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Oxidative Lung Damage Resulting from Repeated Exposure to Radiation and Hyperoxia Associated with Space Exploration

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Abstract

Background: Spaceflight missions may require crewmembers to conduct Extravehicular Activities (EVA) for repair, maintenance or scientific purposes. Pre-breathe protocols in preparation for an EVA entail 100% hyperoxia exposure that may last for a few hours (5-8 hours), and may be repeated 2-3 times weekly. Each EVA is associated with additional challenges such as low levels of total body cosmic/galactic radiation exposure that may present a threat to crewmember health and therefore, pose a threat to the success of the mission. We have developed a murine model of combined, hyperoxia and radiation exposure (double-hit) in the context of evaluating countermeasures to oxidative lung damage associated with space flight. In the current study, our objective was to characterize the early and chronic effects of repeated single and double-hit challenge on lung tissue using a novel murine model of repeated exposure to low-level total body radiation and hyperoxia. This is the first study of its kind evaluating lung damage relevant to space exploration in a rodent model.

Methods: Mouse cohorts (n=5-15/group) were exposed to repeated: a) normoxia; b) >95% O_2 (O_2); c) 0.25Gy single fraction gamma radiation (IR); or d) a combination of O_2 and IR (O_2+IR) given 3 times per week for 4 weeks. Lungs were evaluated for oxidative damage, active TGF-b1 levels, cell apoptosis, inflammation, injury, and fibrosis at 1, 2, 4, 8, 12, 16, and 20 weeks post-initiation of exposure.

Results: Mouse cohorts exposed to all challenge conditions displayed decreased bodyweight compared to untreated controls at 4 and 8 weeks post-challenge initiation. Chronic oxidative lung damage to lipids (malondialdehyde levels), DNA (TUNEL, cleaved Caspase 3, cleaved PARP positivity) leading to apoptotic cell death and to proteins (nitrotyrosine levels) was elevated in all treatment groups. Importantly, significant systemic oxidative stress was also noted at the late phase in mouse plasma, BAL fluid, and urine. Importantly, however, late oxidative damage across all parameters that we measured was significantly higher than controls in all cohorts but was exacerbated by the combined exposure to O_2 and IR. Additionally, impaired levels of arterial blood oxygenation were noted in all exposure cohorts. Significant but transient elevation of lung tissue fibrosis (p<0.05), determined by lung hydroxyproline content, was detected as early as 2 week in mice exposed to challenge conditions and persisted for 4-8 weeks only. Interestingly, active TGF-b1 levels in BAL fluid was also transiently elevated during the exposure time only (1-4 weeks). Inflammation and lung edema/lung injury was also significantly elevated in all groups at both early and late time points, especially the double-hit group.

Conclusion: We have characterized significant, early and chronic lung changes consistent with oxidative tissue damage in our murine model of repeated radiation and hyperoxia exposure relevant to space travel. Lung tissue changes, detectable several months after the original exposure, include significant oxidative lung damage (lipid peroxidation, DNA damage and protein nitrosative stress) and increased pulmonary fibrosis. These findings, along with increased oxidative stress in diverse body fluids and the observed decreases in blood oxygenation levels in all challenge conditions (whether single or in combination), lead us to conclude that in our model of repeated exposure to oxidative stressors, chronic tissue changes are detected that persist even months after the exposure to the stressor has ended. This data will provide useful information in the design of countermeasures to tissue oxidative damage associated with space exploration.

Keywords: Apoptosis; Bronchoalveolar lavage; Caspase 3; Double-hit; Extravehicular activity; Hyperoxia; Inflammation; Lung fibrosis; Lung injury; Mouse model; Nitrotyrosine; Oxidative stress; PARP; Radiation pneumonopathy; Space exploration; TGF-β1; Total body irradiation; TUNEL

Abbreviations: BAL: Bronchoalveolar Lavage; ELISA: Enzyme-linked Immunosorbent Assays; EVA: Extravehicular activity; FI: Fibrotic Index; TBI: Total Body Irradiation; OH-Proline: Hydroxyproline; LP: Lipid Peroxidation; MDA: Malondialdehyde; MPO: Myeloperoxidase; NT: Nitrotyrosine; PARP: Poly (adenosine diphosphate-ribose) polymerase; PMN: Polymorphonuclear Leukocyte; ROS: Reactive Oxygen Species; SEM: Standard Error Means; TLD: Thermoluminescent Dosimeter; TUNEL: Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; WBC: White Blood Cells; DCI: Decompression Illness; SPE: Solar Particle Events; LET: Linear Energy Transfer; GCR: Galactic Cosmic Radiation

Introduction

Space travel requires Extravehicular Activities (EVA) at which time

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astronauts are exposed to challenges that may pose potential health risks [1-4]. Planned EVAs, which include routine maintenance aboard the International Space Station, are critical aspects of daily space flight. However, unplanned EVAs are often required resulting in further exposure to the milieu of outer space [5]. Crewmembers must utilize pressurized spacesuits that create a controlled internal environment outside the spacecraft [6,7], for EVAs typically lasting between 5 and 8 hours [5]. To prevent Decompression Illness (DCI), crewmembers undergo pre-breathing protocols that insure total body nitrogen stores are depleted prior to decompression. Although acute oxygen toxicity is carefully avoided using well-established flight rules (as measured by a 2% reduction in vital capacity), the long term influence on lung function of multiple EVAs and ensuing cyclic exposures to hyperoxic conditions is not known. Radiation exposure limits were proposed by the Radiobiological Advisory Panel of the National Academy of Sciences/National Research Council in 1970 [8,9] with the National Aeronautics and Space Administration (NASA) using that information to help formulate mission limitations for EVA-related radiation exposure. Demand for EVA hours will likely increase with progression of space travel; therefore, it is important to identify potential health risks and optimize protocols to ensure maximal human performance and safety.

Little is known regarding potential lung complications that could occur from prolonged space travel. John West's landmark paper "Pulmonary Function in Space" catalogued numerous changes in physiology observed in Spacelab Life Sciences (SLS) astronauts in the early 1990s [10]; however, EVA risk is compounded by the potential health risk of repeated cycles of 100% O_2 exposure during pre-breathe procedures. Deleterious effects of hyperoxia combined with space-related exposures have long been recognized [10]. Hyperbaric hyperoxic conditions have been extensively studied in relation to DCI in divers [11]. However, exposure to a unique spectrum of radiation including Galactic Cosmic Radiation (GCR) and Solar Particle Events (SPE) [8,12] adds an additional environmental exposure in space that may affect lung tissue primed by hyperoxia during or after an EVA. Radiation exposure can be highly variable with the potential for crewmembers to experience very high doses of radiation. As a result, careful monitoring of radiation exposure dose rates via on board thermoluminescent dosimeters (TLDs) and individual dosimeters are performed with the goals of better understanding risk and minimizing exposure.

