td>
<td>21 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>30 ± 5</td>
<td>29 ± 5</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>VO₂max, ml·kg⁻¹·min⁻¹</td>
<td>42 ± 4</td>
<td>40 ± 4</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>MAP, W</td>
<td>215 ± 37</td>
<td>212 ± 34</td>
<td>213 ± 31</td>
</tr>
</tbody>
</table>

Values are means ± SD. NBR, normoxic bed rest intervention; HBR, hypoxic bed rest intervention; HAMB, hypoxic ambulatory intervention; BMI, body mass index; VO₂max, maximal oxygen consumption; MAP, maximal aerobic power during the cycle ergometer test.

Study outline. The present study was part of a larger research project (FemHab - Planetary Habitat Simulation with female participants) investigating independent and combined effects of bed rest-induced inactivity and exposure to normobaric hypoxia (NBR) on physiological and psychological adaptations in healthy women. The FemHab project was a prospective, randomized, and controlled trial, performed at the Olympic Sport Centre Planica bed rest facility (Rateče, Slovenia). The study protocol was approved by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia. All experimental procedures were conducted according to the above-mentioned European Space Agency recommendations and conformed to the principles of the Helsinki declaration. To determine the independent effect of hypoxia during inactivity and to assess the effect of hypoxia during continuous hypoxic exposure, we randomly assigned the participants to the following three 10-day experimental interventions: 1) normobaric normoxic bed rest [NBR; fraction of inspired O₂ (FIO₂) = 0.209; partial pressure of inspired O₂ (PIO₂) = 132.9 ± 0.3 mmHg], 2) normobaric hypoxic bed rest [HBR; FIO₂ = 0.142 ± 0.001; PIO₂ = 90.4 ± 0.3 mmHg], 3) normobaric hypoxic ambulatory confinement (NBR; FIO₂ = 0.142 ± 0.001; PIO₂ = 90.4 ± 0.3 mmHg).

Each experimental intervention lasted 19 days and comprised the following three phases: 1) the initial testing phase comprising 5 days upon arrival to the facility (all baseline measures were obtained during this period); 2) the 10-day confinement phase (days 1-10) that lasted exactly 240 h (during this phase the participants were exposed to their designated condition, NBR, HBR, or HAMB); 3) a 4-day recovery phase that allowed for gradual reacclimatization of the participants following the bed rest interventions and enabled the researchers to obtain the postconfinement measurements (Post).

Bed rest and hypoxic ambulation protocol. The participants were confined to strict horizontal bed rest, a regularly employed ground-based model to simulate microgravity-induced unloading (30), during the NBR and HBR interventions. The details of the bed rest protocol, frequently employed by our group at the Planica facility, were reported previously (7). Briefly, the participants remained in a horizontal position during all daily activities (i.e., reading, hygiene, watching television). They were allowed to use one pillow for head support. Besides changing body position from supine, prone, and lateral, no static or dynamic activity was permitted during the bed rest phase. Compliance to the protocol was ensured via 24-h closed-circuit television monitoring and permanent supervision of the medical staff.

The normobaric hypoxia was generated using a Vacuum Pressure Swing Adsorption system (b-Cat, Tiel, The Netherlands) that delivered the O₂-depleted air to the designated rooms and hypoxic area. The O₂ and CO₂ room air content was sampled every 15 min and...
examined using calibrated O₂ and CO₂ analyzers. The CO₂ level within the confinement area did not exceed 0.5% throughout the intervention. As a safety precaution, the participants wore portable ambient O₂ concentrators with audible alarms (PGM-1100; RAE Systems, San Jose, CA) throughout both hypoxic interventions. The participants were encouraged to engage in their habitual routines and allowed to move freely in the common hypoxic area during the HAMB intervention. They also performed low-intensity exercise sessions to mimic their habitual physical activity levels. Two 20-min sessions were performed daily, one in the morning and one in the afternoon. To avoid monotony, the exercise mode (stepping, cycling, or dancing) was rotated daily.

