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RESEARCH ARTICLE

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Neurovascular dysfunction associated with erectile dysfunction persists after long-term recovery from simulations of weightlessness and deep space irradiation

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Abstract

There has been growing interest within the space industry for long-duration manned expeditions to the Moon and Mars. During deep space missions, astronauts are exposed to high levels of galactic cosmic radiation (GCR) and microgravity which are associated with increased risk of oxidative stress and endothelial dysfunction. Oxidative stress and endothelial dysfunction are causative factors in the pathogenesis of erectile dysfunction, although the effects of spaceflight on erectile function have been unexplored. Therefore, the purpose of this study was to investigate the effects of simulated spaceflight and long-term recovery on tissues critical for erectile function, the distal internal pudendal artery (dIPA), and the corpus cavernosum (CC). Eighty-six adult male Fisher-344 rats were randomized into six groups and exposed to 4-weeks of hindlimb unloading (HLU) or weight-bearing control, and sham (0Gy), 0.75 Gy, or 1.5 Gy of simulated GCR at the ground-based GCR simulator at the NASA Space Radiation Laboratory. Following a 12–13-month recovery, ex vivo physiological analysis of the dIPA and CC tissue segments revealed differential impacts of HLU and GCR on endothelium-dependent and -independent relaxation that was tissue type specific. GCR impaired non-adrenergic non-cholinergic (NANC) nerve-mediated relaxation in the dIPA and CC, while follow-up experiments of the CC showed restoration of NANC-mediated relaxation of GCR tissues following acute incubation with the antioxidants mito-TEMPO and TEMPOL, as well as inhibitors of xanthine oxidase and arginase. These findings indicate that simulated spaceflight

Abbreviations: 4-HNE, 4-hydroxynonenal; ACh, acetylcholine; Arg1, arginase 1; Arg2, arginase 2; BEC, S-(2-boronoethyl)-l-cysteine; CC, corpus cavernosum; cGMP, cyclic guanosine monophosphate; CVD, cardiovascular disease; DEA, DEA-NONOate; dIPA, distal internal pudendal artery; ED, Erectile dysfunction; EFS, electrical field stimulation; ET-1, endothelin-1; GCR, galactic cosmic radiation; H_2O_2, hydrogen peroxide; HLU, hindlimb unloading; HZE, high-energy protons and high charge and energy; IPA, internal pudendal artery; L-NAME, Nω-Nitro-Larginine methyl ester hydrochloride; MDA, malondialdehyde; NANC, non-adrenergic non-cholinergic; NE, norepinephrine; NO, nitric oxide; NOS, nitric oxide synthase; NSRL, NASA Space Radiation Laboratory; NOx, nitrate/nitrite; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; PE, phenylephrine; SOD, superoxide dismutase; XO, xanthine oxidase.

exerts a long-term impairment of neurovascular erectile function, which exposes a new health risk to consider with deep space exploration.

KEYWORDS

arginase, corpus cavernosum, cosmic radiation, erectile, hindlimb unloading, internal pudendal artery, mito-tempo, myograph, oxidative stress, spaceflight

1 | INTRODUCTION

There has been a recent push by the space industry for expansive deep space exploration. The NASA Artemis 2 and Artemis 3 programs have plans to send astronauts to the Moon as early as 2024 and 2025, while aspirations exist for manned missions to Mars as early as 2040.[1] While manned deep space exploration is a remarkable advancement to society, long trips in space can bring many known and unknown health risks to astronauts. Some known health risks include cardiovascular deconditioning,[2–6] bone loss,[2,7–10] muscle atrophy,[11–14] immune system decrements,[15–17] increased risk of cancer,[18] and cardiovascular disease,[19–21] among others. Astronauts also experience an increased risk of oxidative stress,[22] endothelial and cardiovascular dysfunction[19,21,23] after being exposed to a weightless and radiation environment during spaceflight. Thus, it is of high importance to evaluate the comprehensive health risks associated with long-duration exploration of deep space.

Erectile dysfunction (ED) is the inability to achieve or maintain an erection sufficient for satisfactory sexual performance, and it relies on the disruption of one or more neurovascular signaling pathways that regulate penile vascular tone. Comprehensive aging studies have found the combined prevalence of any level of erectile dysfunction in 52% of men aged 40–70 years.[24,25] The presence of ED has emerged as an early predictor of future cardiovascular disease (CVD) in men.[26–29] While ED and CVD share several common risk factors such as smoking, aging, obesity, and the metabolic syndrome, the common underpinning of endothelial dysfunction is most often attributed as the pathological link between these two diseases.[30] Stimulation of the non-adrenergic, non-cholinergic (NANC) nerve fibers and production of nitric oxide (NO) by the endothelium surrounding the corpus cavernosum (CC) and the small arteries within and supplying the penis are crucial factors to drive erection initiation and maintenance.[31–35] Thus, a physiological stressor that reduces NO bioavailability may have a disproportionately large deleterious effect on erectile function. As prior investigations have demonstrated impairments in endothelial function and NO bioavailability in the central and peripheral vasculature following spaceflight,[19,21,36–38] it is reasonable to speculate that astronauts may be at an elevated risk for ED. With prolonged missions to deep space on the horizon, it is of particular importance to determine the potential long-term impact of spaceflight on the function of the tissues responsible for the erectile response.

The model of hindlimb unloading (HLU)[39] has been widely used as an alternative to spaceflight studies as it simulates important physiological responses to weightlessness from spaceflight, such as differential muscle atrophy, cephalad fluid shifts, unloading of the hindlimbs and limited movement of the forelimbs.[39–43] The model relies on the animal's physical deconditioning and cephalic fluid shifts delivered by the 30-degree head-down unloading of the hindlimbs to develop cardiovascular adaptations similar to those observed under microgravity, such as hypovolemia, tachycardia, reduced ability to elevate peripheral vascular resistance, diminished aerobic exercise capacity and orthostatic hypotension.[43]

During spaceflight, astronauts are also exposed to high levels of galactic cosmic radiation (GCR) which is comprised of high-energy protons and high charge and energy (HZE) ions. This unique form of radiation is distributed through biological tissues in a spatially different manner when compared to those observed on Earth and triggers unique, more severe biological damage.[44] While NASA has strict radiation standard limits for astronaut exposures, their model lacks mention of a HZE ion component due to the limited research on the biological effects of heavy ions.[45–47] The development of the new GCR simulator at the NASA Space Radiation Laboratory (NSRL) was found to better simulate the HZE radiation exposure of deep space travel encountered by astronauts inside the exploration vehicle in space.[44] Initial investigations with this new technology have explored the detrimental effects of deep space radiation on health, including significant impairment of cardiovascular function.[21,48] Since space missions targeting Mars would require long-term exposure to this outer space stressor, prolonged exposure to GCR carries valid concerns about their influence on the body and novel health risks to astronauts.

Of specific interest, the vascular endothelium is significantly affected by radiation exposure, leading to decreases in NO levels, greater incidences of apoptosis, and morphological changes including cell shrinkage, wider

gaps between cell junctions, and detachment from the basement membrane.[21,42,49] Additionally, the elevated levels of xanthine oxidase and enhanced superoxide production following radiation exposure negatively affect endothelium-dependent vasodilation.[21,49–52] The focus of previous studies on the risks of spaceflight have been somewhat limited and little is known about the influence of GCR and microgravity on sexual function. In this study, we simulate and explore the influence of environmental stressors expected to be encountered from long-duration trips into deep space on neurovascular and CC function. The results demonstrate adverse effects of GCR exposure and simulated weightlessness on endothelium and vascular function, even after a period of long-term recovery, and implicate a novel health risk associated with long-duration spaceflights in male astronauts.

2 | MATERIALS AND METHODS

2.1 | Animals

Eighty-six adult (12 month old) male Fisher 344 rats were obtained from the National Institute of Aging (Bethesda, MD, USA). Half the rats were randomly assigned to a 4-week treatment of simulated weightlessness via HLU, while the remaining animals remained in their cages. The HLU and weight-bearing control rats were then exposed to either sham irradiation (0Gy), 0.75 Gy, or 1.5 Gy of simulated galactic cosmic radiation (GCR) using the GCR simulator at the NASA Space Radiation Laboratory (NSRL). Thus, the animals formed six treatment groups: control ($n=17$), 0.75 Gy ($n=17$), 1.5 Gy ($n=15$), HLU ($n=11$), HLU + 0.75 Gy ($n=14$), HLU + 1.5 Gy ($n=11$). Rats were studied following 12–13 months of recovery from the HLU/GCR exposures; thus, all rats were studied at 25–26 months of age. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Florida State University.

2.2 | Hindlimb unloading and radiation treatment

Rats were randomly assigned to groups on day 1 of the experimental study timeline and grouped into weight-bearing (control) or HLU using a modified version of the previously published method.[53] Briefly, rats were lightly anesthetized with isoflurane (2.5–3.0%; 100% oxygen flow rate). While anesthetized, harness tape was placed approximately 1 cm from the base of the tail, spanning distally for approximately the next 3/4ths the length of the tail. The free end of the tape was looped through a catch that was part of a custom plastic ball-bearing swivel. After passing through the swivel, the tape was symmetrically adhered to the other lateral side of the tail in a similar spatial manner. Three pieces of 1/2-in micropore surgical tape (3M, St. Paul, MN, USA) were secured perpendicularly along the traction tape. An oval stainless wire carabiner clip was attached through a hole on the top portion of the plastic swivel. The cord was attached to a clothesline support separator spreader pulley that slides along a solid steel dowel rod. Once the harness had been attached, rats were monitored closely for the next 24 h while being kept ambulatory. After 24 h, the tails were fully suspended, lifting the rat at an angle of 30° relative to the horizontal plane.