Therefore, our study proposed to address the knowledge gap in chronic lung complications resulting from these combined effects by developing and characterizing an *in vivo* model system to test repeated total body irradiation (IR) delivered in the form of γ–radiation and hyperoxia (>95% O_2). We fully recognize that astronauts could experience a cyclic "triple-hit" of hypobaric-to-hyperbaric, hypoxic-to-normoxic-to-hyperoxic, and irradiation. Such a model would be incredibly complex with numerous confounding variables; hence, we focused first on the potential effect of combined cyclic hyperoxia and radiation exposure on cells and tissue. Selecting ground-relevant gamma rays as the radiation stressor, although commonly used [13], as opposed to the isolated or combined effects of Solar Particle Events (SPE) and Cosmic Galactic Radiation (CGR) sources for example [14], may be an oversimplification of the complex charged particle environments in space. Therefore, use of this model should serve as a useful hypothesis-testing model to identify important biological effects and the basis for future studies.

The mouse serves as an excellent model to study the biology of hyperoxia [15]; however, no *in vivo* model currently exists for studying the effects of both hyperoxia and radiation exposure in the lung. Double-hit mouse models of oxidative lung damage resembling clinical scenarios of sepsis [16], trauma, or hemorrhagic shock [15] have been developed but exposure to hyperoxic conditions has never been studied in combination with low-level radiation exposure in murine or other animal models. We developed a novel approach to studying the combined effects of repeated exposures to hyperoxia and low-level total body radiation in murine lungs under controlled atmospheric conditions, hoping to characterize potential deleterious changes in lung tissue and function.

Materials and Methods

In vivo animal exposure study design

Our studies used female C57/BL6 mice, a strain well characterized in our studies of both hyperoxia [17] and pulmonary radiation damage [18,19]. We have been routinely using female mice in our radiation and hyperoxia models as they are less aggressive to work with and to maintain over lengthy studies and they are known to be more sensitive to lung damage/fibrosis as observed by numerous other investigators [20,21]. Mice were obtained from Charles River (Wilmington, MA) and irradiated at 6-8 weeks of age under animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. Animals were housed in conventional cages under standardized conditions with controlled temperature and humidity and a 12–12-h day-night light cycle. Animals had free access to water and chow (Semi-purified AIN-93G diet, Test Diet, Bloomsburg, IN). For this study mouse cohorts (n=5-15 / group) were exposed to: a) normoxia; b) >95% O_2 (O_2); c) 0.25Gy ionizing gamma radiation (IR); or d) a combination of >95% O_2 and 0.25Gy ionizing gamma radiation (O_2+IR).

Based on the "Flight Rules" put forward by NASA-Johnson Space Center in keeping with data from the National Council of Radiation Protection and Measurement (NRCP), constraints have been placed on maximum time crewmembers may breathe 100% oxygen while wearing space suits in tandem with time exposed to galactic cosmic radiation and solar particle events. Our experimental model of 3 cycles of treatment for 8 hours each exposure followed by 16 hours of normoxia simulates the missions an astronaut would be required to perform while exposed to mild background ionizing radiation and still adhering to the Flight Rules of no more than 24 hours weekly of EVA time. 0.25 Gy was chosen based on studies [9,12] as an approximate of the background radiation human tissues would receive under protective shielding in space under conditions of Linear Energy Transfer (LET) – measure of energy transferred to objects as ionizing particles pass through them. Potentially more dangerous levels of different types of radiation are possible while outside the craft, outside proximity to Earth's electromagnetic protection, but 0.25 Gy was chosen as best case scenario exposure. Mice were exposed 3 times a week for 4 weeks and sacrificed at week 1, 2, 4, 8, 12, 16 and 20 (Figure 1).

Radiation exposure of mice

After confirmation of dose uniformity by thermoluminescence dosimetry (TLD), mouse irradiation was performed with a Gammacell 40 ^{137}Cs irradiator (Atomic Energy of Canada Limited, Canada). During irradiation, the animals were held in a circular, well-ventilated custom-made Plexiglas container that minimized their movement so that the whole body would uniformly receive the radiation dose. Mice exposed to 0 Gy served as sham-controls. The average dose rate was 0.43 Gy per minute and was corrected for decay each day. The delivered dose was 0.25 Gy given 3 times a week (Monday, Wednesday, and Friday).

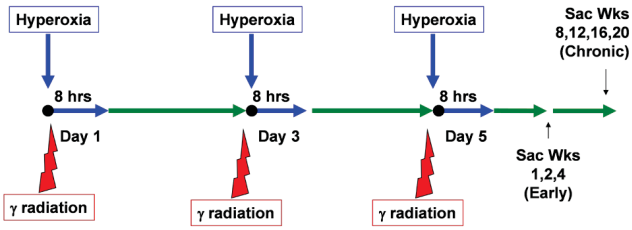

Figure 1: Experimental Plan of *In Vivo* Animal Exposure.

Mouse cohorts (n=5-15 / group) were exposed to a double-hit challenge of 0.25 Gy total body ionizing gamma irradiation (IR) and 100% pO_2 for 8 hours followed by intermittent normoxia (21% pO_2). This cycle was performed 3 times a week for 1, 2, and 4 weeks. Mice were sacrificed at weeks 1, 2, and 4 weeks (early) and 8, 12, 16, and 20 weeks (chronic). A total of 24 hours of hyperoxia / radiation exposure weekly simulates the maximum allowed weekly extravehicular activity (EVA) exposure for crewmembers on lengthy spaceflights.

Mice were immediately placed in hyperoxic conditions for 8 hours following irradiation. The scheme on Figure 1 shows details of the *in vivo* procedure.

Exposure of mice to hyperoxia

Mice were exposed to a continuous flow of pO_2 at 10 L/min in micro-isolator cages after removing the lids and placing the cages in a sealed Plexiglas chamber that allowed the simultaneous exposure of 6 mouse cages, yielding O_2 concentrations of 95-100% [22] for 8 hours followed by intervening normoxia (ambient air containing 21% O_2 and 5% CO_2). This was repeated 3 times a week for 4 weeks (Figure 1).

Bronchoalveolar lavage fluid analysis

Mice were sacrificed using an overdose of ketamine (100 mg/ ml) and xylazine (20 mg/ml) at 1, 2, 4, 8, 12, 16 and 20 weeks from initiation of the challenge (O_2, IR, or O_2+IR). Bronchoalveolar lavage (BAL) was then performed through a 20-gauge angiocatheter (BD Pharmingen, San Diego, CA), with the intra-tracheal instillation of 1 ml of phosphate-buffered saline (PBS) containing an anti-protease cocktail (Sigma) and 5 mM EDTA given in 0.5 ml increments [23-25]. An aliquot was immediately separated to measure total leukocyte cell counts (cells/ml BAL fluid) using a Coulter Cell and Particle Counter (Beckman Coulter, Miami, FL). The remaining lavage fluid was centrifuged at 1,200 rpm for 10 min and the cell-free supernatant was frozen at –80°C for cytokine and protein analysis.

The amount of total protein in the BAL fluid was assayed using the BCA Protein Assay Kit (Pierce, Rockford, IL) as per manufacturer's instructions. Absorbance was read at 560 nm (MRX Microplate Reader, Dynatech Laboratories, Chantilly, VA) and protein levels in mg/ml of BAL fluid were calculated. The results are reported as fold change from control at week 0.