Before each campaign the participants performed one graded cycle-ergometer test in normoxia (FiO₂ = 0.209) to obtain baseline values of maximal oxygen consumption (VO₂max) and maximal aerobic power (MAP) and one in hypoxia (FiO₂ = 0.144) to enable appropriate exercise session intensity calculation for the HAMB participants. The test was based on 25 W/min workload increments until task failure (cadence < 60 revolution/min). The MAP was calculated using the following equation: MAP = Wcompl + [t(60 × 25)–1] (W); Wcompl = fast completed workload; t = seconds during the final uncompleted workload. The prescribed intensity of the exercise sessions during the HAMB intervention was targeted to induce an HR corresponding to that attained at 50% of the hypoxia-specific MAP. To ensure that the targeted HR responses (131 ± 10 beats/min) were achieved and maintained throughout all exercise sessions, the HR and capillary oxyhemoglobin saturation (SpO₂) values were continuously measured using heart rate telemetry (iBody; Wahoo Fitness, Atlanta, GA) and finger pulse oxymetry values were continuously measured using heart rate telemetry (3100 WristOx; Nonin Medicals, Plymouth, MN), respectively.

**Diet.** Individually tailored and standardized diet was provided to the participants throughout the interventions. The participants received the same food items on the same days of the respective interventions. Individualized energy requirements were calculated using the modified Harris-Benedict resting metabolic rate equation (14) multiplied by a physical activity level factor of 1.2 for the NBR and HBR and 1.4 for the HAMB interventions. The targeted macro-nutrient composition was ~55% carbohydrates, ~30% fat, and ~15% protein. The participants were also supplemented with vitamin D3 (1,000 IU/day) throughout all interventions and were encouraged to maintain their daily fluid intake above 28.5 ml/kg.

**Experimental procedures: Daily physiological monitoring.** Daily morning measurements of resting HR and SpO₂ were performed using a short-range telemetry (iBody, Wahoo Fitness) and finger oximetry (3100 WristOx, Nonin Medicals, Plymouth, MN), respectively. To determine the presence and severity of acute mountain sickness (AMS), the participants filled out a self-assessment part of the Lake Louise AMS questionnaire at 08:00 PM daily (43). The Lake Louise score (LLS; 0–15) was calculated by summing the values of the individual questionnaire items. The following two criteria were used to diagnose AMS: 1) LLS ≥3 and 2) presence of headache.

**Blood sampling.** Venous blood samples (10 ml) were obtained before, during, and after each intervention at the following five time points: 1 day before the intervention (Pre), after the first day of the intervention (D2), after 5 days of the intervention (D6), after 10 days of the intervention (D10), and 24 h following the intervention (Post+1). All blood samples were taken from the antecubital vein in the morning before the participants stood up. A 4-ml blood sample was immediately transferred to a nearby clinical laboratory (Jesenice General Hospital, Jesenice, Slovenia) and analyzed for red blood cell count (RBC), reticulocytes, hematocrit, estradiol, and progesterone values. The remaining venous blood was centrifuged (10 min at 3,500 revolution/min; 4°C), stored in six 400-μl aliquots, and immediately frozen to −80°C for subsequent oxidative stress and antioxidant markers analysis performed ≤6 mo after the study within the same time period.

**Biochemical analyses.** The cytochemical impedance (Advia 120; Siemens Hematology, Tarrytown, NY) was used to determine RBC [coefficient of variation (CV) = 2.0%], reticulocyte (CV = 13.3%), and hematocrit (CV = 2.5%) values. Imprecision data for RBC and hematocrit were performed using multiple samples of whole blood (7 runs and 20 aspirations from each sample). For reticulocytes, a total of 18 runs were performed using multiple samples with 25 aspirations from each sample.

 Estradiol and progesterone were measured using electrochemiluminescence immunoassay (ECLIA, Roche Cobas e601; Roche Diagnostics, Mannheim, Germany). The Roche Estradiol II and Progesterone II assays were based on a competitive biotin-streptavidin test principle. Hormone concentrations were determined via the calibration curve, specifically generated by two-point calibration and master curve provided via the reagent barcode. The methods have been standardized by isotope dilution-gas chromatography/mass spectrometry. The intra-assay and interassay CVs for estradiol and progesterone were 6.1%, 2.9%, 7.0%, and 4.8%, respectively.

 Plasma advanced oxidation protein products (AOPP) were measured according to the semiautomated methods developed by Witko-Sarsat et al. (52). The AOPP was determined using spectrophotometry and calibrated with a chloramine-T solution that absorbs at 340 nm in the presence of potassium iodide. The absorbance of the reaction was read immediately at 340 nm on a microplate reader. AOPP activity was expressed as micromoles per liter of chloramine-T equivalents. The intra-assay coefficient CV was 5.4%.