All HLU and weight-bearing control rats were singly housed, and HLU rats were monitored over the next 5 days to ensure the animals had access to food and water and could move smoothly across the cage, despite the hindlimbs not touching the cage surface. Wet rodent chow and gel packs were provided to ensure hydration, with corrugated cardboard bedding supplied to permit burrowing. On Day 5, animals were transferred from HLU or non-HLU housing to a new plexiglass box (holder) drilled with air holes for radiation or sham-radiation exposure. The new holders were placed into a rigid foam frame allowing six rats (in six holders) to be placed into the beamline simultaneously. This process was repeated five more times, such that a total of six rats were HLU and stacked (3 high; with two rats on the bottom, middle, and top levels) within the foam frame. Rats that did not receive HLU were placed into similar plexiglass boxes but remained fully weight-bearing. Their holders were smaller ($8'' \times 4'' \times 4''$) and were aligned in a matrix of three horizontal capsules and four vertical capsules for irradiation on the beamline (or sham-irradiation). Rats received either sham-irradiation or a single radiation exposure, a simplified 5-ion exposure prescribed by NASA and referred to as simGCRsim (consisting of rapid, sequential exposure to protons (1000 MeV/n, LET = 0.2 keV/μm, 35%), ^{28}Si ions (600 MeV/n, LET = 50.4 keV/μm, 1%), ^{4}He ions (250 MeV/n, LET = 1.6 keV/μm, 18%), ^{16}O ions (350 MeV/n, LET = 16.9 keV/μm, 6%), ^{56}Fe ions (600 MeV/n, LET = 173.8 keV/μm, 1%), and protons (250 MeV/n, LET = 0.4 keV/μm, 39%).

2.3 | Isolated corpus cavernosum and internal pudendal artery preparation

Following a 12–13 month recovery from the HLU/irradiation intervention the rats were anesthetized with isoflurane inhalation and euthanized by double thoracotomy and exsanguination. Penile tissue was exposed by freeing the tissue of skin and fascia, and penile tissue

from the base to the proximal glans was harvested. The distal segment of the internal pudendal artery (dIPA) was obtained by dissection from 1 mm distal to the gluteal artery bifurcation to the point where the IPA terminates by dividing into the dorsal and deep penile arteries, as described in further detail.[54] The vessels and the penile tissues were further cleaned of connective tissue under a dissection microscope in ice-cold Kreb's buffer (NaCl 130 mM, KCl 4.7 mM, K_3PO_4 1.18 mM, Mg_2SO_4 1.18 mM, Na_2CO_3 14.9 mM, D-Glucose 5.6 mM, $CaCl_2$ 1.56 mM and EDTA 0.03 mM). The dorsal vein, corpus spongiosum, and connective and adventitial tissues were carefully excised from the penile shaft using a micro spring scissors. The fibrous septum was trimmed from each cavernosal chamber. Each cavernosal chamber was longitudinally bisected, and a total of four strips of CC were obtained from the proximal penile shaft of each animal for ex vivo physiological experimentation, with each segment cut to approximately 1 ×1 ×8 mm strips. The remaining CC from the distal penile shaft was snap-frozen in liquid nitrogen and stored at −80°C for molecular analysis. CC strips were mounted into a muscle strip myograph (820MS, Danish MyoTechnology, Aarhus, Denmark). The dIPA was cut into two 1.5–2.0 mm segments from each animal, which were cannulated with a 40 μm-diameter stainless steel wire and mounted on a multiwire myograph system (620 M, Danish MyoTechnology). The tissues were bathed in Kreb's solution maintained at 37°C and continuously bubbled with a 95% O_2, 5% CO_2 gas mixture for the remainder of the experiment. Following 1 h of equilibration, CC strips were stretched to an optimal resting tension of 4 mN, and the vessels were normalized to optimal dIPA resting tension ($k = 0.9$) using DMT normalization software. All tissues of the study were followed by a 1-hour equilibration period while Kreb's solution was changed every 15 min. Maximum contractile response to high potassium Kreb's solution (120 mM KCl) was assessed before the start of the experiments. Isometric tension values were continuously recorded throughout the experiment with LabChart v8 software (ADInstruments, Sydney, Australia).

2.4 | Ex vivo vascular reactivity assessment

2.4.1 | Cumulative drug concentration response

The dIPA and CC constriction responses were assessed by cumulative-concentration-response curves to phenylephrine (PE, 10^{-9}–10^{-5} mol/L). CC segments underwent additional analysis under vasoconstrictors norepinephrine (NE, 10^{-9}–10^{-5} mol/L) and U46619 (10^{-10}–10^{-6}). Endothelium-mediated vasoconstriction of the dIPA was assessed with endothelin-1 (ET-1, 10^{-11}–10^{-7} mol/L). Likewise, dIPA and CC segments' relaxation responses were assessed by cumulative-concentration-response curve to acetylcholine (ACh, 10^{-9}–10^{-5} mol/L) and DEA-NONOate (10^{-9}–10^{-5} mol/L) following a pre-constriction of 1 μM (dIPA) or 10 μM (CC) phenylephrine. All drugs were washed out for at least 30 min with at least three changes of Kreb's solution to allow tissues to return to their respective basal resting tension prior to the commencement of the subsequent dose–response protocol.

2.4.2 | Electrical field stimulation

CC and dIPA segments underwent electrical field stimulation (EFS) to induce stimulation of local nerve fibers. All EFS followed the same protocol of six, 30 V stimulations with 2 ms pulse width of increasing frequency of 1–32 Hz for 10 s at each frequency with a 2-minute interval between stimulations. EFS was initially applied without pre-constriction to test neurogenic contractility. To test NANC-mediated relaxation, tissues were incubated for 30 min with guanethidine (30 μM) and atropine (1 μM) prior to pre-constriction with PE. NANC relaxation was repeated following a 30 min incubation with N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, NOS inhibitor, 100 μmol/L), *S*-(2-boronoethyl)-l-cysteine (BEC, arginase inhibitor, 100 μmol/L), allopurinol (xanthine oxidase inhibitor, 500 μmol/L), mito-TEMPO (mitochondria-targeted antioxidant, 5 μmol/L), TEMPOL (superoxide dismutase mimetic, 1 mmol/L) and compared to the untreated NANC stimulation for each respective tissue strip. All tissues were washed for 30 min with three changes of Kreb's solution prior to the subsequent drug incubations.

2.5 | Immunoblot analysis

CC tissue segments were homogenized in radioimmunoprecipitation assay buffer (Cell Signaling Technologies, Danvers, MA, USA) with 1 mM phenylmethanesulfonyl fluoride (Sigma Aldrich). Western blot analysis was performed as previously described.[55] Primary antibodies were obtained from Abcam (Waltham, MA, USA) or Protein Tech (Rosemont, IL, USA) and diluted in Superblock T20 Blocking Buffer (ThermoFisher, Waltham, MA, USA) at the following dilutions: malondialdehyde (MDA, 1:1000, ab27641, abcam), 4-hydroxynonenal (4-HNE, 1:2000, ab46545, abcam), xanthine oxidase (XO, 1:3000, 55156-1-AP, Proteintech), arginase 1 (1:1000, 66129-1-Ig,

Proteintech), arginase 2 (1:1000, 14825-1-AP, Proteintech), and GAPDH (1:4000, 60004-1-Ig, Proteintech). Anti-mouse (NA931, Cytiva Life Sciences, Marlborough, MA, USA) or anti-rabbit (NA934, Cytiva Life Sciences) secondary antibodies were diluted 1:4000 in Superblock.

2.6 | Measurement of tissue NO byproducts and cyclic GMP levels

Tissue levels of the NO metabolic byproducts nitrate (NO_3^-) and nitrite (NO_2^-) were measured with a nitrate/nitrite (NOx) colorimetric assay kit (#780001, Cayman Chemical, Ann Arbor, MI, USA). Thirty micrograms of total protein of CC tissue homogenate was diluted in sufficient assay buffer to bring the total sample to 80 μL. The assay was performed according to manufacturer instructions with each sample measured in duplicate, with absorbance read at 540 nm using an Epoch 2 spectrophotometer (BioTek Instruments, Winooski, VT, USA). Tissue levels of cyclic GMP (cGMP) were measured using a cGMP ELISA kit (#581021, Cayman Chemical). Thirty micrograms of total protein of CC tissue homogenate was diluted in sufficient assay buffer to bring the total sample to 50 μL. The assay was performed according to manufacturer instructions with each sample measured in triplicate, with absorbance read at 412 nm using a spectrophotometer (BioTek Instruments).

2.7 | Data and statistical analysis

Vascular reactivity data analysis was performed using LabChart v8 software. Vascular constriction data is reported as a percentage of the maximum KCl-mediated constriction of each individual tissue or artery segment. Vascular relaxation data is reported as a percentage restoration to the resting tension from the PE pre-constricted value. Statistical differences between groups were determined by two-way repeated measures ANOVA followed by Sidak's multiple comparisons post hoc analysis if main effects were detected. The pharmacologic concentration-response curves were further analyzed by non-linear regression with GraphPad Prism v9 (GraphPad, La Jolla, CA, USA) for the determination of E_{max} and pEC_{50}. Western blot images were quantified by densitometry using Image J software (National Institutes of Health, Bethesda, MD, USA). Band densities were normalized to GAPDH levels and expressed as fold-change relative to the control group. Statistical differences for E_{max}, pEC_{50}, Western blot relative band density, NOx, and cGMP levels were determined by two-way ANOVA followed by Sidak's multiple comparisons post hoc analysis if main effects were detected. Statistical significance was set as $p < .05$ in all cases. All data are expressed as mean ± standard error of the mean. Statistical analyses were performed with GraphPad Prism.

3 | RESULTS

3.1 | Body Mass

At the time of experimentation, body mass of the weight-bearing control (401 ± 9 g), 0.75 Gy (421 ± 12 g), 1.5 Gy (399 ± 8 g), HLU (407 ± 13 g), HLU + 0.75 Gy (400 ± 10 g), and HLU + 1.5 Gy (409 ± 8 g) did not differ among groups.