Tissue harvesting and evaluation of oxidative lung injury

For histological studies, mouse lungs were instilled prior to removal from the animal with 0.75 ml of buffered formalin through a 20-gauge angiocatheter placed in the trachea, immersed in buffered formalin overnight and processed for conventional paraffin histology. Sections were stained with hematoxylin and eosin and examined by light microscopy. Malondialdehyde (MDA), an indicator of oxidative stress [26] was measured in homogenized lung tissues using a commercially available kit (OXIS International, Portland, OR) according to manufacturer's protocol. The results were recorded as µmol MDA/g of lung protein and are reported as fold change from control at week 0.

Mouse plasma, BAL fluid, and urine was evaluated at week 20 for lipid peroxidation (an indicator of oxidative stress) using the TBARS Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Specifically, levels of thiobarbituric acid reactive substances (TBARS) were quantified by measuring the fluorescence of malondialdehyde-thiobarbituric acid adducts in BAL fluid samples. According to manufacturer instructions, MDA-TBA adducts were formed via acid hydrolysis at 100°C and measured fluorometrically with an excitation wavelength of 530 nm and an emission wavelength of 550 nm. Levels of lipid peroxidation in plasma, BAL fluid, and urine are reported as the concentration (µM) of MDA.

Immunohistochemistry

Paraffin embedded lungs were sectioned and processed for routine immunohistochemistry as described earlier [27]. The following antibodies were used: anti-nitrotyrosine (rabbit polyclonal, a kind gift from Dr Harry Ischiropoulos, University of Pennsylvania, PA) [3]. The same antibody was also used for western blot validation of NT levels in lung homogenates. Details on the methodology are provided below.

Apoptosis detection in lung tissues by TUNEL staining

In order to identify the extent of potential cell apoptosis/necrosis as a consequence of DNA damage, we performed Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL)-staining on histological lung sections from mice exposed as described above. Lungs were excised and processed for paraffin embedding. Lung sections were deparaffinized and hydrated before blocking of endogenous hydrogen peroxide with hydrogen peroxide-methanol. TUNEL staining was performed using an ApopTag kit obtained from Oncor (Gaithersburg, MD), as previously described [28]. Data is presented as number of TUNEL- positive cells/high power field (400X). A minimum of 10 fields were measured per lung.

Apoptosis detection by western blotting

Lung tissue apoptosis was determined by caspase-3 and poly (adenosine diphosphate-ribose) polymerase (PARP) cleavage seen using immunoblotting as described earlier [29]. Briefly, lungs were homogenized in PBS containing protease inhibitors using a Dounce homogenizer. Immunoblot analysis of total lung homogenates was then performed using 10 well SDS 12% NuPAGE gel (Invitrogen, Carlsbad California). Electrophoresis was performed at 200V for 1 hour. Transfer to PolyScreen PV transfer membrane (PerkinElmer Life Sciences, Boston, MA) was performed for 1 hour at 25 volts. Membrane was blocked overnight in 5% non-fat dry milk in phosphate buffered saline. The non-fat dry milk was then discarded and the membrane was incubated with primary antibody. Cleaved caspase-3 was detected using manufacturer recommended dilutions of a rabbit anti-mouse cleaved caspase-3 (Asp175), monoclonal antibody and a rabbit polyclonal anti-cleaved PARP (214/215) cleavage site specific antibody (Cell Signaling Technology, Danvers, MA). The membrane was washed five times and then incubated in secondary antibody conjugated to horseradish peroxidase for 45 minutes at room temperature. Membranes were developed using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA) and quantified by densitometry scanning of specific bands (17/19 kDa for cleaved caspase-3 and 89 kDa for cleaved PARP) that were adjusted for loading using ß-actin expression level detected by specific secondary antibody (Sigma, St. Louis, MO). Four mouse lungs were evaluated per cohort and time-point.

Determination of active TGF-β1 in bronchoalveolar lavage fluid

Quantitative measurement of active TGF-β1 in lung BAL fluid was performed by Enzyme-linked immunosorbent assays (ELISA) according to manufacturer's recommendations (Biolegend, San Diego, CA). Specimens were run in duplicate. TGF-β1 was expressed as pg/mg protein. Assay sensitivity was 2.3 pg/mL for TGF-b1.

Evaluation of cardiopulmonary function parameters

During the late phase of the study, at 8, 12, 16, and 20 weeks post-initiation of exposure, pulse oximetry was performed prior to sacrifice on conscious mice (n=5/ group) using a MouseOx non-invasive vital signs monitor (STARR Life Sciences Corp., Oakmont, PA) as described previously [30]. A mouse collar sensor was used to obtain measurements for arterial oxygen saturation, pulse distension, breath rate, and heart rate. To minimize stress and maintain body temperature, mice were placed on a heating pad. True measurements were recorded for a minimum of 3 minutes and values reported as mean ± SEM for the total recording.

Flow cytometry

Analysis of leukocytes was performed using blood combined with EDTA obtained from anesthetized mice exposed to one cycle of exposure (8 hours hyperoxia, 0.25Gy or combination treatment followed by 16 hours of normoxia) added to test tubes containing a proprietary preservative (Cyto-Chex BCT from Streck, Inc., Omaha, NE). Assay methods were as described in a previous publication [31] using FITC-conjugated anti-mouse myeloperoxidase (MPO) (Becton Dickinson), R-phycoerythrin (PE)-conjugated anti-mouse CD41 (e-Biosciences), and Alexa 647-conjugated anti-mouse CD18 (Serotec).

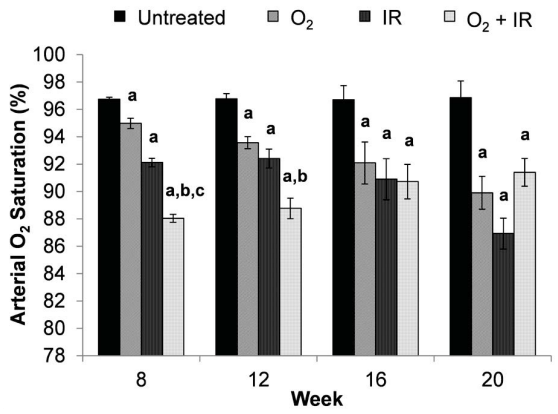

Figure 2: Kinetic study of the effect of hyperoxia, radiation, or double-hit combination on blood oxygenation.

Separate cohorts of mice (n=5-15 / group) were exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (IR) only, or a double-hit combination of both challenges (O_2+IR) followed by intermittent normoxia (21% pO_2) for repeated cycles 3 times a week for 4 weeks. Mice were sacrificed at weeks 1, 2, and 4 weeks (acute) and 8, 12,,16, and 20 weeks (chronic). Arterial blood oxygenation was determined prior to sacrifice 8, 12, 16, and 20 weeks. Data is represented as mean ± SEM. Letter a indicates p< 0.05 for all challenges vs. untreated at each respective time point. Letter b indicates p< 0.05 for O_2+IR vs. O_2 at each respective time point. Letter c indicates p< 0.05 for O2+IR vs IR at each respective time point.