 Concentrations of plasma malondialdehydes (MDA), as reactive thiorbarbituric acid substances, were determined as previously described (33). The pink chromogen was extracted with N-butanol, and its absorbance was measured at 532 nm by spectrophotometry with 1,1,3,3-tetraethoxypropan as standard. Although methodological limitations have been shown for MDA assay, it remains one of the most widely used markers of lipid peroxidation (19). The intra-assay CV was 2.2%.

 Concentrations of nitrotyrosine, the end-product of protein nitration by ONOO –, were determined by a commercially available ELISA kit (Sigma-Aldrich, St. Louis, MO), as described previously (12). The intra-assay CV was 6.8%.

 The superoxide dismutase (SOD) activity assessment was performed using the Oberley and Spitz method (28). SOD activity was determined by the degree of inhibition of the reaction between superoxide radicals, produced by a hypoxanthine-xanthine oxidase system and nitroblue tetrazolium. The intra-assay CV was 5.6%.

 Catalase activity was determined using the Johansson and Borg method (15), with hydrogen peroxide (H₂O₂) acting as a substrate and formaldehyde as a standard. The formaldehyde formation rate, induced by the reaction of methanol and H₂O₂, was used to determine the catalase activity. The intra-assay CV was 5.6%.

 Ferric-reducing antioxidant power (FRAP) plasma concentrations were determined by spectrophotometry using the manual Benzie and Strain method (2). FRAP concentrations were calculated at a wavelength of 593 nm using a standard aqueous solution of known Fe²⁺ concentration (FeSO₄, 7H₂O). The intra-assay CV was 2.9%.

 Glutathione peroxidase (GPX) activity was determined by the modified method of Paglia and Valentine (29) as the rate of oxidation of NADPH to NADP⁺ after the addition of NADPH, reduced glutathione, and glutathione reductase, using H₂O₂ as a substrate. The intra-assay CV was 6.6%.

 The uric acid (UA) concentration was determined using a commercially available kit (Bio-Quant, San Diego, CA). The limits of detection for this assay are 1–100 μmol/l. As a product of purine metabolism during reoxygenation, the UA concentration reflects ROS production via xanthine oxidase pathway activation. The intra-assay CV was 0.9%.

 NO metabolism was quantified as the sum of nitrate and nitrite (NOX) concentrations as previously described (9). After nitrate reduction by nitrate reductase, the fluorometric quantification of NOX was
Table 2. *Select hematological and hormonal markers assessed before, during, and after experimental campaigns*