3.2 | HLU and GCR altered vasorelaxation responses in both dIPA and CC

Prior to the evaluation of relaxation responses, tissues were pre-constricted with phenylephrine (PE). The PE-mediated increases in tension normalized to the length of the dIPA for each group were: weight-bearing control (4.34 ± 0.41 mN/mm), 0.75 Gy (4.73 ± 0.45 mN/mm), 1.5 Gy (3.98 ± 0.42 mN/mm), HLU (4.60 ± 0.49 mN/mm), HLU + 0.75 Gy (4.30 ± 0.39 mN/mm), and HLU + 1.5 Gy (4.30 ± 0.41). The PE-mediated increases in tension of the CC normalized to tissue weight for each group were: weight-bearing control (0.208 ± 0.013 mN/mg), 0.75 Gy (0.222 ± 0.021 mN/mg), 1.5 Gy (0.188 ± 0.014 mN/mg), HLU (0.183 ± 0.010 mN/mg), HLU + 0.75 Gy (0.181 ± 0.015 mN/mg), HLU + 1.5 Gy (0.202 ± 0.018 mN/mg). There were no significant effects of HLU or GCR on pre-contraction force development for either the dIPA or the CC.

Cumulative addition of acetylcholine (ACh) revealed impaired endothelium-dependent vasodilation responses of the CC tissues in the groups under HLU intervention relative to that in weight-bearing control rats (Figure 1; representative tracings Figure S1), while cumulative addition of the nitric oxide donor DEA-NONOate (DEA) showed a subtle but statistically significant augmentation of endothelium-independent relaxation of the dIPA following HLU (Figure 2; representative tracings Figure S2). In contrast, higher radiation exposure decreased relaxation responses to ACh in the dIPA of HLU and weight-bearing control groups (Figure 1), whereas the endothelium-independent relaxation responses to DEA were only impaired by HLU in the CC (Figure 2). Non-linear regression analysis similarly reveals a decrease in E_{max} of the CC associated with HLU for the ACh response and a decrease in E_{max} of the dIPA associated with GCR for the ACh response (Table 1). Analysis of the DEA responses reveals

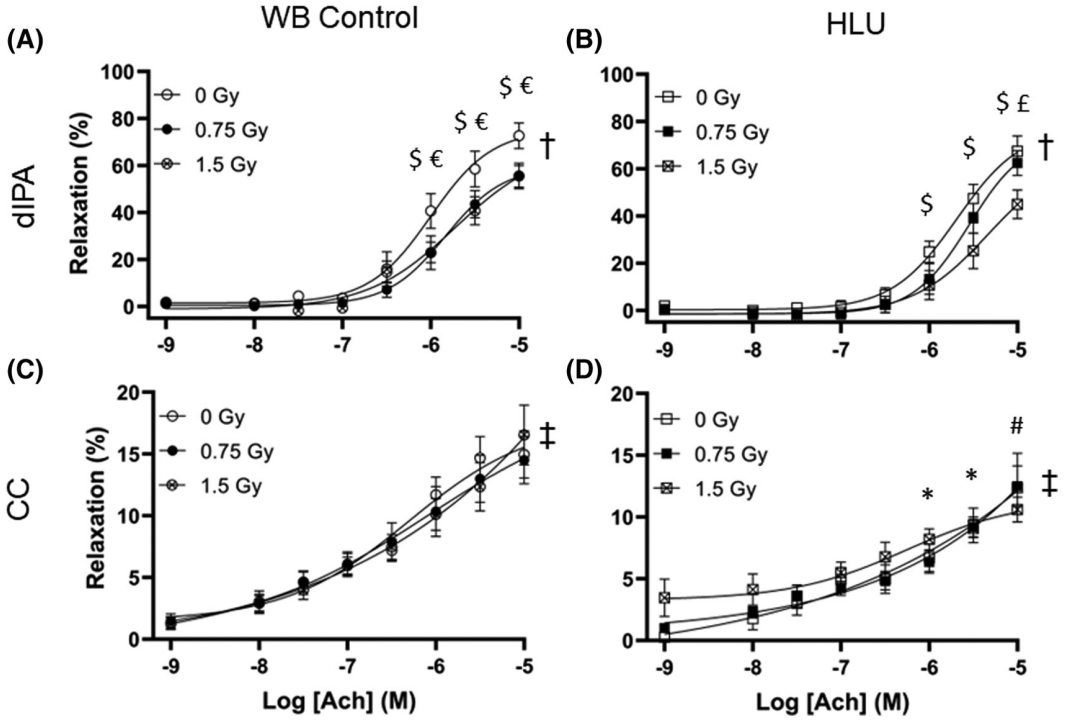

FIGURE 1 Influence of hindlimb unloading (HLU) and galactic cosmic radiation (GCR) on endothelium-dependent relaxation of the distal internal pudendal artery (dIPA) and corpus cavernosum (CC) assessed by cumulative dose–response to acetylcholine (ACh). Relaxation responses for the dIPA are presented for animals following (A) weight-bearing (WB) control, and (B) HLU. Relaxation responses for the CC are presented for animals following (C) WB control, and (D) HLU. All data values are presented as mean ± SEM for $n = 11–17$ per group. $^{‡}p < .05$ two-way ANOVA main effect of hindlimb unloading and $^{†}p < .05$ two-way ANOVA main effect of GCR. The following indicate a significant difference between groups at individual concentrations via post-hoc analysis: $^{\$}$0 Gy versus 1.5 Gy; $^{€}$0 Gy versus 0.75 Gy; $^{£}$0.75 Gy versus 1.5 Gy; *HLU effect at 0 Gy; $^{\#}$HLU effect at 1.5 Gy.

an augmentation in E_{max} for the dIPA and a decrease in E_{max} for the CC associated with HLU (Table 1). Collectively, these data demonstrate that long-duration simulated microgravity impairs endothelium-dependent and -independent vasodilation in the CC and cosmic radiation exposure impairs endothelium-dependent vasodilation in the dIPA, both of which could adversely affect sexual function.

3.3 | HLU alters vasoconstriction responses in the CC

Analysis of the CC and dIPA adrenergic response to increasing concentrations of PE revealed a marginally decreased constrictor response of the CC following irradiation, while no differences were observed following HLU (Figure S3). Additional experiments to investigate the α-adrenoreceptor-mediated vasoconstriction in the CC with cumulative addition of NE showed a significant decrease in constriction for the groups under HLU treatment (Figure 3A–C; representative tracings Figure S4A). Parallel experiments with the addition of the thromboxane A2 receptor agonist U46619 similarly showed decreased CC constriction in the groups under HLU intervention (Figure 3D–F; representative tracings Figure S4B). Nonlinear regression analysis revealed a significant decrease in the E_{max} of the CC to both NE and U46619, as well as a decreased sensitivity (pEC_{50}) to U46619 (Table 1) following the HLU intervention. However, dIPA tissue segments did not show any significant differences among groups in the vasoconstriction response to PE (Figure S3), nor to the endothelial-secreted vasoconstrictor endothelin-1 (ET-1) (Figure S5). Neurogenic contractile responses of both CC and dIPA to electrical field stimulation (EFS) of increasing frequencies (1–32 Hz) showed a clear frequency–dependent response curve, but with no significant differences among groups and with no clear influence of either HLU or GCR (Figure 4; representative tracings Figure S6).

3.4 | GCR elicits oxidative stress and penile neurovascular dysfunction

To specifically analyze the function of NANC nerve fibers on penile vasoreactivity, we incubated the tissues with the anticholinergic drug atropine and the sympatholytic drug

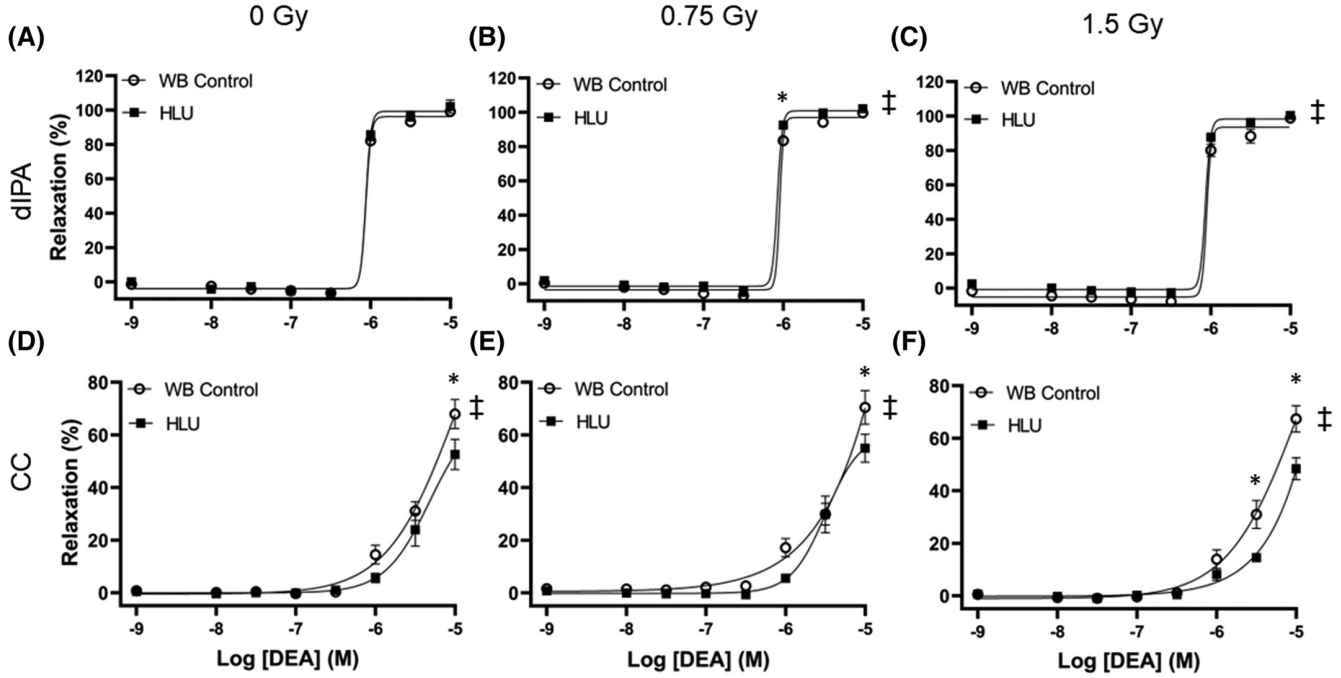

FIGURE 2 Influence of hindlimb unloading (HLU) and galactic cosmic radiation (GCR) on endothelium-independent relaxation of the distal internal pudendal artery (dIPA) and corpus cavernosum (CC) assessed by cumulative dose–response to the nitric oxide donor DEA-NONOate (DEA). Relaxation responses for the dIPA are presented for animals following (A) no GCR (0 Gy), (B) 0.75 Gy GCR, and (C) 1.5 Gy GCR. Relaxation responses for the CC are presented for animals following (D) no GCR (0 Gy), (E) 0.75 Gy GCR, and (F) 1.5 Gy GCR. All data values are presented as mean ± SEM for $n = 11–17$ per group. $^{‡}p < .05$ two-way ANOVA main effect of HLU. *Indicates a significant effect of HLU at individual concentrations via post-hoc analysis.

