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Flaxseed Mitigates Acute Oxidative Lung Damage in a Mouse Model of Repeated Radiation and Hyperoxia Exposure Associated with Space Exploration

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Abstract

Background: Spaceflight missions may require crewmembers to conduct extravehicular activities (EVA). Pre-breathe protocols in preparation for an EVA entail 100% hyperoxia exposure that may last for a few hours and 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. We have developed a mouse model of total body radiation and hyperoxia exposure and identified acute damage of lung tissues. In the current study we evaluated the usefulness of dietary flaxseed (FS) as a countermeasure agent for such double-hit exposures.

Methods: We evaluated lung tissue changes 2 weeks post-initiation of exposure challenges. Mouse cohorts (n=5/group) were pre-fed diets containing either 0% FS or 10% FS for 3 weeks and exposed to: a) normoxia (Untreated); 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) 3 times per week for 2 consecutive weeks, where 8-hour hyperoxia treatments were spanned by normoxic intervals.

Results: At 2 weeks post challenge, while control-diet fed mice developed significant lung injury and inflammation across all challenges, FS protected lung tissues by decreasing bronchoalveolar lavage fluid (BALF) neutrophils ($p<0.003$) and protein levels, oxidative tissue damage, as determined by levels of malondialdehyde (MDA) ($p<0.008$) and nitrosative stress as determined by nitrite levels. Lung hydroxyproline levels, a measure of lung fibrosis, were significantly elevated in mice fed 0% FS ($p<0.01$) and exposed to hyperoxia/radiation or the combination treatment, but not in FS-fed mice. FS also decreased levels of a pro-inflammatory, pro-fibrogenic cytokine (TGF-ß1) gene expression levels in lung.

Conclusion: Flaxseed mitigated adverse effects in lung of repeat exposures to radiation/hyperoxia. This data will provide useful information in the design of countermeasures to early tissue oxidative damage associated with space exploration.

Keywords: Bronchoalveolar lavage; Double-hit; extravehicular activity; Hyperoxia; Inflammation; Lung fibrosis; Acute lung injury; Mouse model; Nitrosative stress; Oxidative stress; Radiation pneumonopathy; Space exploration; TGF-β1; Total body irradiation

Abbreviations: BALF: Bronchoalveolar Lavage Fluid' ELISA: Enzyme-linked Immunosorbent Assays; EVA: Extravehicular Activity; FI: Fibrotic Index; TBI: Total Body Irradiation; LP: Lipid Peroxidation; MDA: Malondialdehyde; NT: Nitrotyrosine; PMN: Polymorphonuclear Leukocyte; ROS: Reactive Oxygen Species; SEM: Standard Error Means; WBC: White Blood Cells; DCI: Decompression Illness

Introduction

Extravehicular activities (EVAs) for assembly and maintenance activities at the International Space Station (ISS) [1], or EVAs during interplanetary missions expose astronauts to challenges that may pose potential health risks [2-5]. Studies such as the MATROSHKA experiment [6,7] was designed to determine, evaluate and if possible even foresee, as accurately as possible the radiation exposures that crew members will experience during such an EVA in order to minimize risks and to establish exposure limits. In addition to radiation exposure dangers, in order to prevent decompression illness (DCI), crewmembers undergo pre-breathing protocols that insure total body nitrogen stores are depleted prior to decompression. EVA risk is thus 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 [8]. In addition, exposure to a unique spectrum of radiation including galactic cosmic radiation (GCR) and solar particle events (SPE) [9,10] adds an additional environmental exposure in space that may affect lung tissue primed by hyperoxia during or after an EVA. Well-established flight rules ensure that acute oxygen toxicity is avoided, however, the long term effects on lung function of multiple EVAs and ensuing cyclic exposures to hyperoxic conditions is not known.

In a recent study, Pietrofesa et al. [11] developed a novel in vivo rodent model system of combined repeated total body irradiation (IR) delivered in the form of γ-radiation and hyperoxia (>95% O_2) aimed to identify possible pulmonary complications associated with EVA-relevant exposures. While astronauts in reality may experience a much more complex interplay of cyclic "triple-hit" of hypobaricto-hyperbaric, hypoxic-to-normoxic-to-hyperoxic, and irradiation exposures the double hit radiation/hyperoxia model used in the study provided a useful hypothesis-testing model to identify potential harmful effects and to test countermeasures. The study successfully

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identified pulmonary complications which included inflammation, fibrosis, oxidative tissue damage and apoptotic cell death.