<table>
<thead>
<tr>
<th>NBR</th>
<th>Pre</th>
<th>D2</th>
<th>D6</th>
<th>Post</th>
<th>Post +1</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC, 10^12/l</td>
<td>4.71 ± 0.36</td>
<td>4.94 ± 0.36</td>
<td>4.99 ± 0.28</td>
<td>5.09 ± 0.26†</td>
<td>4.63 ± 0.23</td>
</tr>
<tr>
<td>Reticulocytes, 10^9/l</td>
<td>73.5 ± 17.9</td>
<td>87.1 ± 20.9</td>
<td>95.1 ± 24.2</td>
<td>87.1 ± 28.4</td>
<td>75.7 ± 27.9</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>0.39 ± 0.04</td>
<td>0.41 ± 0.04</td>
<td>0.42 ± 0.03</td>
<td>0.42 ± 0.03*</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td>Estradiol, pg/ml</td>
<td>75 ± 118</td>
<td>97 ± 153</td>
<td>61 ± 73</td>
<td>41 ± 54</td>
<td>/</td>
</tr>
<tr>
<td>Progesterone, μg/l</td>
<td>2.1 ± 3.2</td>
<td>1.3 ± 1.3</td>
<td>2.3 ± 2.9</td>
<td>1.9 ± 2.0</td>
<td>/</td>
</tr>
<tr>
<td>HRR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC, 10^12/l</td>
<td>4.40 ± 0.30</td>
<td>4.88 ± 0.45†</td>
<td>5.17 ± 0.48†</td>
<td>5.29 ± 0.36†</td>
<td>4.81 ± 0.26†</td>
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<tr>
<td>Reticulocytes, 10^9/l</td>
<td>69.3 ± 13.9</td>
<td>85.9 ± 20.9</td>
<td>126.7 ± 24.6†</td>
<td>107.1 ± 30.7†</td>
<td>93.4 ± 20.1*</td>
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<tr>
<td>Hematocrit, %</td>
<td>0.38 ± 0.03</td>
<td>0.42 ± 0.04†</td>
<td>0.44 ± 0.04†</td>
<td>0.46 ± 0.03†</td>
<td>0.42 ± 0.03†</td>
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<tr>
<td>Estradiol, pg/ml</td>
<td>35 ± 45</td>
<td>38 ± 49</td>
<td>50 ± 56</td>
<td>24 ± 25</td>
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</tr>
<tr>
<td>Progesterone, μg/l</td>
<td>2.4 ± 4.6</td>
<td>2.5 ± 4.3</td>
<td>2.2 ± 4.2</td>
<td>1.0 ± 0.9</td>
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<tr>
<td>HAMB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC, 10^12/l</td>
<td>4.54 ± 0.34</td>
<td>4.71 ± 0.25</td>
<td>4.93 ± 0.33†</td>
<td>5.03 ± 0.30†</td>
<td>4.63 ± 0.17</td>
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<tr>
<td>Reticulocytes, 10^9/l</td>
<td>68.7 ± 7.0</td>
<td>88.0 ± 12.0</td>
<td>138.0 ± 28.2‡</td>
<td>120.0 ± 43.9†</td>
<td>105.6 ± 31.7*</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>0.39 ± 0.03</td>
<td>0.40 ± 0.03</td>
<td>0.43 ± 0.05</td>
<td>0.42 ± 0.03*</td>
<td>0.40 ± 0.03*</td>
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<tr>
<td>Estradiol, pg/ml</td>
<td>40 ± 54</td>
<td>38 ± 26</td>
<td>74 ± 57</td>
<td>158 ± 167*</td>
<td>/</td>
</tr>
<tr>
<td>Progesterone, μg/l</td>
<td>0.7 ± 0.3</td>
<td>0.7 ± 0.4</td>
<td>1.4 ± 2.4</td>
<td>1.0 ± 1.1</td>
<td>/</td>
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</table>

Values are means ± SD. *P < 0.05, †P < 0.01 denotes significant difference compared with before the intervention (Pre value); ‡P < 0.05 denotes significant difference compared with the corresponding NBR value. RBC, red blood cell count; D2, day 2 of the intervention; D6, day 6 of the intervention; Post, immediately after the 10-day intervention; Post +1, 1 day after the end of the intervention.

RESULTS

General adaptation. Apart from headaches and backaches, observed during the initial few days of the NBR and HBR, all participants finished their respective interventions without any significant adverse health-related issues. The participants also tolerated the hypoxic condition without significant problems during both HBR and HAMB although AMS was diagnosed in four and three participants on D1 and in six and four participants on D2 of the HBR and HAMB, respectively.

Hematological and hormonal markers. Compared with Pre, the RBC count (P < 0.01) and hematocrit (P < 0.05) were increased at Post in the NBR, at all samplings in the HBR and at D6 and Post in the HAMB (Table 2). Reticulocyte count did not change during NBR (P = 0.37) but was significantly increased in both the HBR and HAMB interventions on D6, Post, and Post +1 compared with Pre (P < 0.05; Table 2). No differences were observed between different interventions for both RBC (P = 0.56) and reticulocyte count (P = 0.21). Estradiol was only significantly increased at Post in the HAMB (P < 0.05). No other changes were observed in both estradiol and progesterone concentration within and between interventions (P > 0.33; Table 2).

Oxidative stress markers. AOPP was increased at D2 (+14%, +12%) and at D6 (P < 0.05) in the NBR and HBR, respectively (P < 0.05; Fig. 1A) with no differences observed in HAMB. Compared with Pre, the MDA increased at Post +1 in NBR (+116%; P < 0.01) and at D2 in both HBR (+114%; P < 0.01) and HAMB (+95%; P < 0.05; Fig. 1B). Except for reduction at Post +1 (-45%) in HAMB, no other significant within- or between-intervention differences were noted in nitrotyrosine (Fig. 1C).