guanethidine prior to pre-constriction and EFS application. The data indicated impaired relaxation responses of the dIPA that was related to radiation dose and a slight increase in responses due to HLU (Figure 5A,B; representative tracings Figure S7A). The results also demonstrated a significant radiation-induced impairment of the CC relaxation responses, but without an effect from HLU (Figure 5C,D; representative tracings Figure S7B).

To verify the role of nitric oxide in the NANC relaxation response, the CC tissues were incubated with the NOS inhibitor L-NAME for 30 min before undergoing another round of EFS. The presence of L-NAME completely inhibited tissue response to EFS in all groups, supporting that the relaxation was mainly driven by the action of endogenous nitric oxide (Figure S8). The inhibitory role of high levels of arginase in NO synthesis was also examined. Upon treatment with the arginase inhibitor BEC, the groups under high radiation exposure and radiation exposure combined with HLU showed a significant improvement in NANC-mediated relaxation (Figure 6).

To assess the possible adverse interaction of NO with increased superoxide levels following intervention, the CC tissues were incubated with the xanthine oxidase inhibitor allopurinol, the mitochondria-targeted antioxidant mito-TEMPO, and the superoxide dismutase (SOD) mimetic TEMPOL. Allopurinol incubation induced a modest augmentation of relaxation at lower frequency stimulations and a modest decrement in relaxation at the highest frequency stimulation in the sham irradiated tissues (Figure 7). However, allopurinol induced more consistent increases in relaxation at the lowest three stimulation frequencies for the 0.75 Gy GCR groups, while allopurinol increased relaxation through the entire EFS frequency range for animals exposed to 1.5 Gy GCR. These effects of allopurinol appeared to be independent of HLU status.

Treatment with mito-TEMPO significantly improved the NANC relaxation response for all groups (Figure 8). While mito-TEMPO augmented the NANC relaxation response for the sham radiation groups at the lower and middle frequencies, the mito-TEMPO-induced improvements were more pronounced and were present throughout the frequency range for the groups exposed to high radiation levels (1.5 Gy) and the combination of radiation and HLU (0.75 Gy + HLU and 1.5 Gy + HLU). Interestingly, while treatment with the superoxide dismutase (SOD) mimetic TEMPOL also improved relaxation responses for the groups exposed to high radiation levels (1.5 Gy) and radiation and HLU (0.75 Gy + HLU), it induced opposite effects on the sham radiation groups in which it led to a small decrease in relaxation responses (Figure S9).

Western blot analysis of the CC tissues confirmed increased levels of oxidative stress and lipid peroxidation in

TABLE 1 Non-linear regression analysis of the pharmacologic concentration-response curves for acetylcholine (ACh), DEA-NONOate (DEA), norepinephrine (NE), and U46619.

	WB			HLU		
	0 Gy	0.75 Gy	1.5 Gy	0 Gy	0.75 Gy	1.5 Gy
dIPA—ACh						
pEC_{50}	5.89 ± 0.43	5.85 ± 0.34	5.75 ± 0.52	5.69 ± 0.33	5.64 ± 0.36	5.71 ± 0.33
E_{max} †	72.7 ± 19.6	55.3 ± 18.5	55.6 ± 20.7	67.4 ± 23.5	62.4 ± 18.3	44.9 ± 21.7§
CC—ACh						
pEC_{50}	6.64 ± 0.56	6.44 ± 0.58	6.31 ± 0.35	6.29 ± 0.67	6.09 ± 0.60	6.66 ± 1.10
E_{max} ‡	18.4 ± 6.3	16.2 ± 8.8	17.9 ± 7.6	12.6 ± 9.1	12.5 ± 5.7	10.8 ± 3.3
dIPA—DEA						
pEC_{50}	6.08 ± 0.06	6.06 ± 0.01	6.10 ± 0.06	6.10 ± 0.09	6.09 ± 0.06	6.11 ± 0.08
E_{max} ‡	99.0 ± 2.4	99.5 ± 2.6	98.7 ± 4.5	101.1 ± 9.1	102.1 ± 4.1	100.4 ± 2.6
CC—DEA						
pEC_{50}	5.35 ± 0.46	5.23 ± 0.54	5.69 ± 0.37	5.21 ± 0.86	5.42 ± 0.41	4.75 ± 0.83*
E_{max} ‡	70.0 ± 21.9	66.3 ± 30.5	68.0 ± 19.8	52.5 ± 19.0	57.2 ± 20.2	48.4 ± 14.1
CC—NE						
pEC_{50}	5.38 ± 0.76	5.49 ± 0.58	5.40 ± 0.60	5.30 ± 0.30	5.41 ± 0.32	5.04 ± 0.47
E_{max} ‡	112 ± 34.8	115 ± 27.8	105 ± 22.5	90 ± 29.8	96 ± 21.0	81 ± 12.5
CC—U46619						
pEC_{50} ‡	7.15 ± 0.20	7.05 ± 0.30	6.97 ± 0.27	6.94 ± 0.26	6.78 ± 0.63	6.76 ± 0.27
E_{max} ‡	112 ± 42.9	116 ± 17.2	112 ± 20.9	87 ± 18.9*	102 ± 21.2	75 ± 22.3*

Note: Values are presented as mean ± SD.

*Indicates a difference ($p < .05$) between groups exposed to the same GCR dose.

†$p < .05$ two-way ANOVA main effect of GCR exposure.

‡$p < .05$ two-way ANOVA main effect of HLU.

§Indicates a difference ($p < .05$) between groups within the same HLU exposure compared to the 0 Gy group.

the corpus cavernosum on the groups under radiation exposure by assessment of 4-hydroxynonenal (4-HNE), but with no changes in MDA levels among groups (Figure 9A–D). Xanthine oxidase protein expression in the CC was not different among groups (Figure 9E,F). Western blot analysis of CC showed increased levels of cytosolic arginase 1 (Arg1) and mitochondrial arginase 2 (Arg2) in the CC in the groups under radiation exposure (Figure 9E–H). Assessment of CC tissue levels of the NO metabolism byproducts NO_2^- and NO_3^- (NOx), as well as CC tissue cGMP levels, revealed decreases in both measures associated with both GCR and HLU (Figure 10).

4 | DISCUSSION

To our knowledge, this study is the first investigation into the effects of real or stimulated spaceflight on tissues relevant to erectile function. While erectile dysfunction affects more than half of men over the age of 40 and represents an important factor for life satisfaction, the consequences of space travel on erectile function are still obscure.[25] With the growing interest in expanding manned missions into deep space, it is fundamental to analyze the holistic physiological effects of deep space travel to ensure that astronauts can return to normal life on Earth. This research demonstrates the long-term consequences of HLU and simulated GCR exposure on the vascular reactivity of the distal internal pudendal artery and corpus cavernosum. The 4-week HLU intervention was coupled with GCR exposure to mimic the environment astronauts would face in an exploratory mission to the Moon or Mars. The results demonstrate that the most deleterious alterations to vascular reactivity were produced primarily by GCR, which were observed after a prolonged recovery period following simulated spaceflight. Collectively, these results suggest that neurovascular function of the erectile tissues may be impaired throughout the remainder of the astronauts' sexual health span following return to Earth from prolonged deep space exploration.

Erectile dysfunction can be caused by any of the multi factors acting on the regulation of the smooth muscle contraction and relaxation in the vessels supplying the penis. The internal pudendal arteries (IPA) have been shown to