We have shown that FS decreased radiation-induced inflammation and oxidative stress in micein different clinically-related scenarios [12,13]. Importantly, dietary FS mitigated thoracic radiationinduced pneumonitis and fibrosis in experimental rodent models [14]. It has been reported, that the protective effects of FS against various types of cancer such as breast [15], prostate [16], and colon cancer [17] are attributed to the presence of plant lignans. FS is the richest known source of the mammalian lignan precursor, secoisolariciresinol diglucoside (SDG). As a plant phenolic, SDG was shown in vitro to have direct hydroxyl radical scavenging properties and to inhibit lipid peroxidation [18-20]. Flaxseed lignan SDG has been reported to provide potential health benefits in several disease conditions (for review see [21]) related to hypercholesterolemia [22], diabetes [23], postmenopausal symptoms [24,25], cardiovascular health [26], metabolic syndrome and bone health [27] and other diseases. Our group has performed extensive research in characterizing the beneficial effects of FS and its lignan component in experimental models of Acute Respiratory Distress Syndrome (ARDS), Ischemia-Reperfusion Injury (IRI), radiation pneumonopathy and hyperoxia [12,13,28,29]. In the current study, using the mouse model of combined effects of repeated exposures to hyperoxia and low-level total body radiation under controlled atmospheric conditions, we evaluated dietary administration of whole grain flaxseed (FS) as a countermeasure to the identified deleterious changes in lung tissue such as inflammation and oxidative/nitrosative damage.

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 [30] and pulmonary radiation damage [31,32]. 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 (Semipurified 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). Mice were exposed 3 times a week for 2 weeks and sacrificed for evaluation (Figure 1).

Diets and dietary treatments

Semi-purified AIN-93G diet was used as the base diet which was supplemented with 10% (w/w) FS as described in our previous publications [33-35]. Control and experimental diets were isocaloric and identical in physiological fuel value.

Analytical evaluation of lignan content in murine plasma

Circulating plasma levels of the flaxseed lignans ED and EL at time of sacrifice were determined by liquid chromatography/tandem mass spectrometry (LC/MS/MS) as described earlier [14,36] using commercially available standards in 95% purity (Chromadex, Inc., Santa Ana, CA). Plasma flaxseed lignan metabolite levels were evaluated in 3 randomly selected mice per cohort.

Radiation Exposure of Mice

Mouse irradiation was performed with a Gammacell 40 137Cs irradiator (Atomic Energy of Canada Limited, Canada) as described in our previous work [11]. 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 shamcontrols.

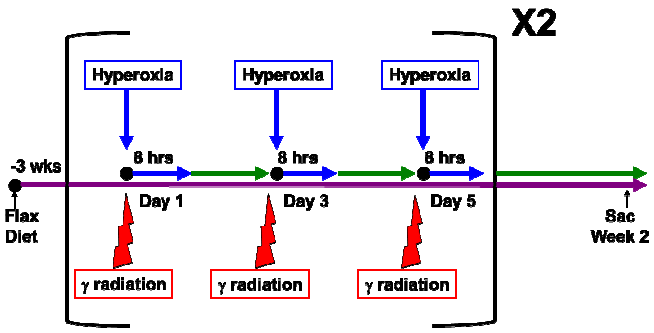

Figure 1: Experimental Plan of *In Vivo* Animal Exposure. Mouse cohorts (n=5/group) were fed 0% or 10% FS diet for 3 weeks prior to exposure and remained on test diets throughout the course of the study. Mice were exposed to a double-hit challenge of 0.25 Gy total body ionizing gamma irradiation (IR) and 100% pO_2 for 8 hours followed by intermittent normoxia (21% pO_2). This cycle was performed 3 times a week for 2 weeks. Mice were sacrificed after 2 weeks of challenge exposure. A total of 24 hours of hyperoxia/radiation exposure weekly simulates the maximum allowed weekly extravehicular activity (EVA) exposure for crewmembers on lengthy spaceflights.

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). 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 [11] 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% [37] 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 2 weeks (Figure 1).

Bronchoalveolar lavage fluid analysis

Mice were sacrificed using an overdose of ketamine (160 mg/ Kg) and xylazine (25 mg/Kg) at 2 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 [13,14,36]. 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 cellfree 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.

Gene expression analysis by real-time qPCR

Real-time quantitative Polymerase Chain Reaction (qPCR) was performed using a TaqMan® Probe-Based Gene Expression Assay for transforming growth factor-beta1 (TGF-ß1) supplied by Applied Biosystems, Life Technologies (Carlsbad, CA). Briefly, total RNA was isolated from lung tissue of mice fed 0% FS or 10% FS and exposed to challenge conditions using a commercially available kit: RNeasy Plus Mini Kit, supplied by Qiagen (Valencia, CA). Total RNA was quantified using a NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA). Reverse transcription of RNA to cDNA was then performed on a Veriti® Thermal Cycler using the high capacity RNA to cDNA kit supplied by Applied Biosystems, Life Technologies. qPCR was performed using 25 ng of cDNA per reaction well on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Gene expression data was normalized to 18S ribosomal RNA and was calibrated to untreated 0% FS samples according to the ΔΔCT method.

Determination of BALF TGF-β) and IL-1ß

Levels of pro-inflammatory cytokines, interleukin-1 beta (IL-1ß) and tumor necrosis factor alpha (TNF-α), were determined in BALF after 2 weeks of challenge exposure using enzyme-linked immunosorbent assays (ELISA). ELISA assay kits were purchased from BD biosciences (BD OptEIA Mouse TNF-α and IL-1ß ELISA Kit). BALF samples were run undiluted and assay was performed according to manufacturer's instructions.