Antioxidant markers. The plasma SOD values were significantly increased at D6 (+82%; P < 0.05) and Post +1 (+67%; P < 0.05) in HAMB with no changes observed in both bed rest interventions (Fig. 2A). Whereas no significant changes in catalase activity were observed in NBR, it was increased at D6 (128%; P < 0.05) and at Post +1 (146%; P < 0.05) in HBR and HAMB, respectively (Fig. 2B). No significant within- or between-intervention differences were observed for FRAP (Fig. 2C).
GPX values were significantly reduced at D6 (−20%, \( P < 0.01 \)) and at Post (−18% \( P < 0.05 \)) in HBR with no differences noted in NBR and HAMB (Fig. 3A). Whereas UA values were not significantly different within all three interventions (Fig. 3B), the UA HBR value at Post was significantly higher than the corresponding HAMB value (\( P < 0.05 \)).

**NOx and nitrites.** No significant changes in any of the interventions were noted in NOx (Fig. 4A). Compared with Pre, significantly higher values of nitrites were observed at Post +1 (+46%; \( P < 0.05 \)) in HAMB only. The Post +1 HAMB nitrates value was significantly higher than the corresponding NBR and HBR value (\( P < 0.05 \); Fig. 4B).

**Correlations.** Estradiol and progesterone values did not significantly correlate to the oxidative stress/antioxidant marker values either at baseline or at any other sampling points (\( P > 0.13 \)). Significant correlations were noted between the pooled changes in SpO2, two oxidative stress markers (AOPP, \( r = 0.35, P < 0.01 \); nitrotyrosine, \( r = 0.23, P < 0.05 \)), and two antioxidant status markers (FRAP, \( r = 0.25, P < 0.05 \); GPX, \( r = −0.29, P < 0.05 \)) during both hypoxic interventions.

**DISCUSSION**

This study investigated the acute and prolonged effects of unloading and hypoxia on oxidative stress and antioxidant status. The effects of habitual levels of activity during hypoxic exposure, as opposed to complete inactivity, were also investigated. Our main findings were that 1) exposure to 4,000-m simulated altitude during inactivity impairs prooxidant/antioxidant balance, as demonstrated by the MDA and GPX responses during the early phases of HBR; 2) the changes observed in AOPP throughout the interventions, and in nitrotyrosine, SOD, and GPX at the end of interventions, suggest that, in contrast to bed rest-induced unloading, habitual activity levels stimulate the antioxidant system and consequently blunt hypoxia-induced oxidative stress.

Increases in oxidative stress levels as a consequence of acute (42) or prolonged (1, 6, 41) inactivity are well established. The present data confirm that inactivity per se increases oxidative

![Fig. 1. Advanced oxidation protein products (AOPP; A), malondialdehyde (MDA; B) and nitrotyrosine (C) plasma values (means ± SD) before (Pre), during [day 2 (D2); day 6 (D6)], immediately after (Post), and 1 day after (Post +1) the normoxic bed rest (NBR), hypoxic bed rest (HBR), and hypoxic ambulatory (HAMB) intervention. **\( P < 0.01 \), *\( P < 0.05 \) denotes significant difference compared with Pre value.](image1)

![Fig. 2. Superoxide dismutase (SOD; A), catalase (B), and ferric-reducing antioxidant power (FRAP; C) plasma values (means ± SD) before (Pre), during (D2 and D6), immediately after (Post), and 1 day after (Post +1) the NBR, HBR, and HAMB intervention. *\( P < 0.05 \) denotes significant difference compared with Pre value.](image2)
stress because AOPP was significantly increased immediately following the NBR intervention. Also, and in line with previous reports (41), the MDA was increased following the NBR. The fact that no changes during or after the NBR were observed in any of the measured antioxidant markers further suggests that inactivity per se does not worsen the antioxidant status in healthy women. This is in contrast to previous data from male cohorts indicating significant inactivity-provoked antioxidant status reduction (22, 36). Although the unchanged antioxidant status following normoxic unloading could be related to antioxidant properties of estrogen (44), this seems unlikely given that no association between the sex hormones and any of the measured antioxidant markers was noted.