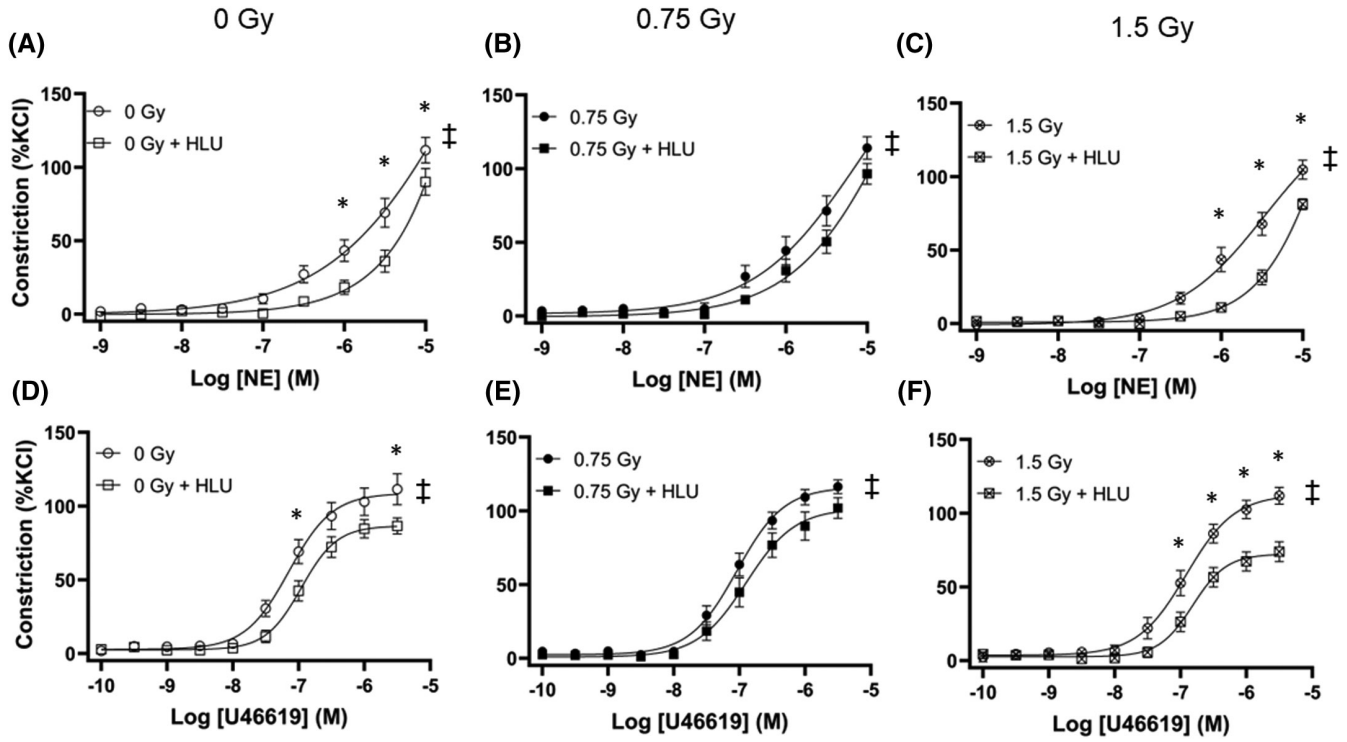

FIGURE 3 Influence of hindlimb unloading (HLU) and galactic cosmic radiation (GCR) on constriction of the corpus cavernosum (CC). Adrenergic constriction was assessed by cumulative dose–response to the α-adrenoreceptor norepinephrine (NE) and presented for animals following (A) no GCR (0 Gy), (B) 0.75 Gy GCR, and (C) 1.5 Gy GCR. Constriction was further tested by cumulative dose–response to the thromboxane A2 receptor agonist U46619 and present for animals following (D) no GCR (0 Gy), (E) 0.75 Gy GCR, and (F) 1.5 Gy GCR. All data values are presented as mean ± SEM for $n = 11–17$ per group. $^{‡}p < .05$ main effect of HLU. *Indicates a significant effect of HLU at individual concentrations via post-hoc analysis.

be the primary arteries supplying blood to the penis and contributing most of the vascular resistance to the penis, with the distal IPA displaying neurovascular regulation that most resembles that of the penis.[56,57] The vascular endothelium plays an important role in regulating penile vascular tone and promoting erection mainly through the production of nitric oxide, which mediates smooth muscle relaxation and increased blood flow to the penis. Notably, endothelial dysfunction has been shown to reduce the bioavailability of nitric oxide, blood inflow, and to be a prominent factor in erectile dysfunction.[58,59]

Additionally, non-adrenergic non-cholinergic (NANC) nerve fibers play an important role in erectile function, which stimulates the release of NO into the corpus cavernosum smooth muscle cell upon sexual stimulus leading to its relaxation and penile erection.[32,34,35,60] Initial data on the drug-elicited vasoreactivity and electrical stimulation of the dIPA and CC showed that GCR significantly impaired endothelial function and NANC-mediated relaxation in the tissues of study. We also observed the complete inhibition of tissue relaxation using nitric oxide synthase (NOS) inhibitor L-NAME, confirming that NANC relaxation is highly dependent on NO activity. These effects appear to be driven by increased oxidative stress and diminished NO bioavailability in the CC upon NANC stimulation following GCR exposure. Follow-up analysis on CC tissue conferred a decrement in NO metabolism byproducts (NOx) and cGMP levels following GCR, which further implicates a likely defect in the NOS-sGC-cGMP signaling pathway that is critical for normal erectile function.

Arginase is an enzyme responsible for converting L-arginine to urea and ornithine in the final step of the urea cycle. While important, high levels of arginase compete with NOS for L-arginine as the precursor for the synthesis of NO, demonstrating that elevated arginase activity may reduce NO bioavailability and lead to vascular endothelial dysfunction.[61,62] Previous studies have shown the potential role of arginase inhibition for the treatment of erectile dysfunction by enhancing NO-dependent smooth muscle relaxation and CC function.[63,64] Correspondingly, data from the present study show that arginase inhibition significantly improved relaxation responses for the groups exposed to 1.5 Gy of radiation, and 0.75 and 1.5 Gy in combination with HLU, suggesting the role of arginase upregulation in decreasing the NO-mediated smooth muscle relaxation in the penis following radiation. This observation is further supported by increased protein expression

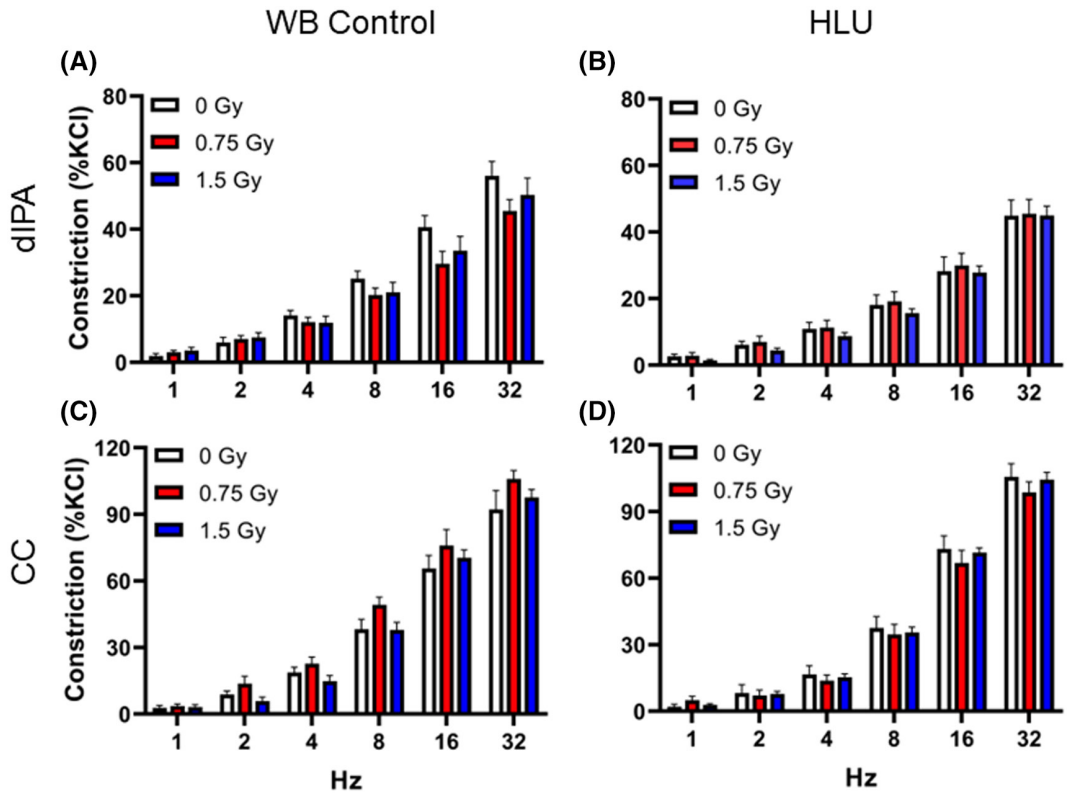

FIGURE 4 Influence of hindlimb unloading (HLU) and galactic cosmic radiation on neurogenic constriction of the distal internal pudendal artery (dIPA) and corpus cavernosum (CC) assessed by electrical field stimulation. Constriction responses for the dIPA are presented for animals following (A) weight-bearing (WB) control, and (B) HLU. Relaxation responses for the CC are presented for animals following (C) WB control, and (D) HLU. All data values are presented as mean ± SEM for $n = 10–17$ per group.

for cytosolic arginase 1 and mitochondrial arginase 2 in the CC of groups exposed to increased levels of radiation, indicating a sustained radiation-induced upregulation of arginase. While investigation of the possible upstream mediators of arginase regulation was beyond the scope of this project, prior research indicates that arginase expression and activity in endothelial cells is induced by the ROS peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2) through a protein kinase C-RhoA/Rho kinase-arginase signaling pathway.[65] It is possible that GCR-induced oxidative stress in the CC caused an upregulation of arginase through a similar pathway, which would be a worthy area of further investigation.