Determination of plasma and BALF nitrite concentration

Levels of nitrite were determined in mouse plasma and BALF using the Greiss Reagent System supplied by Promega (Madison, WI). The Greiss Reagent System determines nitrite (a stable breakdown product of nitric oxide) concentration in biological specimens. BALF and plasma samples were run undiluted and assay was performed according to manufacturer's instructions.

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 withhematoxylin and eosin (H&E) and Mason's Trichrome Blue and examined by light microscopy. Malondialdehyde (MDA), an indicator of oxidative stress [38] 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.

Mouse plasma and BAL fluid were evaluated at 2 weeks post exposure 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 BAL fluid are reported as the concentration (µM) of MDA and in lung tissues as µM/g lung tissue [11].

Determination of active TGF-β1 in bronchoalveolar lavage fluid

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

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

Challenges associated with space travel, whether hyperoxia [30] or radiation [39], induce architectural lung tissue changes and lung oxidative tissue damage [11]. We have previously developed a murine model that permits the study of the combination of the two challenges and in the current study we determined the usefulness of wholegrain FS in preventing the lung damaging effects associated with such exposures. Mice were fed 0% or 10% FS and exposed to short-duration hyperoxia (8 hours), low-level irradiation (0.25 Gy), or the combination of both challenges. We evaluated the usefulness of wholegrain FS in this murine model system after 2 weeks of challenge exposure as we have previously observed significant acute lung injury and inflammation (Figure 1).

Detection of the mammalian lignans, enterodiol and enterolactone, in mice fed wholegrain flaxseed

Wholegrain FS is an abundant source of the plant lignan secoisolariciresinol diglucoside (SDG). Following ingestion, SDG is converted by intestinal bacteria to the mammalian lignin enterodiol (ED) and enterolactone (EL), which then enter the circulation and can be detected in plasma. Mice were pre-fed 0% or 10% FS for 3 weeks prior to challenge exposure and for the duration of challenge exposure. After 2 weeks of exposure, we determined plasma levels of ED (Figure 2A) and ED (Figure 2B). We detected significantly high plasma levels of the mammalian lignans in all mouse cohorts fed 10% FS. Levels of ED and EL were below the lower limit of detection in plasma from all mouse cohorts fed 0% FS. Compared to untreated mice fed 10% FS (ED=1991.1nM; EL=536.3nM), mouse cohorts exposed to O_2, IR, and O_2+IR displayed plasma enterodiol levels ranging from 58.9-1066.21 nM and plasma enterolactone levels ranging from 118.21-583.4 nM. We observed no significant changes in mouse bodyweight after 2 weeks of challenge exposure (data not shown).

Lung inflammation associated with exposure to repeated cycles of hyperoxia, low-level total body radiation and combination challenge is improved by wholegrain flaxseed

Pulmonary inflammation is routinely evaluated by determining inflammatory cell influx inthe BAL fluid. After 2 weeks of exposure, mice fed 0% FS has significant lung inflammation as determined by PMN cell counts in bronchoalveolar lavage fluid (BALF). While no PMN cells were detected in untreated mice fed 0% FS, all challenges induced significant PMN influx ($p<0.05$) in mice fed 0% FS (968, 1401, and 467 PMN cells per ml BALF for O_2, IR, and O_2+IR respectively) (Figure 3A). Notably, PMN cells were entirely absent in BALF from mice fed 10% FS. Alternatively, no significant differences were determined in the total number of white blood cells in BALF and in BALF protein levels across all study cohorts (data not shown).

The inflammatory response was further characterized by determining the levels of proinflammatory cytokines, interleukin-1 beta (IL-1ß) and tumor necrosis factor alpha (TNF-α), in BALF after