Previous studies clearly show that both short-term (20, 35) and long-term (10, 25, 46) hypoxic exposures, similarly to inactivity, increase oxidative stress and may also blunt antioxidant status. The comparison of the NBR and HBR outcomes, which enables determination of the independent effect of hypoxia during unloading, suggests that the inactivity-induced oxidative stress is exacerbated by concomitant exposure to NBR. In particular, the acute increase in MDA values (D2) observed during the HBR suggests an additive effect of hypoxia on inactivity-induced oxidative stress during the initial bed rest phase. In addition, higher oxidative stress levels observed in the HBR than the NBR might also be secondary to a decreased antioxidant status, as evidenced by a significant decrease in GPX in the HBR only. The fact that MDA significantly increased at Post+1 during the NBR only is rather surprising. It could be speculated that this is a consequence of increased ROS mitochondrial production following reambulation and that hypoxia stimulation, noted in the early stages of both HBR and HAMB, blunted this response in both hypoxic interventions. Although higher nitrotyrosine levels were previously reported following prolonged hypoxic exposures (8), no differences were observed either during the courses of or between the NBR and HBR interventions. Given that changes in nitric oxide activity directly affect nitrotyrosine levels via peroxynitrite-mediated tyrosine nitration (40), the lack of changes in NOx and nitrates throughout all interventions could explain the unchanged nitrotyrosine levels.

The fact that MDA was increased, not only in HBR, but also in the HAMB confirms previous studies showing augmented MDA values as a result of hypoxic exposure (35) and suggests that light physical activity, known to stimulate antioxidant enzyme activity, did not affect the MDA production. Indeed, the MDA is a result of polyunsaturated fatty acids forming peroxidation within the cell membranes and is primarily protected against ROS by vitamin E. Similarly to MDA, catalase increased in both hypoxic interventions, indicating an acute effect of hypoxia on catalase stimulation. Significantly higher UA values at Post, observed in the HBR, than in the HAMB might suggest that moderate exercise limits ROS production via the xanthine oxidase pathway (54). The increased nitrite concentration observed at Post+1 in the HAMB confirms that physical activity enhances NO metabolism. The observed improvement of NO metabolism might result from the decreased oxidative stress that may reflect lower superoxide content. Indeed, peroxynitrite (ONOO⁻) results from the inhibitive superoxide action on NO.
observed concomitant decrease in the nitrotyrosine, an index of ONOO⁻ nitritative activity, and an increase in NO at the same sampling point (Post +1) in HAMB. The observed association between pooled FRAP values and the degree of participants’ hypoxic stress (i.e., SpO₂ decrease during HBR and HAMB) suggests that the level of hypoxemia significantly modulates redox status. This is in line with previous studies demonstrating correlations between oxidative stress marker changes (i.e., MDA) and desaturation levels during acute hypoxia in elite athletes known to have excellent antioxidant status (35). Collectively, this lends further support to the concept that hypoxia affects oxidative stress and antioxidant status in a dose-dependent fashion (9). Furthermore, the lack of significant correlation between estrogen and progesterone and oxidative/antioxidant markers confirms previous reports suggesting that sex hormones may not directly modulate oxidative stress in healthy women (5, 47).

Collectively, the HBR and HAMB data are in line with our recent study (8) investigating the redox balance responses to 10-day hypoxic confinement with or without moderate-intensity exercise. In particular, the HBR intervention outcomes confirm that hypoxic confinement per se increases oxidative stress also in women. The antioxidant system upregulation observed in the HAMB is similar to that noted with moderate-intensity exercise training (2 × 60 min/day) performed during hypoxic confinement (8) and might have buffered the oxidative stress response to hypoxia in the HAMB (i.e., no changes in AOPP) during moderate-intensity exercise training performed during continuous hypoxic exposure improves antioxidant status (8), the present data suggest that even lower physical activity levels might upregulate antioxidant enzyme activity. Importantly, the levels of continuous hypoxia employed in the present and previous study (8) were identical (simulated altitude of 4,000 m), and the hematomatological and systemic effects of hypoxia are clearly reflected in increased hematomatological markers and profoundly reduced SpO₂ in both HBR and HAMB compared with the NBR. The effect of hypoxia is also reflected in the AMS incidence that only occurred during both hypoxic interventions and was slightly higher in the HBR than the HAMB.