Several studies have now indicated a central role for oxidative stress as a mediator of ED, in which a decrease in NO bioavailability has been connected to decreased cardiovascular function and ED due to its interaction with the superoxide anion (O_2^-), leading to the production of peroxynitrite ($ONOO^-$).[33,52,66–70] Likewise, results from the present study demonstrate increased levels of oxidative stress and lipid peroxidation in the CC of irradiated groups through elevated levels of 4-hydroxynonenal (4-HNE). While we did not observe any changes in malondialdehyde (MDA) levels among groups, 4-NHE is a more sensitive biomarker of lipid peroxidation in rats.[71] Thus, the prolonged elevation in oxidative stress and ROS production resulting from radiation exposure presents a risk to the erectile function of astronauts.[49,72–77]

Previous work simulating weightlessness and deep space irradiation in rodents have reported increases in xanthine oxidase (XO) enzyme levels and XO-dependent ROS production that corresponded with impaired endothelium-dependent vasodilation in the abdominal aorta and coronary and skeletal muscle resistance arteries.[21,49,50] Furthermore, these pro-oxidant alterations were evident soon after radiation exposure,[49] as well as following recovery periods of 4–8 months,[21,50] or the human equivalent of approximately 20 years. In the present study, XO inhibition improved NANC-mediated relaxation responses in rats exposed to the higher radiation level (1.5 Gy), as well as in animals undergoing the combined treatments of HLU and the lower and higher doses of irradiation (0.75 Gy + HLU and 1.5 Gy + HLU). Thus, these results indicate that alterations in XO and/or XO-derived ROS production induced by simulations of GCR produce dysfunction of penile vascular function, even after a prolonged period of recovery. Prior research indicates that ROS production by xanthine oxidase is induced by H_2O_2

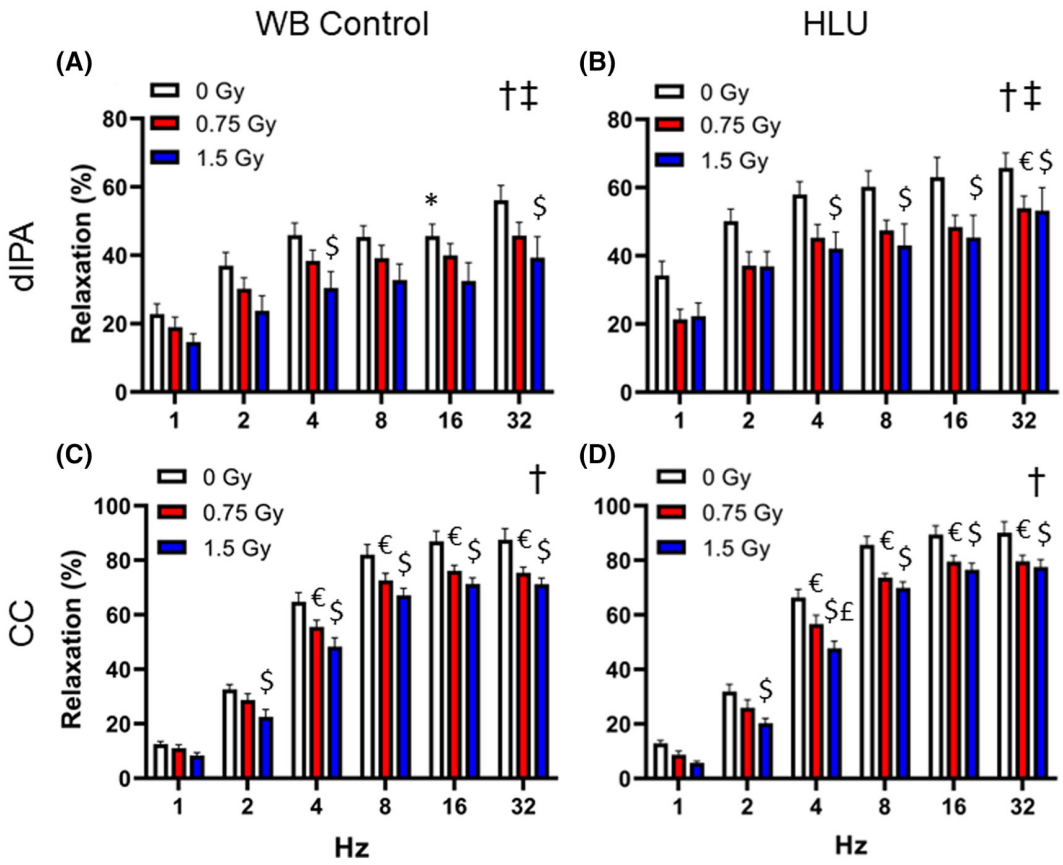

FIGURE 5 Influence of hindlimb unloading (HLU) and galactic cosmic radiation (GCR) on non-adrenergic, non-cholinergic (NANC) nerve-mediated relaxation of the distal internal pudendal artery (dIPA) and corpus cavernosum (CC) assessed by electrical field stimulation following treatment with atropine and guanethidine and constriction with phenylephrine. Relaxation responses for the dIPA are presented for animals following (A) weight-bearing (WB) control, and (B) HLU. Relaxation responses for the CC are presented for animals following (C) WB control, and (D) HLU. All data values are presented as mean ± SEM for n = 12–17 per group. $^{‡}p < .05$ two-way ANOVA main effect of HLU and $^{†}p < .05$ two-way ANOVA main effect of GCR exposure. The following indicate a significant difference between groups at individual concentrations via post-hoc analysis: $^{\$}$0 Gy versus 1.5 Gy; $^{€}$0 Gy versus 0.75 Gy; $^{£}$0.75 Gy versus 1.5 Gy; *HLU effect at 0 Gy.

through oxidation of key cysteine residues in the xanthine oxidoreductase/dehydrogenase complex.[78,79] It is certainly possible that other sources of ROS, such as the mitochondria, NADPH oxidase, or uncoupled NOS contributed to the chronic oxidative stress observed following GCR, while it is plausible that the general oxidative stress was a contributing factor to the XO component of the functional decline in NANC-mediated relaxation following GCR.

While the mitochondria play an important role in cellular energy production, it is also a major source of ROS synthesis in most tissues, in which the mitochondrial respiratory complexes leak electrons to oxygen molecules producing superoxide anion (O_2^-).[80,81] In our studies, treatment with the mitochondrial-targeted antioxidant mito-TEMPO showed the most significant restorative effects in relaxation responses for the groups exposed to the higher radiation level (1.5 and 1.5 Gy + HLU) and the combined HLU and irradiation at the lower level (0.75 Gy + HLU), indicating an adverse effect of mitochondrial ROS production to impair CC function following simulated deep space flight. This conclusion is supported by recent research reporting the incidence and impacts of mitochondrial dysfunction resulting from the dysregulation of ROS production and antioxidant defense after spaceflight.[17,77,82] Overall, these observations suggest that mitochondrial dysfunction is present in the CC following GCR that leads to decreased vasoreactivity and function and represents an important area for further exploration in future investigations.

The hindlimb unloading model to mimic weightlessness observed in space was used to address the role of microgravity on vascular function. Following the HLU intervention, the data showed impaired endothelium-dependent and -independent vasodilation in the CC following exposure to HLU. Given the stark decline in relaxation to the NO donor DEA following HLU, the decrement in endothelium-dependent relaxation may have potentially resulted from decreased smooth muscle sensitivity to NO.

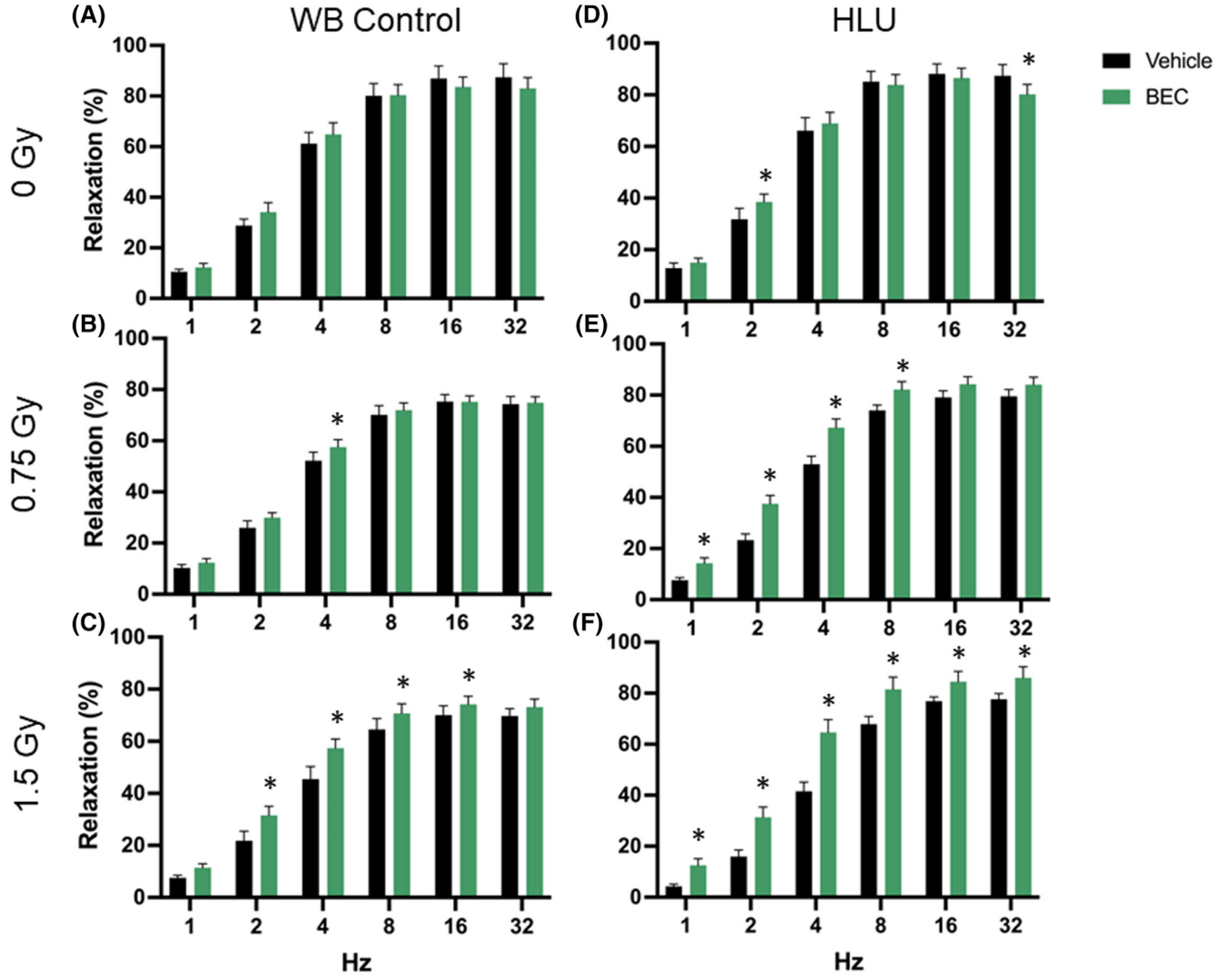

FIGURE 6 Effect of acute arginase inhibition on NANC-mediated relaxation of the corpus cavernosum. Tissues were incubated with atropine and guanethidine and pre-constricted with PE to assess NANC-mediated relaxation in response to electrical field stimulation in the absence (black) and presence of 100 μM BEC (green). Relaxation responses are presented for weight-bearing (WB) control animals following (A) no GCR (0 Gy), (B) 0.75 Gy GCR, and (C) 1.5 Gy GCR, and for animals following hindlimb unloading (HLU) and (D) no GCR (0 Gy), (E) 0.75 Gy GCR, and (F) 1.5 Gy GCR. All data values are presented as mean ± SEM for $n = 9–15$ per group. *$p < .05$ versus vehicle treatment.