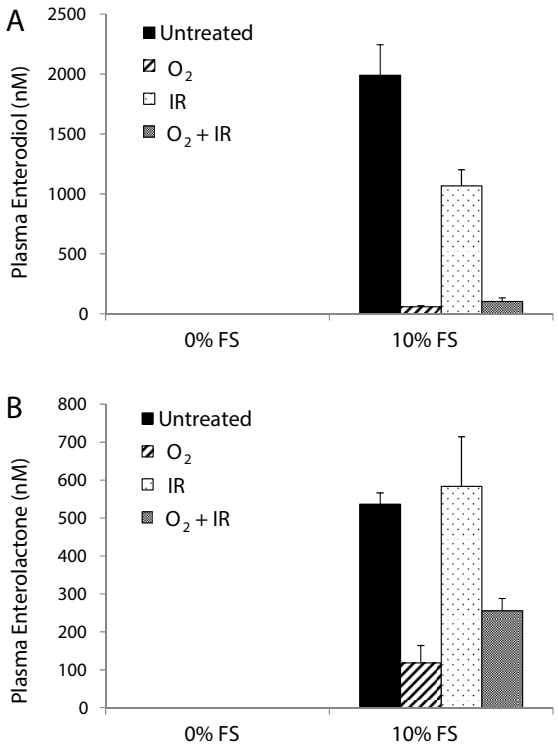

Figure 2: Detection of Mammalian Lignans in Mouse Plasma. Separate cohorts of mice (n=5/group) were fed 0% or 10% FS diet and exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (IR) only, or a double-hit combination of both challenges (O_2+IR) followed by intermittent normoxia (21% pO_2) for repeated cycles 3 times a week for 2 weeks. Mice were sacrificed after 2 weeks of challenge exposure and plasma analyzed for levels of circulating mammalian lignans enterodiol (Panel A) and enterolactone (Panel B) using GC/MS/MS. Data is represented as mean ± SEM.

2 weeks of challenge exposure using enzyme-linked immunosorbent assays (ELISA). Exposure to challenge conditions led to significant increases ($p<0.05$) in the levels of IL-1ß in mice fed 0% FS (Figure 3B). 10% FS significantly reduced levels of IL-1ß by 39, 47, and 66% in cohorts exposed to O_2, IR, and O_2+IR respectively. Similarly, elevated levels of BALF TNF-α in mouse cohorts fed 0% FS and exposed to all challenge conditions were significantly ($p<0.05$) blunted by 10% FS (77, 78, and 96% reduction in 10% FS fed mice exposed to O_2, IR, and O_2+IR respectively, compared to mouse cohorts fed 0% FS) (Figure 3C).

Flaxseed reduces hyperoxia and radiation induced oxidative stress in murine lung tissue

Oxidative tissue damage relevant to spaceflight has been identified and confirmed recently in both lipids and DNA [40]. Specifically, lipid peroxidation plays a major role in mediating oxidativedamage 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 [14,36]. In the current study, we determined significant ($p<0.05$) increases in MDA concentration in both lung tissue (Figure 4A) and BALF (Figure 4B) of mice fed 0% FS and exposed to all challenge conditions. Additionally, we determined no significant increase in lung tissue MDA concentration over untreated control in mice fed 10% FS and exposed to O_2 or O_2+IR. Furthermore, BALF MDA concentrations were significantly reduced by 52, 43, and 37% in mice fed 10% FS and exposed O_2, IR, and O_2+IR, respectively, relative to 0% FS fed mouse cohorts.

Hyperoxia, low-level total body radiation and double-hit combination challenge induce acute elevations in nitrosative stress that is improved by wholegrain flaxseed

We further explored the significant elevation in oxidative stress and oxidative tissue damage observed in mouse cohorts exposed to challenge conditions by determining the presence of nitrosative stress in both BALF and plasma. Acting along with reactive oxygen species,

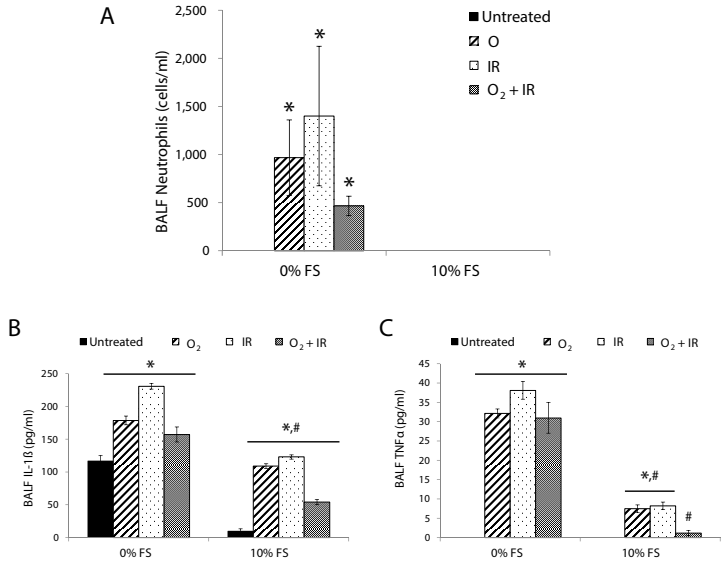

Figure 3: Flaxseed ameliorates increased Hyperoxia, Radiation, or Double-Hit Combination Challenge Induced Lung Inflammation and Proinflammatory Cytokine Release. Separate cohorts of mice (n=5/group) were fed 0% or 10% FS diet and exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing 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 2 weeks. Mice were sacrificed after 2 weeks of challenge exposure. BALF was evaluated for total neutrophil cell counts (Panel A) and levels of pro-inflammatory cytokines, IL-1ß (Panel B) and TNFα (Panel C). Data is represented as mean ± SEM. *$p< 0.05$ for O_2, IR, and O_2+IR versus respective untreated control mice in each diet cohort. #$p< 0.05$ for respective 0% FS fed mice exposed to O_2, IR, and O_2+IR versus 10% FS fed mice exposed to O_2, IR, and O_2+IR.