These findings, obtained under hypoxic conditions, are in line with previous studies investigating the effects of different exercise countermeasures (i.e., endurance and resistance exercise) on bed rest-induced physiological changes. In particular, resistance exercise has been shown to upregulate the antioxidant system and blunt oxidative stress responses following 60 and 90 days of NBR (22). It is of note, however, that the beneficial effect of moderate-intensity exercise on antioxidant status seems highly dependent on the individual’s thermal and hydration status, as emerging evidence indicates that both hyperthermia and dehydration significantly blunt this adaptation (17). Nonetheless, although it seems clear that even habitual activity levels are sufficient to upregulate antioxidant status under hypoxic conditions, the dose-response relationship to different activity levels and modes need to be further investigated under hypoxic and normoxic conditions.

The key strengths of the present study include a strictly controlled experimental protocol, standardized activity levels and diet, and the acute and prolonged assessment of redox status changes using a number of oxidative stress and antioxidant markers. Furthermore, given that the vast majority of research in this area is performed on men, the present study provides a unique insight into the redox balance modulation in a female population under conditions of unloading and hypoxia. Nevertheless, there are some limitations that should be acknowledged. In particular, this study was performed in normobaric hypoxia, and, because differences in oxidative and nitrosative stress responses to normobaric and hypocbaric hypoxia have previously been demonstrated (11), the present results should not be generalized to terrestrial altitudes. Second, the nonenzymatic dietary antioxidants have also been shown to play an important role in oxidative stress responses to hypoxia and different levels of physical activity (45, 51). However, the differences in oxidative stress levels observed in the interventions could not result from different dietary antioxidant intakes because all participants received the same, individually tailored diet throughout all interventions. Third, this study was performed in women, and, although the potential differences in oxidative stress responses between the sexes are currently not firmly established (5), the outcome might have been different in a male population. Given that marker-specific differences in oxidative stress responses were demonstrated between males and females (47), further studies on the combined effects of hypoxia and unloading should include mixed and male cohorts. Finally, the assessment of redox balance was performed from blood plasma samples. Although previous studies (50) and a recent systematic review (21) suggest that the majority of the redox biomarkers measured in plasma adequately reflect the redox status of different bodily tissues, there are some limitations to this approach. For example, it is well established that the activity of antioxidant enzymes is significantly higher in the erythrocytes than in plasma (26). Moreover, the fact that plasma and blood cells autonomously generate significant amounts of ROS during rest and exercise (27) and potential confounding factors associated with blood handling (e.g., hemolysis) should also be taken into account.

As noted earlier, the combined exposure to unloading and hypoxia will be a prominent challenge during future space exploration, which is envisaged to base on permanent planetary/lunar habitation. To minimize the risk of decompression sickness during preparations for extravehicular activities on the moon or Mars surface, the envisaged habitats will be hypobaric and hypoxic. Given the well-known importance of redox balance for long-term wellbeing (55) and operational ability of the astronauts, the present study provides important information for planning of future space missions. It has to be mentioned that the present study did not test high-fidelity analog conditions envisaged during long-term space explorations. On such missions, the astronauts will probably be exposed to a combination of musculoskeletal unloading due to reduced gravity, hypoxia, and activity/exercise. The latter will comprise habitual and extravehicular physical activities as well as countermeasure exercise protocols, such as those currently performed during missions at the International Space Station. Accordingly, future investigations should focus on investigating combined effects of all three factors (i.e., unloading, hypoxia, and exercise).

Besides space-related application, the obtained data are also relevant in a clinical setting because the combination of unloading and hypoxia is often encountered in patients with COPD and HF rendered inactive and hypoxic by their illness (48, 49). Present results suggest that it is important to, in both

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settings, recognize the potentially deleterious effects of combined inactivity and hypoxia on redox balance and to promote physical activity to counteract oxidative stress.

In conclusion, the obtained data suggest that exposure to NBR (~4.000 m) might exacerbate inactivity-related oxidative stress in healthy females. Furthermore, even habitual activity levels seem to blunt hypoxia-provoked oxidative stress via antioxidant system upregulation. Future investigations should scrutinize the potential dose-response relationships between different levels of activity and hypoxia and the resulting oxidative and nitrosative stress.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: T.D., V.P., O.E., I.B.M., and G.P.M. conception and design of research; T.D., O.E., and I.B.M. performed experiments; T.D., V.P., NDe) and the Swedish National Space Board (grant no. 109/11:2).

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