We observed a modest decrement in CC tissue NOx and cGMP that implicates a deficiency in the NO-sGC-cGMP signaling pathway associated with HLU. However, we did not observe any apparent differences in the CC NANC-mediated relaxation responses due to HLU. In contrast, we observed an enhanced propensity for relaxation of the dIPA with an increase in endothelium-independent and NANC-mediated relaxation, suggesting a slight sensitization to NO in the dIPA of the groups under HLU intervention. We did not observe any effect of HLU on neurogenic constriction or endothelium-dependent relaxation in the dIPA. Collectively, these findings indicate a greater adverse impact of HLU on the corpus cavernosum than the dIPA after exposure to HLU.

While we observed an increased propensity for relaxation of the dIPA following HLU, there were no HLU-mediated changes in vasoconstriction for any of the mechanisms investigated. In contrast, we observed an impairment in NE and U46619-mediated constriction of the CC following HLU. Similarly, previous work with rodent mesenteric and gastrocnemius muscle arteries has shown reduced vasoconstrictor responses following HLU and actual spaceflight with a decrease in intracellular calcium release via decreased RyR-2 and RyR-3 expression in smooth muscle cells.[83–85] These factors have been suggested to underlie the lower peripheral vascular resistance and orthostatic intolerance after spaceflight. Collectively, the data in these experiments are suggestive of an HLU-mediated

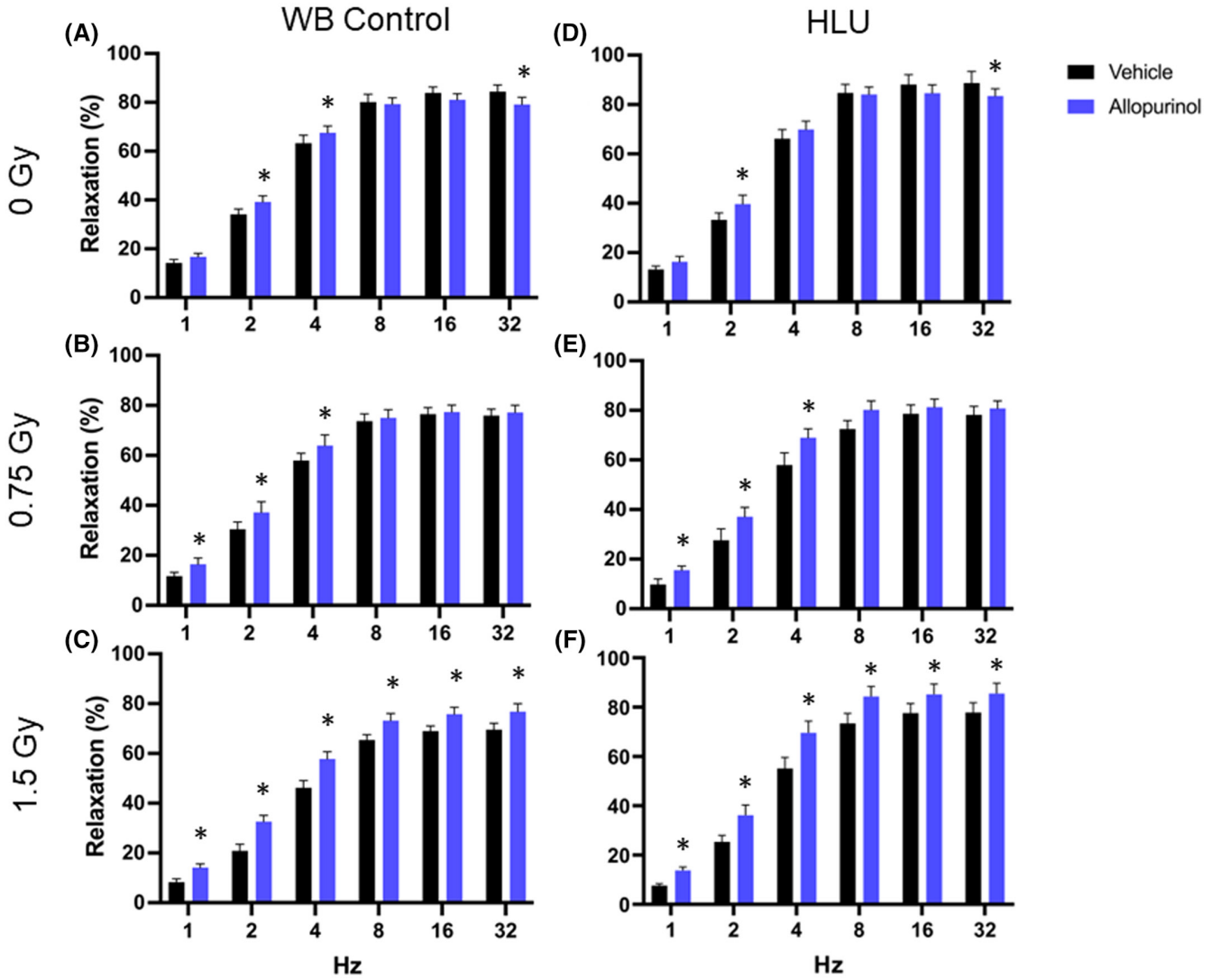

FIGURE 7 Effect of acute xanthine oxidase inhibition on NANC-mediated relaxation of the corpus cavernosum. Tissues were incubated with atropine and guanethidine and pre-constricted with PE to assess NANC-mediated relaxation in response to electrical field stimulation in the absence (black) and presence of 500 μM allopurinol (blue). Relaxation responses are presented for weight-bearing (WB) control animals following (A) no GCR (0 Gy), (B) 0.75 Gy GCR, and (C) 1.5 Gy GCR, and for animals following hindlimb unloading (HLU) and (D) no GCR (0 Gy), (E) 0.75 Gy GCR, and (F) 1.5 Gy GCR. All data values are presented as mean ± SEM for $n = 11–16$ per group. *$p < .05$ versus vehicle treatment.

disruption in the smooth muscle function of the corpus cavernosum.

There were several limitations present in this study. The use of the HLU rodent model to simulate weightlessness, while a useful and widely used ground-based model, HLU is not a robust model of microgravity and does not always reproduce the vascular alterations associated with the microgravity component of spaceflight. Additionally, experiments were conducted following a long recovery period after the intervention to ascertain the long-term consequences of deep space travel on the vasculature associated with erectile function. Other, and perhaps more severe effects on penile vascular function may have been found following a more short-term recovery from GCR irradiation and HLU. Future research should investigate simulated spaceflight at shorter time points following GCR exposures to gain a more complete understanding of the temporal nature of spaceflight and recovery on sexual function. As this was an initial study into the potential impacts of spaceflight on penile neurovascular function, this study was rather descriptive in nature. Further study will be needed to determine alterations in signaling pathways that lead to changes in arginase and xanthine oxidase activity, as well as to pinpoint the site of impairment in the e/nNOS-sGC-PKG-PDE5 signaling pathway. There are several