reactive nitrogen species are generated upon the reaction of nitric oxide and superoxide. In the current study, we determined the levels of BALF and plasma nitrite, which is a stable and measurable breakdown product of nitric oxide. BALF nitrite was significantly ($p<0.01$) elevated 10.7, 8.8, and 9.5 fold over untreated in mouse cohorts fed 10% FS and exposed to O_2, IR, and O_2+IR, respectively (Figure 5A). Additionally, 0% FS fed mice exposed to hyperoxia only displayed a significant ($p<0.05$) elevation in plasma nitrite concentration (Figure 5B). Levels of BALF and plasma nitrite were significantly ($p<0.05$) reduced in mouse cohorts exposed to all challenge conditions and fed 10% FS.

Acute fibrotic lung changes in mice exposed to repeated cycles of hyperoxia, low-level total body radiation and double-hit combination challenge are mitigated by flaxseed

Single or fractionated exposure of lung tissues to ionizing radiation [41] as well as to high oxygen levels [42] is associated with excess deposition of extracellular collagen fibers implicated in fibrotic lung changes. We have previously determined pulmonary fibrosis resulting from repeated lowlevel radiation challenge, in combination with hyperoxic exposure that was associated with acute elevations in BALF levels of active TGF-ß1, a pro-fibrogenic cytokine. To first test this, we evaluated the total hydroxyproline content of murine lungs after 2 weeks of challenge exposure (Figure 6A). 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. Untreated mice fed 0% FS had hydroxyproline levels equivalent to 73.2 µg/g of lung tissue. Mice exposed to O_2, IR, and O_2+IR had significantly ($p<0.05$) increased levels of hydroxyproline equal to 92.6, 116.1, and 114.8 µg/g of lung tissue; a 26.5, 58.6, and 56.9% increase from control. Alternatively, levels of hydroxyproline in mouse cohorts fed 10% FS were significantly lower than the respective mouse cohorts fed 0% FS. Compared to the average level of hydroxyproline (54.7 µg/g of lung) in untreated mice fed 10% FS, mouse cohorts exposed to O_2, IR, and O_2+IR had comparable levels of hydroxyproline equal to 56.7, 69.5, and 56.3 µg/g of lung tissue.

We explored this observation of increased collagen deposition in lung tissue by evaluating lungspecific mRNA changes in transforming growth factor-beta1 (TGF-ß1), which is a key activator of tissue repair and the synthesis of extracellular matrix proteins (Figure 6B). We determined a significant ($p<0.05$) 1.61- and 1.55-fold induction of TGF-ß1 over control in mice fed 0% FS and exposed to hyperoxia or radiation, respectively. Notably, the double-hit challenge of hyperoxia

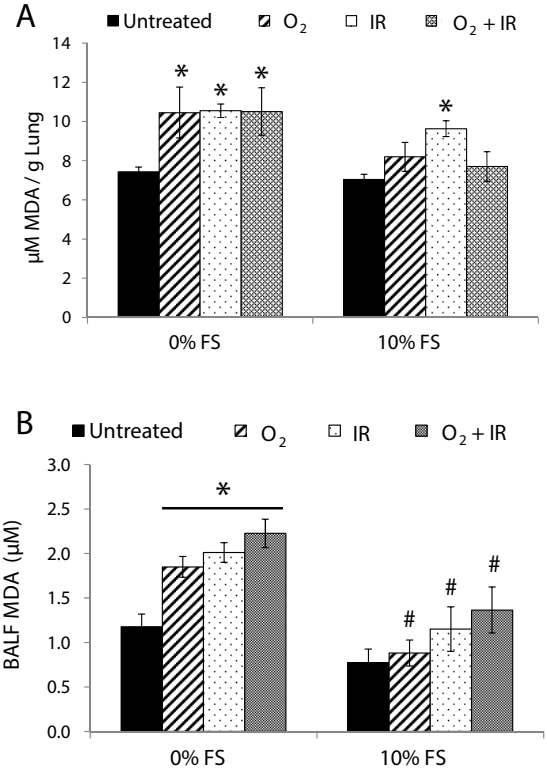

Figure 4: Flaxseed mitigates increased Oxidative Stress in Lung Tissue and BALF due to Hyperoxia, Radiation, or Double-Hit Combination Challenge Separate cohorts of mice (n=5/group) were fed 0% or 10% FS diet and exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (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 2 weeks. Mice were sacrificed after 2 weeks of challenge exposure. Levels of malondialdehyde, a marker of lipid peroxidation, were determined in mouse lung tissue (Panel A) and BALF (Panel B). Data is represented as mean ± SEM. *$p< 0.05$ for O_2, IR, and O_2+IR versus respective untreated control mice in each diet cohort. #$p< 0.05$ for 0% FS fed mice exposed to O_2, IR, and O_2+IR versus respective 10% FS fed mice exposed to O_2, IR, and O_2+IR.