Quantitative assessment of pulmonary fibrosis

Collagen content of mouse lung tissue was evaluated quantitatively by determining hydroxyproline (OH-Proline) content using acid hydrolysis [29] according to Woessner et al. [31]. The data is expressed as μg hydroxyproline/ g of lung tissue.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical differences among groups were determined using one-way analysis of variance (ANOVA) or Student's T-Test. When statistically significant differences were found (p<0.05) individual comparisons were made using the Bonferoni/Dunn test (Statview 4.0).

Results

Selected oxidative stress-inducing challenges to the lung, whether hyperoxia [17] or radiation [29] have been associated with adverse responses in the lung; however, a knowledge gap exists in that no *in vivo* model system permits the study of the combination of the two challenges. Short-duration hyperoxia (8 hours), low-level irradiation (0.25 Gy), or the combination of both challenges in mice was evaluated to simulate weekly exposures of spaceflight crewmembers during EVAs; determined and defined in accordance with the maximum allowance of no more than 24 hours of EVA per week per crewmember (Figure 1).

Changes in mouse bodyweight and arterial blood oxygenation associated with challenge conditions

Mouse cohorts exposed to challenge conditions displayed significantly lower bodyweights when compared to untreated controls (Table 1) during the early phase of the study which did not persist during the late phase. This significant (p<0.05) difference was noted at week 4 and 8 of the study.

Pulse oximetry analysis was performed at 8, 12, 16, and 20 weeks of the study. Arterial blood oxygenation was greater than 96% in untreated mice at all time points (Figure 2). All mouse cohorts exposed to O_2, IR, or O_2+IR had significantly (p<0.05) reduced oxygen saturation; 89%, 86%, and 91%, respectively at 20 weeks post-initiation of challenge

Mouse Bodyweight (g)								
	Time Point (Week)							
	0	**1**	**2**	**4**	**8**	**12**	**16**	**20**
Untreated	19.2 ± 0.1	19.6 ± 0.1	20.0 ± 0.1	21.7 ± 0.0	23.8 ± 0.3	25.7 ± 0.2	29.1 ± 0.2	30.2
O_2	19.4 ± 0.3	19.6 ± 0.2	19.8 ± 0.2	21.2 ± 0.3	**22.6 ± 0.3** [a]	24.9 ± 0.4	27.1 ± 1.1	31.3
IR	18.8 ± 0.7	19.0 ± 0.7	19.3 ± 0.5	**20.3 ± 0.3** [a]	**22.8 ± 0.4** [a]	26.1 ± 1.1	30.3 ± 5.1	37.5
O_2 + IR	18.8 ± 0.5	19.0 ± 0.5	19.1 ± 0.4	**20.1 ± 0.3** [a]	**21.9 ± 0.3** [a]	24.1 ± 1.3	29.6 ± 1.0	33.5

[a] p<0.05 for all challenges versus untreated at respective time point Values reported as mean ± standard error of the mean
Separate cohorts of mice (n=5-15 / group) were exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (IR) only, or a double-hit combination of both challenges (O_2+IR) followed by intermittent normoxia (21% pO_2) for repeated cycles 3 times a week for 4 weeks. Mice were sacrificed at weeks 1, 2, and 4 weeks (acute) and 8, 12, 16, and 20 weeks (chronic). (Panel A) Body weight was recorded weekly throughout the course of the study. At 4 and 8 weeks, mouse cohorts exposed to challenge conditions had significantly lower bodyweight compared to untreated mice at respective time points. Letter a indicates p< 0.05 for all challenges vs. untreated

Table 1: Effect of challenge conditions on mouse bodyweight.

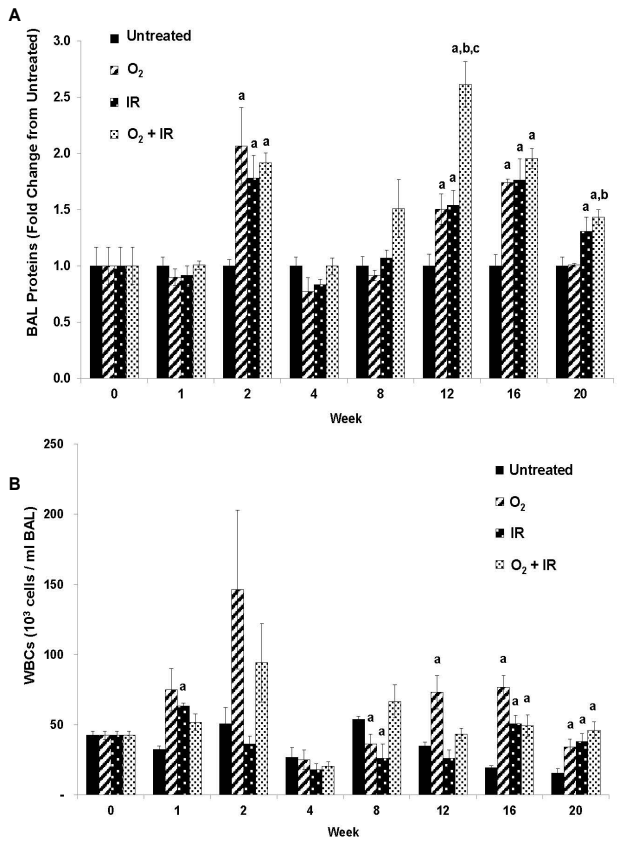

Figure 3: Evaluation of the effect of hyperoxia, radiation, or double-hit combination on lung inflammation and damage/edema.

(Panel A) Separate cohorts of mice (n=5-15 / group) were exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (IR) only, or a double-hit combination of both challenges (O_2+IR) followed by intermittent normoxia (21% pO_2) for repeated cycles 3 times a week for 4 weeks. Mice were sacrificed at weeks 1, 2, and 4 weeks (acute) and 8, 12, 16 and 20 weeks (chronic). BAL white blood cell counts were evaluated as a measure of lung injury. **(Panel B)** BAL was performed at the time of sacrifice and BAL proteins determined as a measure of lung damage.

Data is represented as mean fold change from untreated at each respective time point ± SEM. Letter a indicates $p< 0.05$ for all challenges vs. untreated at each respective time point. Letter b indicates $p< 0.05$ for O_2+IR vs. O_2 at each respective time point. Letter c indicates $p< 0.05$ for O_2+IR vs IR at each respective time point.

conditions. O_2 saturation was evaluated at later time points (>8 weeks) as we did not anticipate mice to desaturate at early stages of exposure. Statistical comparisons were made for each cohort and the respective age-matched untreated control for each time-point, as well as between the double-hit (O_2+IR) and each of the single hits (O_2 or IR).

Exposure to repeated cycles of hyperoxia, low-level total body radiation and combination challenge induces bi-phasic lung inflammation and lung injury

Pulmonary inflammation is routinely evaluated by determining inflammatory cell influx in the BAL fluid. Bronchoalveolar lavages were performed at various time points of radiation and hyperoxia exposure. We observed significant lung inflammation, determined by BAL WBC counts, in the animals exposed to all challenges for both the early and chronic phases of the study. The WBC influx was bi-phasic, occurring early and again during the chronic phase. Specifically, WBCs were significantly ($p<0.05$) elevated across mouse cohorts exposed to challenge conditions at one week of exposure and again during the late phase of the study starting at 8 weeks (Figure 3A).