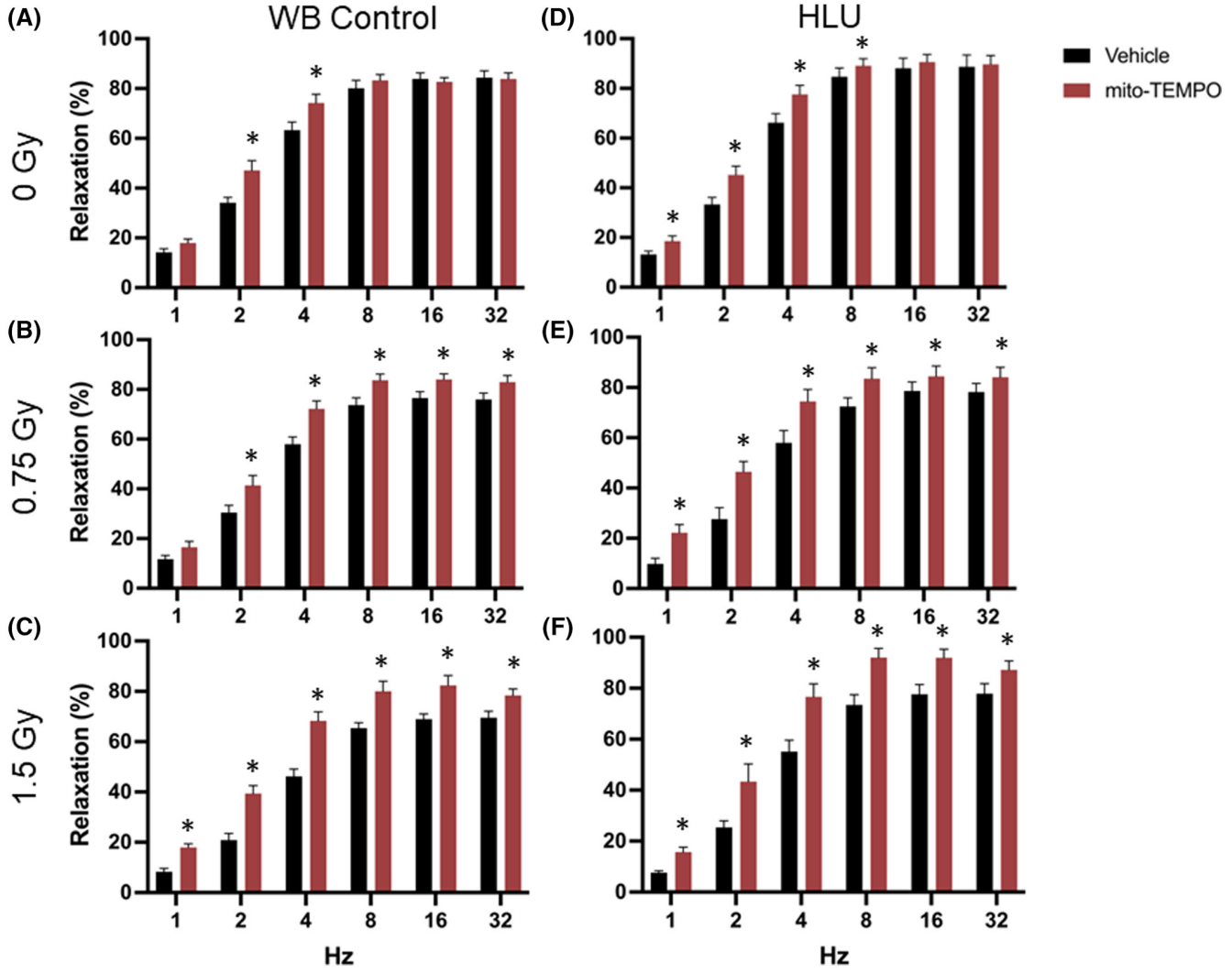

FIGURE 8 Effect of acute mitochondria-targeted antioxidant on NANC-mediated relaxation of the corpus cavernosum. Tissues were incubated with atropine and guanethidine and pre-constricted with PE to assess NANC-mediated relaxation in response to electrical field stimulation in the absence (black) and presence of 5 μM mito-TEMPO (red). Relaxation responses are presented for weight-bearing (WB) control animals following (A) no GCR (0 Gy), (B) 0.75 Gy GCR, and (C) 1.5 Gy GCR, and for animals following hindlimb unloading (HLU) and (D) no GCR (0 Gy), (E) 0.75 Gy GCR, and (F) 1.5 Gy GCR. All data values are presented as mean ± SEM for $n = 11–16$ per group. *$p < .05$ versus vehicle treatment.

post-translational modifications of eNOS and nNOS that may be induced by oxidative stress that impede NO production that could conceivably be involved in the GCR response. Future research should also investigate the role of mitochondrial dysfunction in the CC following GCR as the mitochondria-targeted antioxidant elicited the most striking improvement in NANC-mediated relaxation among the drugs investigated. Additionally, future studies should include an assessment of in vivo erectile function via measures of intracavernous pressure and mean arterial pressure in response to electrical stimulation of the cavernous nerve for a more comprehensive assessment of spaceflight on erectile function. Lastly, as space exploration missions in the coming years include female astronauts, continuing work in this area should investigate the potential impacts of spaceflight on female sexual function.

5 | CONCLUSION

This study is the first to explore the chronic health effects of simulated microgravity and deep space radiation on vascular reactivity and sexual health in males. The data demonstrate that impairment of vasoreactivity of internal pudendal arteries and corpus cavernosum occurs through the decrease in endothelium-dependent and -independent pathways, even after a prolonged

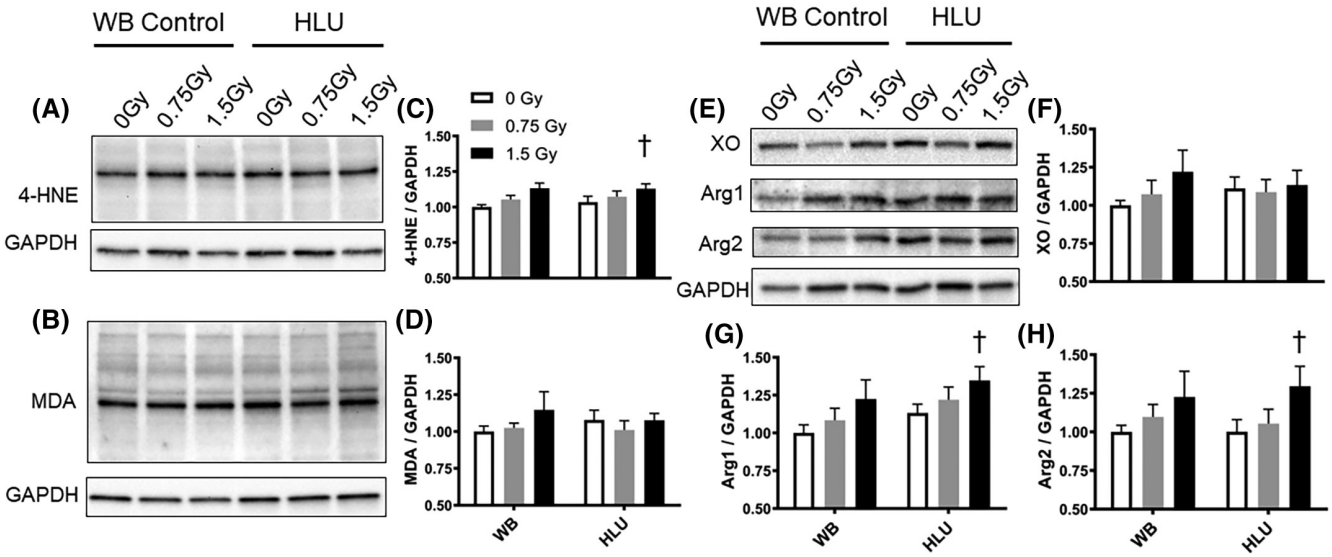

FIGURE 9 Influence of hindlimb unloading (HLU) and galactic cosmic radiation (GCR) on oxidative stress and arginase protein content in the corpus cavernosum (CC). Representative immunoblot images for the markers of chronic oxidative stress and lipid peroxidation (A) 4-hydroxynonenal (4-HNE) and (B) malondialdehyde (MDA). Quantification of band intensity density normalized to the loading control GAPDH for (C) 4-HNE and (D) MDA. (E) Representative immunoblot images for xanthine oxidase (XO), arginase 1 (Arg1), and arginase 2 (Arg2). Quantification of band intensity density normalized to GAPDH for (F) XO, (G) Arg1, (H) Arg2. All data values are presented as mean ± SEM for $n = 12$ per group. $^{\dagger}p < .05$ two-way ANOVA main effect of GCR.

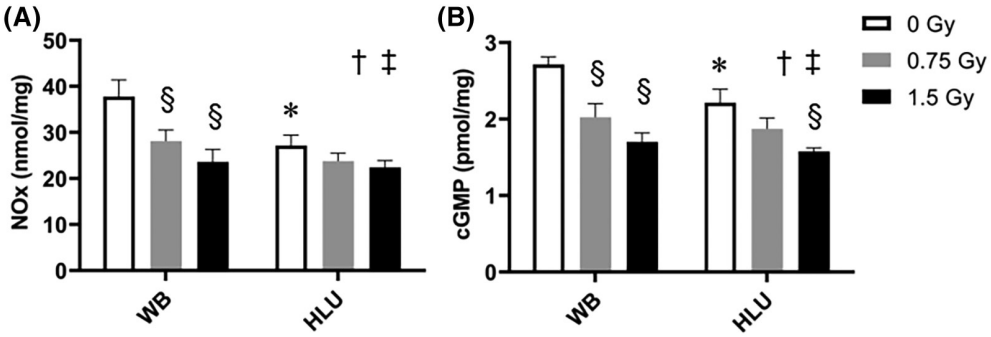

FIGURE 10 Influence of hindlimb unloading (HLU) and galactic cosmic radiation (GCR) on nitric oxide and cGMP levels in the corpus cavernosum (CC). (A) Assessment of the nitric oxide metabolic byproducts nitrate and nitrite (NOx). (B) Assessment of tissue cGMP levels. All data values are presented as mean ± SEM for $n = 11$–14 per group (A) and $n = 8$ per group (B). $^{\ddagger}p < .05$ two-way ANOVA main effect of HLU and $^{\dagger}p < .05$ two-way ANOVA main effect of GCR exposure. *Indicates a difference ($p < .05$) between groups exposed to the same GCR dose. §Indicates a difference ($p < .05$) between groups within the same HLU exposure compared to the 0 Gy group.

recovery period. These vascular alterations are induced by relatively low doses of GCR and to a lesser extent simulated weightlessness, primarily through increases in oxidative stress. The resulting degradation of the relaxation capacity of CC likely elevates the risk for developing erectile dysfunction. Acute treatment of vascular tissue with different antioxidants improved relaxation responses to EFS in the radiation-exposed groups. These findings also suggest that increased oxidative stress and arginase activity from radiation exposure limit NO action and impair corpus cavernosum vasoreactivity, uncovering new factors to consider with long-duration space exploration to ensure that astronauts can return to normal life after deep space missions.

AUTHOR CONTRIBUTIONS

Justin D. La Favor, Michael D. Delp, S. Anand Narayanan1, and Jeffrey S. Willey conceived and designed the study. Manuella R. Andrade1, Tooyib A. Azeez, McLane M. Montgomery, Jacob T. Caldwell, Hyerim Park, S. Anand Narayanan1, Andy T. Kwok, Alexander M. Borg, and Justin D. La Favor performed the research. Manuella

R. Andrade1, Tooyib A. Azeez, and McLane M. Montgomery acquired and analyzed data. All authors discussed and interpreted the data. Manuella R. Andrade wrote the initial manuscript draft. Justin D. La Favor and Michael D. Delp revised the manuscript. All authors reviewed and approved the final manuscript.

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DISCLOSURES

The authors have no competing interests to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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