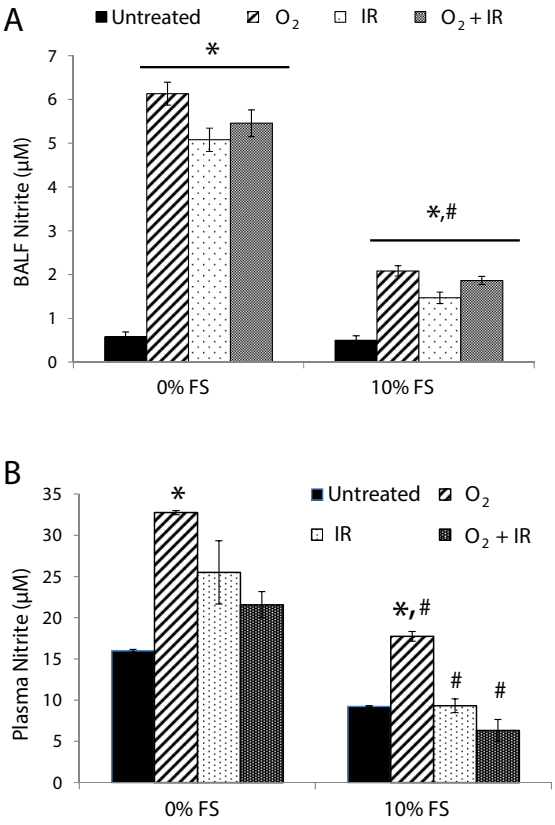

Figure 5: Flaxseed Abrogates increased Nitrosative Stress from Exposure to Hyperoxia, Radiation, or Double-Hit Combination Challenge Separate cohorts of mice (n=5/group) were fed 0% or 10% FS diet and exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing gamma irradiation (IR) only, 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 2 weeks. Mice were sacrificed after 2 weeks of challenge exposure. Levels of nitrite, a stable breakdown product of nitric oxide, were determined in mouse BALF (Panel A) and plasma (Panel B). Data is represented as mean ± SEM. *$p< 0.05$ for O_2, IR, and O_2+IR versus respective untreated control mice in each diet cohort. #$p< 0.05$ for 0% FS fed mice exposed to O_2, IR, and O_2+IR versus respective 10% FS fed mice exposed to O_2, IR, and O_2+IR.

and radiation exposure further induced mRNA levels of TGF-ß1 2.55-fold higher, relative to control. These elevations in lung mRNA levels of TGF-ß1 from mice exposed to challenge conditions and fed 0% FS were absent from mice fed 10% FS.

To determine whether the detected fibrotic changes and increased TGF-ß1 mRNA content in lung tissue are associated with secretion