Damage to lung tissues is detectable by determining the level of plasma proteins that leak into the airways. BAL fluid is analyzed for protein levels as a direct measure of vascular leakiness and lung damage. Similarly with the WBC influx shown above (Figure 3A), the lung damage (edema) also followed a bi-phasic pattern, occurring in all exposed cohorts during the early phase, i.e., at 2 weeks of exposure to challenge ($p<0.05$) and again later, starting at 12 weeks post exposure (Figure 3B) and persisted throughout the late phase of the study at week 12, 16 and 20 in mice exposed to all challenge conditions.

The inflammatory response was further characterized by determining the expression of neutrophil activation markers present on the PMN surface [32] using flow cytometry. Figure 4A shows measurements of markers of neutrophil activation and cell surface injury, obtained from mice subjected to O_2, IR or O_2+IR as compared to untreated controls. Blood cells were used for evaluation of surface expression of CD18, CD41, and myeloperoxidase (MPO). After

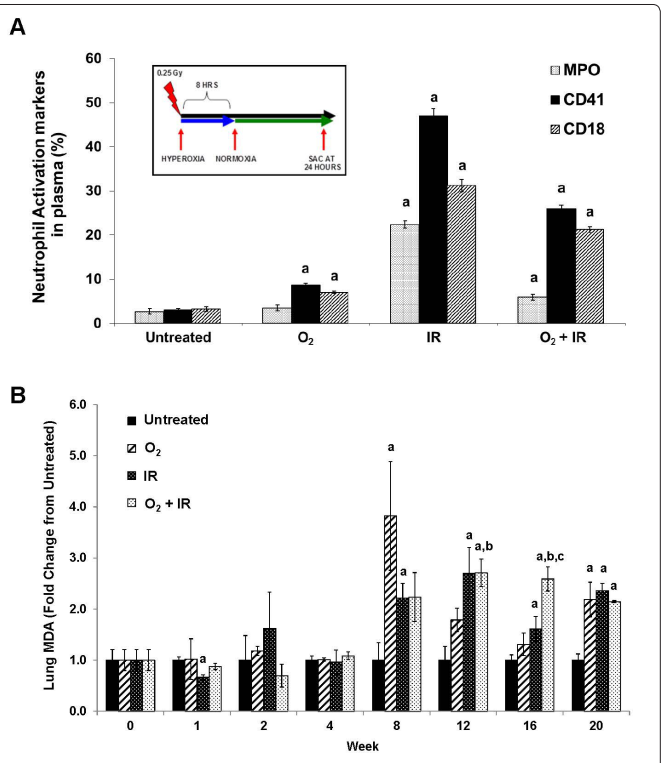

Figure 4: Kinetics of inflammatory cell activation and oxidative damage in mouse lungs after repeated cycles of O_2, IR or O_2+IR exposure.

(Panel A) Mice were exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (IR) only, or a double-hit combination of both challenges (O_2+IR) followed by 16 hours of normoxia (21% pO_2) for a single cycle. Whole blood was collected and leukocyte analysis of MPO, CD41, and CD18 was performed using flow cytometry. **(Panel B)** Separate cohorts of mice (n=5-15 / group) were exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (IR) only, or a double-hit combination of both challenges (O_2+IR) followed by intermittent normoxia (21% pO_2) for repeated cycles 3 times a week for 4 weeks. Mice were sacrificed at weeks 1, 2, and 4 weeks (early) and 8, 12, 16, and 20 weeks (chronic). Oxidative modification of lung tissues was measured by determining MDA levels in lung. Data is represented as mean fold change from untreated at each respective time point ± SEM. Letter a indicates $p< 0.05$ for all challenges vs. untreated at each respective time point. Letter b indicates $p< 0.05$ for O_2+IR vs. O_2 at each respective time point. Letter c indicates $p< 0.05$ for O_2+IR vs IR at each respective time point.

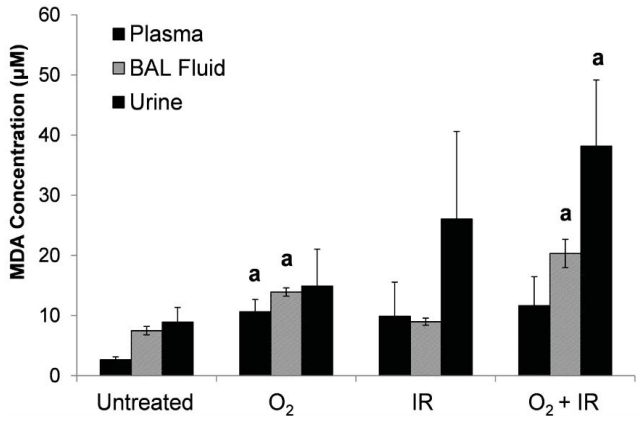

Figure 5: Determination of oxidative stress in mouse plasma, BAL fluid, and urine at week 20 post-initiation of repeated cycles of O_2, IR or O_2+IR exposure.

Separate cohorts of mice (n=5-15 / group) were exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (IR) only, or a double-hit combination of both challenges (O_2+IR) followed by intermittent normoxia (21% pO_2) for repeated cycles 3 times a week for 4 weeks. At week 20, mouse plasma, BAL fluid, and urine were collected. Oxidative modification was determined by measuring MDA levels. Data is represented as mean ± SEM. Letter a indicates $p< 0.05$ for each challenge condition cohort vs. untreated.

one cycle of exposure (8 hours hyperoxia, 0.25 Gy or combination treatment followed by 16 hours of normoxia), fluorescence intensity on the PMN cell surface on all mice was increased for CD41 (from platelet fragments), CD18 (a component of β_2-integrins), and MPO indicating intravascular PMN activation.

Oxidative stress correlates with exposure to repeated cycles of hyperoxia, low-level total body radiation and double-hit combination challenge in murine tissues

Oxidative tissue damage relevant to spaceflight has been identified and confirmed recently in both lipids and DNA [33]. Specifically, lipid peroxidation plays a major role in mediating oxidative-damage in tissues, is a qualitative indicator of oxidative stress within tissues and cells, and can be measured by determining the amount of malondialdehyde (MDA), a product of lipid peroxidation in lung tissues [24,25]. In this study, we recorded a significant ($p<0.01$) increase in lung MDA levels in mice exposed to all challenge conditions during the chronic phase, namely at weeks 8, 12, 16 and 20, as compared to unchallenged controls (Figure 4B). There was no significant increase in lung tissue MDA levels determined in the early phase of exposure (weeks 1-4). Importantly, at the late phase (20 weeks) post-initiation of radiation and hyperoxia exposure, significantly elevated systemic oxidative stress was detected by quantifying the levels of MDA in three important biological fluids; namely plasma, BAL fluid, and urine (Figure 5).