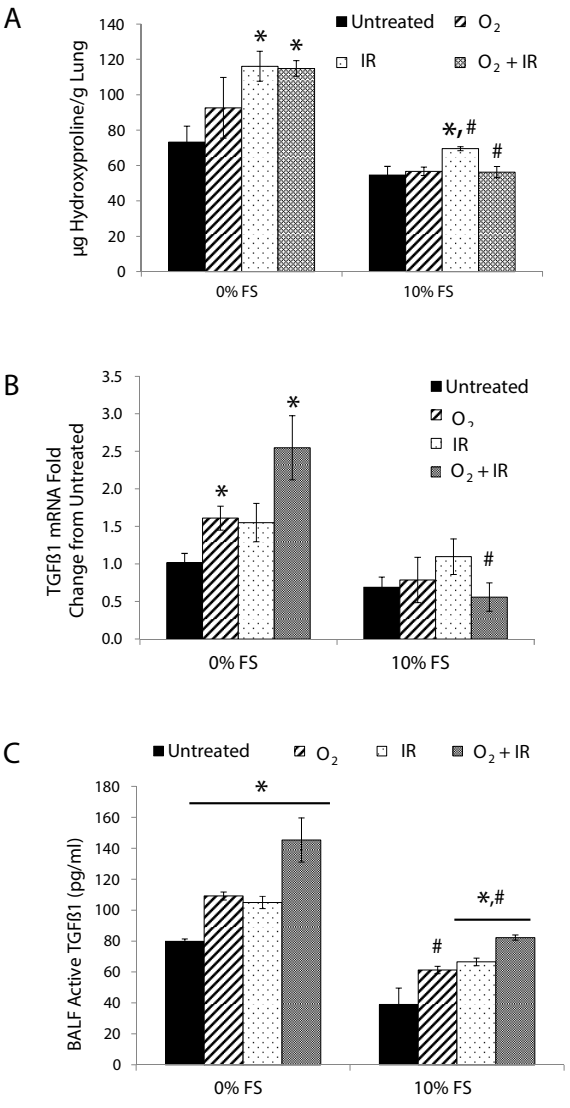

Figure 6: Flaxseed blunts increased Lung Fibrosis and Profibrogenic Cytokine Levels induced by Exposure to Hyperoxia, Radiation, or Double-Hit Combination Challenge Separate cohorts of mice (n=5/group) were fed 0% or 10% FS diet and exposed to 100% O_2 for 8 hours only (O_2), 0.25 Gy total body ionizing 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 2 weeks. Mice were sacrificed after 2 weeks of challenge exposure. Lung tissue was harvested and evaluated for hydroxyproline content, a measure of fibrosis (Panel A). Changes in lung tissue mRNA levels of profibrogenic TGFß1 were determined quantitative real-time PCR analysis (Panel B). Analysis was performed in duplicate and gene expression normalized to 18S ribosomal RNA. Levels of active TGFß1 were determined in mouse BALF using ELISA (Panel C). All data is represented as mean ± SEM. *p< 0.05 for O_2, IR, and O_2+IR versus respective untreated control mice in each diet cohort. #p< 0.05 for 0% FS fed mice exposed to O_2, IR, and O_2+IR versus respective 10% FS fed mice exposed to O_2, IR, and O_2+IR.

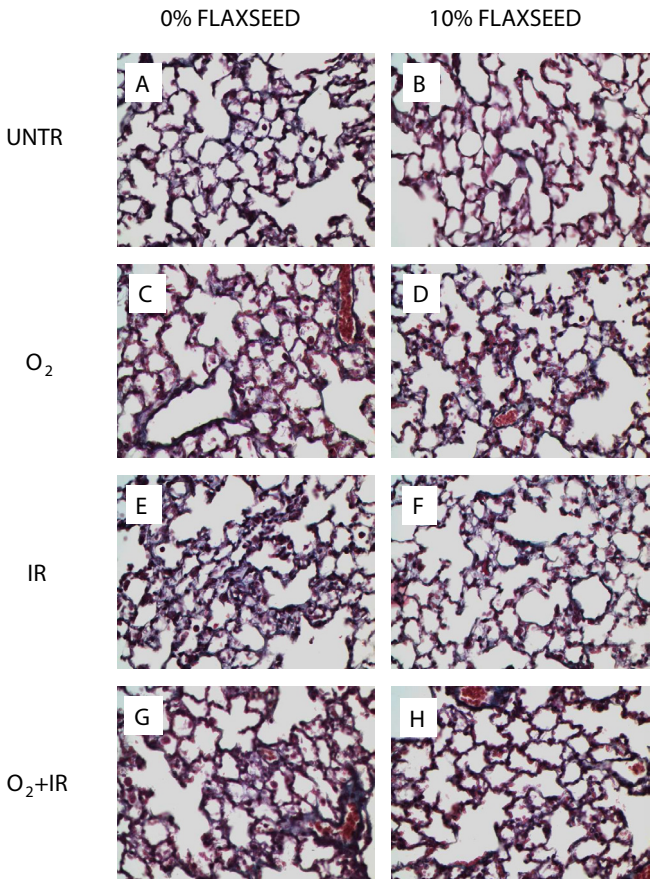

Figure 7: Flaxseed blunts increased Lung Fibrosis induced by Exposure to Hyperoxia, Radiation, or Double-Hit Combination Challenge-A histological evaluation Representative lung photomicrographs are shown here, from the mouse cohorts described in Figure 6. Sections are stained with Mason's Trichrome Blue to identify collagen deposition. Magnification, 400X.

of the pro-fibrogenic cytokines, the levels of active TGF-ß1 were determined in the BALF of mice at using ELISA (Figure 6C). Significant elevations ($p<0.05$) in active TGF-ß1 was detected in all cohorts fed 0% FS and exposed to O_2, IR, or O_2+IR as compared to untreated controls. Levels were significantly ($p<0.05$) reduced across all mouse cohorts fed 10% FS. Notably, the levels of BALF active TGF-ß1 were comparable to the level determined in untreated 0% FS fed mice and confirm previous findings relating to fibrotic changes and TGF-ß1 mRNA induction in lung tissues of 0% FS fed mice. using Mason's Trichrome Blue staining (Figure 7) displays a mild increase in collagen deposition (blue color) in mouse cohorts fed 0% FS and exposed to O_2, IR, and O_2+IR (Panels C, E, and G) as compared to 10% FS-fed mice (Panels D, F, and H). This corroborates the hydroxyproline findings (Figure 6A).

Discussion

Using a novel in vivo system of combined repeated exposure to low-level total body γ-irradiation and hyperoxia, as a simplified model of a pre-breathe protocol that crewmembers may be subjected prior to an EVA, our group identified oxidative lung damage associated with systemic effects [11]. We demonstrated a significant impairment of blood oxygenation and systemic oxidative stress, related to repeated radiation and hyperoxia exposure as well as increased pulmonary inflammation and bronchoalveolar lavage protein levels indicating the development of significant lung tissue injury. In the current study, we

used the same model to evaluate dietary administration of wholegrain flaxseed, a grain with potent antioxidant, anti-inflammatory and anti-fibrotic properties, shown by our group to be protective in lung tissues [12-14,34,43]. We report here for the first time that in a novel model of repeat hyperoxia and radiation exposure, flaxseed intake mitigated adverse effects in lung, thus proving to be a promising countermeasure to potential health risks associated with space exploration.