To determine whether oxidative damage at the late phase post exposure to O_2 and radiation was present in other classes of macromolecules, not just lipids (MDA) we evaluated nitrotyrosine (NT) production, a biomarker of protein nitrosative stress [34], shown to be associated with radiation [30] -and hyperoxia [35] -induced lung injury in rodent models. Lung sections were processed immunohistochemically as shown in representative lung sections in Figure 6A-D and lung homogenates by western blotting (Figure 6E and F). As anticipated, NT staining of histological lung sections revealed increased positivity in sections from hyperoxia (6B) and radiation (6C) exposed mice as compared to control, untreated (6A). Interestingly, NT positivity was significantly more intense in the double-hit lung sections (6D). Staining was identified in the alveolar wall as well as in alveolar macrophages. In addition to the qualitative immunohistochemical detection of NT, we performed semi-quantitative western blot validation (Figure 6E) followed by densitometric analysis (6F) (n=4 mice per group). This confirmed the immunohistochemical NT profile of lungs, indicating a robust, and statistically significant 2.04-, 3.01 and 3.3-fold increase of NT as compared to untreated control, for O_2,

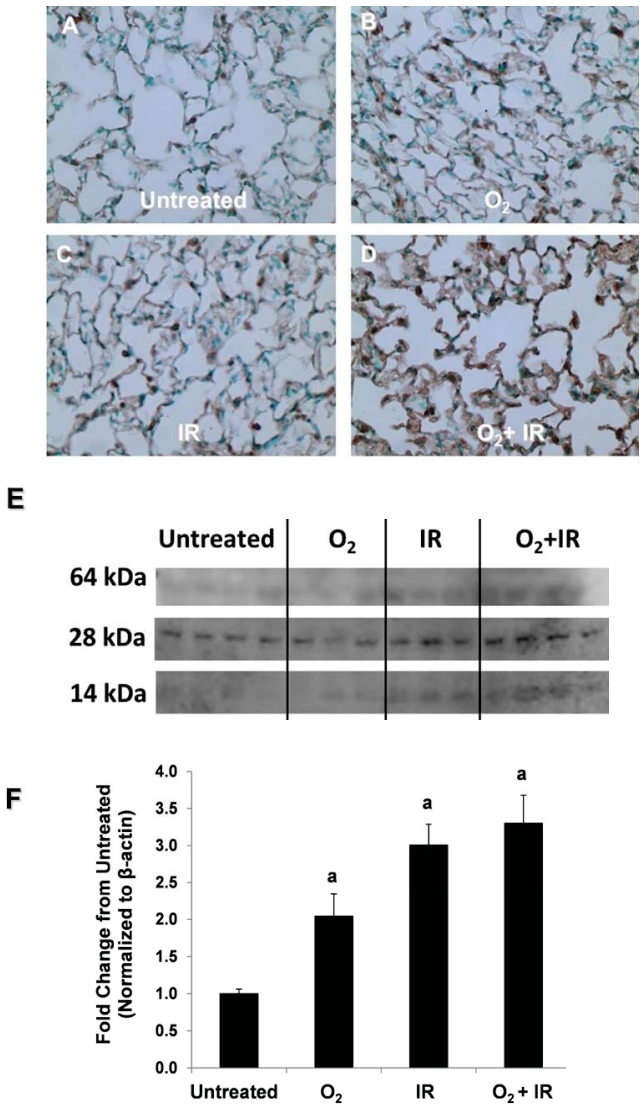

Figure 6: Effects of repeated O2, IR and combined challenge on Nitrotyrosine formation, during the late phase post termination of the challenge exposure.

Separate cohorts of mice were exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (IR) only, or a double-hit combination of both challenges (O_2+IR) followed by intermittent normoxia (21% pO_2) for repeated cycles 3 times a week for 4 weeks. Mice were sacrificed at 20 weeks (chronic). **Panels A-D** Representative sections from paraffin lung sections stained for nitrotyrosine (brown staining) from untreated (A), O2 (B), IR (C) and O_2+IR (D) animals. Sections were counterstained with crystal blue. **Panel E** Representative western blot validation of NT formation in lung homogenates from the same mouse cohorts as above. **Panel F** Densitometric analysis with beta actin normalization of the combined levels of all 3 major NT bands for each cohort. Data is represented as mean fold change from untreated at each respective time point ± SEM. Letter a indicates $p< 0.05$ for all challenges vs. untreated at each respective time point.

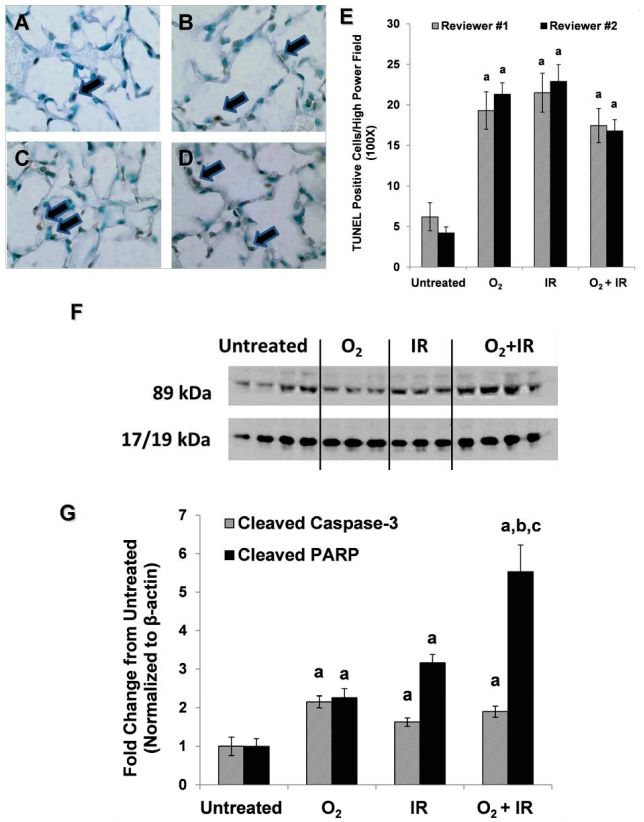

Figure 7: Determination of oxidative DNA damage using TUNEL staining on lung sections at various times post-initiation of repeated cycles of O2, IR or O2+IR exposure.

Panels A-D Histological lung sections of representative animals from each cohort (O2, IR, O2+IR) processed for TUNEL positivity (late phase, 20 weeks). Negative controls (performed simultaneously but without TdT) were examined at the same time. **Panel E** Cells were counted as % positive cells per high power field (400X) in 10 fields per condition. Data is represented as mean ± SEM. $p< 0.05$ for each challenge condition cohort vs. untreated as determined by two independent reviewers. **Panel F** Western blot for cleaved caspase 3 and cleaved PARP. **Panel G** Densitometric analysis of representative blot. Data is represented as mean fold change from untreated at each respective time point ± SEM. Letter a indicates $p< 0.05$ for all challenges vs. untreated at each respective time point. Letter b indicates $p< 0.05$ for O_2+IR vs. O_2 at each respective time point. Letter c indicates $p< 0.05$ for O_2+IR vs IR at each respective time point.

IR and O_2+IR, respectively. Three distinct bands were detected (6E) representing groups of nitrated proteins. The graphed data represents the combined expression levels of all 3 bands for each cohort.

Apoptotic cell death in lung tissues, associated with late phase post exposure to hyperoxia, radiation and the combination of the two challenges

TUNEL positivity (Figure 7A-E) is widely accepted as an indication of apoptotic cell death and is associated with radiation exposure [36] and hyperoxia [37]. TUNEL staining was increased 20 weeks (late phase) in lungs of mice exposed to each condition as compared to untreated controls confirming that repeated exposure to hyperoxia, radiation or the combination of the two causes damage detectable even months post termination of the challenge.

Using western blot analysis of lungs exposed to O_2, IR or O_2+IR, the processed form of poly (ADP-ribose) polymerase (PARP), which is among the characteristic substrates of caspase-3 and caspase-7 activated during apoptosis was expressed at higher levels than in untreated control lungs 20 weeks (chronic phase) after exposure. Significant expression increase for cleaved Caspase 3 (2.15-, 1.63- and 1.9-fold increase) and cleaved PARP (2.26-, 3.16- and 5.53-fold increase) as compared to untreated control, for O_2, IR and O_2+IR, respectively (Figure 7F and G) confirmed that such challenge conditions induce apoptosis in lung associated with the activation of caspase-3 detectable even as late as 20 weeks post exposure.

Fibrotic lung changes in mice exposed to repeated cycles of hyperoxia, low-level total body radiation and double-hit combination challenge

Single or fractionated exposure of lung tissues to ionizing radiation [25] as wells as to high oxygen levels [27] is associated with excess deposition of extracellular collagen fibers implicated in fibrotic lung changes. Pulmonary fibrosis resulting from repeated low-level radiation challenge, in combination with hyperoxic exposure, however, has never been demonstrated in animal model systems. To test this, we evaluated the total hydroxyproline content of murine lungs at 1, 2, 4, 8, 12, 16, and 20 weeks post-exposure to challenge (Figure 8). Analysis of hydroxyproline content in the lung is a quantitative measure of irreversible collagen deposition and fibrosis, which is expressed as µg of hydroxyproline/lung. We selected to include an untreated group of mice for each time-point, so that we would have an age-matched cohort for each selected time of observation. This is important since the levels of collagen increase as mice age and thus a comparison to time "0" would not reveal meaningful information. As early as 1 week (3 cycles of repeated challenge) post treatment, mice exposed to the combination of hyperoxia and radiation led to a significant ($p<0.05$) increase in hydroxyproline content in mouse lung tissue in comparison to unchallenged controls. Significant differences ($p<0.05$) in collagen levels were detectable in the lungs of mice exposed to IR only after 4 weeks of repeated exposure (12 cycles of challenge). Notably, at 8 weeks post-initiation of exposure, increased collagen ($p<0.05$) was detected in mouse lungs exposed to all challenge conditions. Although fibrotic

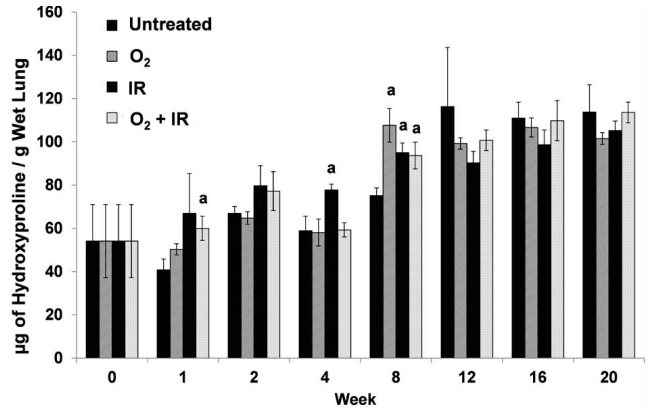

Figure 8: Kinetics of pulmonary fibrotic changes in mouse lungs after repeated cycles of O_2, IR or O_2+IR exposure.

Separate cohorts of mice (n=5-15 / group) were exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (IR) only, or a double-hit combination of both challenges (O_2+IR) followed by intermittent normoxia (21% pO_2) for repeated cycles 3 times a week for 4 weeks. Mice were sacrificed at weeks 1, 2, and 4 weeks (acute) and 8, 12, 16 and 20 weeks (chronic). Lung fibrosis was determined by measuring lung hydroxyproline content. Data is represented mean ± SEM. Letter a indicates $p< 0.05$ each challenge cohort as it compares with the untreated control at the respective time point.

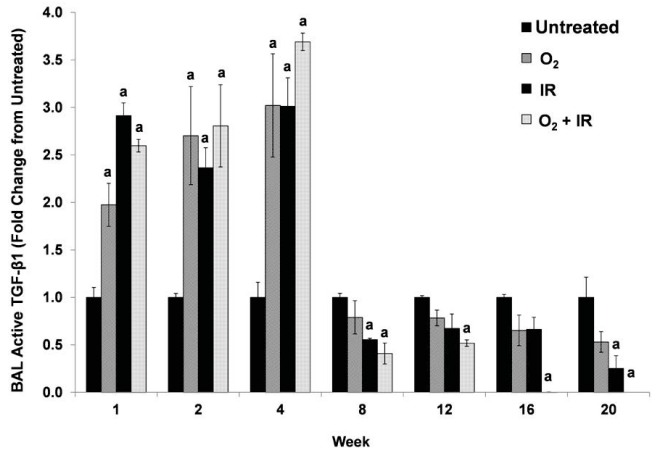

Figure 9: Determination of Active TGF-β1 levels in mouse BAL fluid at various times post-initiation of repeated cycles of O_2, IR or O_2+IR exposure.

Separate cohorts of mice (n=5-15 / group) were exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (IR) only, or a double-hit combination of both challenges (O_2+IR) followed by intermittent normoxia (21% pO_2) for repeated cycles 3 times a week for 4 weeks. At 1,2,4,8,12,16 and 20 weeks post exposure, BAL fluid were collected. Active TGF-β1 levels were determined by ELISA. Data is represented as mean ± SEM. Letter a indicates $p< 0.05$ for each challenge condition cohort vs. untreated.

changes in lung tissues were significant as measured by the chemical analysis of total hydroxyproline content, upon histological analysis of lung fibrosis with Mason's trichrome blue staining (data not shown), mild and not readily visible differences were noted.

To determine whether the detected fibrotic changes in lung during the early phase post exposure are associated with secretion of pro-fibrogenic cytokines, the levels of active TGF-β1 were determined in the BAL fluid of mice at all time points (n=4 per time point) using ELISA (Figure 9). Significant elevation ($p<0.05$) of active TGF-β1 was detected in all cohorts as compared to untreated controls, during the duration of the exposure phase (1-4 weeks) to each challenge. Levels dropped to baseline and even below that, for all time points thereafter, confirming the hydroxyproline findings and the trichrome-blue staining.