Data collected by NASA suggest that lung cancer is a major risk for space travel mainly due to space radiation exposure [44,45]. While focus thus far has been on cancer development in lung post exposure to space radiation, important risks to lung function and tissue pathophysiology have been poorly studied and understood. Our study aimed to close the knowledge gap by identifying acute tissue degenerative processes that might lead to impaired lung function long before cancer develops. In addition, we aimed to abrogate this degenerative process by evaluating a potential countermeasure that has shown efficacy in hyperoxic and radiation exposure of lung tissues.

Several nutritional approaches have been suggested as countermeasures to space travel-related pathologies. A nutritional nucleotide supplement has been proposed for ameliorating microgravity induced impairment of the immune system [46] while selenomethionine [47,48], a known antioxidant, was proposed to ameliorate radiation toxicity. Nutritional antioxidant cocktails have also been shown to be radioprotective of hematopoietic cells and even boost animal survival [49] in mice exposed to spacerelevant radiation such as protons and gamma radiation. Wholegrain flaxseed (FS) and its bioactive metabolic components have been extensively studied in other organ systems and have proven to be beneficial, mostly in regards to cancer including breast [50], colon [51,52], ovarian [53,54] or prostate [55] cancer. Our group was the first to show that dietary FS supplementation could also reduce lung inflammation and lipid peroxidation in murine models of acid aspiration and hyperoxia [12] and more recently, in lung ischemia/reperfusion injury related to transplantation damage [13]. Our group pioneered the studies establishing the radioprotective and radiation mitigating properties in lung of FS and FS lignan component [14,33-35,43]. Evaluating the usefulness of this nutritional agent in blunting adverse effects in lung by the cycling and combinatorial effects of hyperoxia and radiation exposures is novel as this agent has never been tested as countermeasure relevant to space travel complications.

The information presented here by our group is quite unique and applicable to ongoing research in human space missions. Importantly, while prior studies [30] have characterized the effects where by hyperoxia alone or radiation alone affects pulmonary tissues, no previous research has investigated the role of a) both exposures (double-hit), b) repeated insults (cycling hyperoxia/normoxia) or c) evaluated countermeasures to the tissue damage resulting from such exposures. Additionally, models designed to test such complex interactions on a tissue and cellular/molecular level are unique to our approach. Research in the field of space travel and changes in the anatomy, physiology, and genome of animal tissues is limited and thus, its planned application to the propagation of manned space travel is unique and appealing. Our data show that a number of markers of acute radiation effects are altered by FS. One such marker is the level of TGF-β1 expression. Indeed, TGF-β1 mRNA levels have been demonstrated to surge soon after irradiation and that this elevation is reversible with a single dose of radioprotective antioxidant enzymes (AOEs) administered intravenously [56,57]. The results from the current study are consistent with this previous data in that creating an antioxidant-rich environment by FS feeding at the time of radiation (and or hyperoxia) exposure that abrogates the early rise in the proliferative cytokine TGF-β1.

During the acute phase of radiation-, hyperoxia- and the combination treatment-induced lung injury, FS inhibited oxidative and nitrosative stress as well as inflammatory cell influx. Studies by Lee at al. [13] showed that inflammatory cells isolated from lung lavage of FS-fed animals are inhibited in respiratory burst and ROS release when stimulated. In addition, a decrease in the release of TNF-a and IL1b pro-inflammatory cytokine by FS shown in this study, may help explain the combinatorial antioxidant/anti-inflammatory, protective effects of FS as an effective countermeasure in this injury model. Nitrosative stress is a hallmark of both radiation and hyperoxic [58] lung damage. Protective effects of FS in irradiated lung tissues have been associated with inhibition of nitrotyrosine formation [35].

Conclusion

In summary, this study evaluates a non-toxic, widely available nutritional supplement that offers long term tissue radioprotective effects in the setting of repeated cycles of total body radiation and hyperoxia exposure associated with an EVA. Potential mechanisms of the effects of prolonged FS feeding include altering immediate radiation- and hyperoxia-induced markers of lung damage, creating a baseline tissue-protective state prior to exposure, and providing high levels of circulating antioxidants from the continued metabolism is of bioactive flaxseed lignans. We have identified a novel countermeasure agent to the pulmonary complications from space travel which could include inflammation, fibrosis, and oxidative tissue damage. In conclusion, we have identified flaxseed as a promising protective agent for adverse effects associated with prolonged space travel using a novel murine model of double-hit repeated low-level radiation and hyperoxia exposure.

Authors' contributions

RAP performed animal experiments, biochemical tissue assays and conducted data analysis. CCS performed pathology assessment of histological specimens. MCS designed the study and individual experiments, analyzed data, wrote the manuscript and supervised lab personnel.

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