Discussion

We have identified oxidative lung damage associated with systemic effects, such as impaired blood oxygenation and systemic oxidative stress, related to repeated radiation and hyperoxia exposure. Using a novel *in vivo* model system of combined repeated exposure to low-level total body γ-irradiation and hyperoxia, we attempted to recreate conditions relevant to those of a pre-breathe protocol that crewmembers may be subjected to prior to an EVA. We have identified by looking both early and at later times, increased bronchoalveolar lavage protein levels in all treatment groups pointing to the development of significant lung tissue injury. Importantly, significant levels of pulmonary inflammation was noted in all treatment groups of single and combined exposure, that was associated with increases in activation markers on the surface of PMN. Although oxidative tissue damage was persistent at all time points that we evaluated, fibrotic changes in lung were transient, occurring only during the early phase. We report here for the first time a novel model of repeat hyperoxia and radiation exposure that identify a new risk relative to space exploration.

The information presented here by our group is quite unique and applicable to ongoing research in human space missions. While prior studies [17] have characterized the effects whereby hyperoxia alone or radiation alone affects pulmonary tissues, no research has investigated the dual role of both exposures. Additionally, models designed to test that complex interaction on a tissue and cellular molecular level are unique to our group. Research in the field of space travel and changes in the anatomy, physiology, and genome of animal tissues is limited and its planned application to the propagation of manned space travel is unique and appealing.

Our protocol was engineered to approximate potential conditions crewmembers could face during EVAs typically limited by NASA to 6-10 hours 3 times weekly but not to exceed 24 hours in a week. In the field of radiation protection, such a stipulation is grouped under the name of ALARA (As Low As Reasonably Achievable) Principle [9,38-41]. Where appropriate, these steps take into account possible countermeasures, which can generally be classified as avoidance, mitigation, and intervention. Readers may think that there is a degree of arbitrariness when establishing radiation limits. In reality, of course, the term refers to the flexibility allowed in good radiation protection practice to ensure that radiation limits are never exceeded and held to an achievable minimum. Dose relationship studies [42] on rodent and other mammalian tissues from low dose fractionation (250 cGy over 9 weeks) to sub-lethal several Gy doses revealed wide ranges of biologic responses [43] ranging from temporary, reversible damage to death in a broad spectrum of tissues. The decision to use 0.25 Gy in 3 to 12 doses could have put the exposure levels into the range seen in studies by Kennedy and Ainsworth, where high dose low fraction number and low dose high fraction number induced notable changes including permanent neurologic impairment to decreases in serum antioxidants [44]. Furthermore, studies by Ainsworth [45] showed that a single dose of 0.3 Gy caused radiologic changes to femurs and depressed counts of platelets and other hematopoietic progenitor cells in mouse neonates.

It has been reported in the literature that repetitive hyperoxia exposure associated with changes in oxygen levels results in periodic generation of free radical signals which in turn triggers induction of antioxidant enzyme protein synthesis that induces important adaptations, according to Conchar and Mankovska, 2012 [46]. Rocco et al. [47] in a recent paper, made a similar observation relevant to repeated changes in oxygen availability as a trigger to induce HIF-1a and subsequent gene regulation. This is referred to the "normobaric oxygen paradox". Tissues, sense the return to normoxia after hyperoxia as a "shortage of oxygen" and induce HIF-1-regulated gene synthesis associated with an increase of matrix metalloproteinases activity which results in decreased fibrosis. This may in part explain the abrogation of lung fibrosis seen after the challenge was terminated. In Figure 10, PMN activation in the double hit cohort appears significantly less than that of IR alone suggesting that perhaps O_2 may be protective. It is important to note, however, that the data reflect just one 24-hour cycle of hyperoxia exposure, and not repeated cycling levels of O_2, so it is unlikely that adaptation responses were triggered. Additional experiments are needed to confirm the involvement of HIF-1α and to confirm that indeed O_2 may mitigate the effects of radiation. The observation that O_2 may provide some level of protection to the irradiated tissues would have been valid if it was persistent across multiple parameters of damage, which is not supported by the data presented here.

We recognize the limitations of our experimental design and realize our choice of radiation (in and of itself) is but one type of galactic cosmic radiation and may not be completely representative of what the astronauts may experience. Radiation applied through a shielding material yields additional energy that scatters through tissues of different densities at different rates with different outcomes. The order

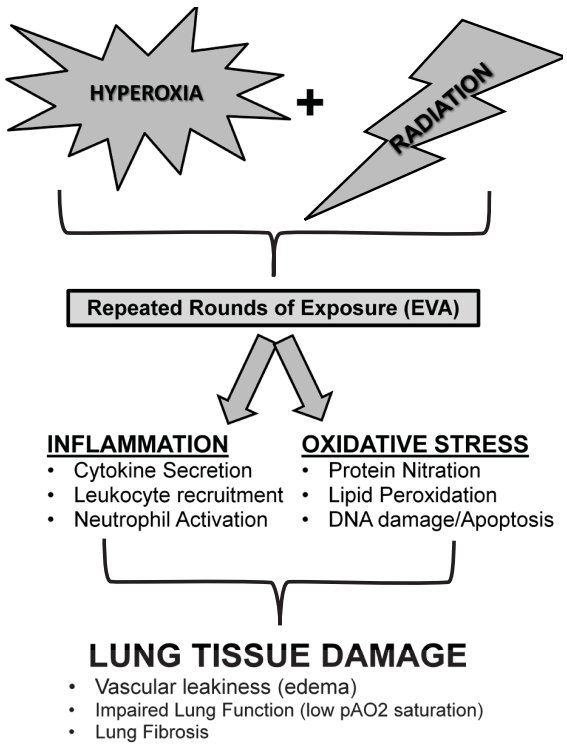

Figure 10: Mechanism of damage in mouse lungs after repeated cycles of O_2, IR or O_2+IR exposure.

of exposure to the combined treatments of hyperoxia and radiation may not be analogous to what the astronauts would experience; but our study aimed to identify possible adverse effects of cycling changes of in the inspired partial pressure of oxygen in combination with a radiation exposure effect. In our model, mice were irradiated with a constant 0.25 Gy 3 times per week for 1 to 4 weeks, then transferred to their hyperoxic chambers under normal gravity conditions for 8 hours, followed by 16 hours of normoxia. In contradistinction, astronauts undergo a "pre-breathing" protocol prior to EVA then while inhaling 100% oxygen in a zero gravity environment in non-uniform, non-standardized fashion, based on probabilities, the astronaut is exposed to GCR and/or SPE during the 6-8 hours of the scheduled or unscheduled extravehicular activity. A complex "triple-hit" occurs in reality with changes in pressure, changes in inhaled oxygen content, and potential radiation exposure – the interaction of which could be a model for future experiments using more space-relevant radiation sources [14]. Indeed, the effects of cycling barometric pressure and inspired partial oxygen pressure have long been recognized as being relevant in causing adverse effects in lung tissues [48].

Conclusion

We have helped bridge the knowledge gap in terms of pulmonary complications from space travel through the development and testing of a novel *in vivo* model system of combined exposure to total body γ-radiation and hyperoxia. Pulmonary complications from space travel could include inflammation, fibrosis, oxidative tissue damage and apoptotic cell death. In conclusion, we have identified lung injury associated with prolonged space travel using a novel murine model of double-hit repeated low-level radiation and hyperoxia exposure.

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