In Vitro Modeling of Microgravity-Induced Muscle Atrophy and Spaceflight Radiation

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Abstract
Muscular atrophy, defined as the loss of muscle tissue, is a serious issue for immobilized patients on Earth and in human spaceflight, where microgravity prevents normal muscle loading. A major factor in muscular atrophy is oxidative stress, which is amplified not only by muscle disuse, but also by the increased levels of ionizing radiation in spaceflight. Additionally, elevated radiation exposure can damage DNA, increasing cancer risk.

To model oxidative stress and DNA damage generated by conditions on the International Space Station, murine C2C12 myoblasts were cultured in a rotary cell culture system irradiated by cesium-137. Changes due to the spaceflight model were characterized with fluorescent imaging, amino acid analysis, and enzyme linked immunosorbent assay for heme oxygenase 1. Fluorescent imaging was performed to assess viability, lipid peroxidation, and DNA damage.

Minor DNA damage was observed in cells exposed to 20 µCi cesium-137 for 15 days. No significant differences in viability or lipid peroxidation were noted. Exposure to radiation decreased intracellular heme oxygenase 1 and extracellular alanine, but did not affect branch chain amino acids. Investigation of stronger radiation sources and extended culture time is ongoing. We anticipate that radiation will exacerbate the atrophic effects of microgravity on muscle cells. Simulation of microgravity and spaceflight radiation will provide a valuable platform for drug discovery and an understanding of the progression from normal to disease state.

Introduction
Muscular atrophy due to disuse is a serious issue for individuals subject to bed rest or prolonged immobilization, as well as in human spaceflight, where microgravity prevents normal muscle loading. Atrophy affects cardiac muscle in addition to skeletal muscle, with a decrease in left ventricular mass of 8% and 12% following 6 weeks of bed rest and 10 days of spaceflight, respectively1. Preventing cardiac and skeletal muscle atrophy would preserve the strength and endurance of patients subject to extended bed rest and immobilized limbs, as well as enable longer duration space travel and exploration.

A major factor in muscular atrophy is increased oxidative stress, where reactive oxygen species (ROS) induce degradation of muscle fibers2. As astronauts travel past the protection of the Earth’s magnetic field, they experience increased radiation levels which generate ROS by the radiolysis of water3. Even after returning to Earth, ROS generated by microgravity and irradiation can continue to disrupt cellular systems.

Developing countermeasures for atrophy and radiation in spaceflight will require extensive screening of promising pharmaceuticals for efficacy, safety, contraindications, and dosage schedule. Due to the cost of spaceflight and limited crew time aboard the International Space Station, high throughput screening of pharmaceuticals in actual microgravity conditions is not economically feasible. We propose a ground-based model, detailed herein, to mimic both microgravity and radiation and provide a low cost platform for drug discovery and testing. Anti-atrophy
treatments will not only help immobilized patients on Earth, but will also be critical for future spaceflight missions beyond low Earth orbit.

**Background**

Atrophic conditions can be generated in ground-based laboratories with a rotary cell culture system (RCCS), developed by Synthecon Inc. in conjunction with NASA to simulate microgravity. Microgravity is simulated by the rotational motion of the vessel maintaining cells in a constant state of free fall, similar to what astronauts experience in orbit around Earth. The RCCS has been used to simulate microgravity in a variety of cell types, including lymphocytes, osteoblasts, and myoblasts. The C2C12 cell line used here is a mouse myoblast line that can differentiate into contractile skeletal muscle fibers. First cultured in 1977, the C2C12 cell line produces many of the same proteins as human muscle tissue, making it a suitable analog investigating radiation and atrophic conditions.

Harmful radiation in space primarily comes in two forms, gamma rays and high energy heavy ions. Radiation intensity fluctuates depending on solar activity and proximity to the Earth. The lowest radiation dose rates occur when in close proximity to the Earth and during the solar maximum. Gamma rays are a form of electromagnetic radiation with no mass while high energy heavy ions are atomic nuclei stripped of electrons and moving at relativistic velocities. Some high energy heavy ions are produced by the Sun, but most come from distant supernovae.

Relative biological effectiveness (RBE) is a measure of how damaging a particular type of radiation is to living tissue. Gamma radiation is assigned an RBE of 1 by definition. High energy heavy ions can have RBE values ranging from 20-40, indicating significantly more tissue damage for an equivalent dose. Although high energy heavy ions present a greater health hazard in spaceflight, they are difficult to simulate in a laboratory and require large particle accelerators to produce. In contrast, gamma radiation sources are relatively inexpensive and a large body of data exists regarding epidemiological studies in radiation-exposed humans, rodents, and non-human primates.

NASA limits astronaut radiation exposure to levels that correspond with a 3% increase in fatal cancer risk. Long term missions beyond low Earth orbit, such as a journey to Mars, exceed this limit within a single mission and increase the risk of developing cancer. Due to the highly penetrating nature of high energy ions in cosmic radiation, shielding the spacecraft is not economically feasible. Biological countermeasures to radiation damage and increased cancer risk provide an attractive alternative to heavy and costly shielding.

One cellular response that counters radiation damage is the up-regulation of heme oxygenase-1 (HO-1). HO-1 is induced in response to oxidative stress and combats this stress in part by degrading heme into biliverdin-IXα, a natural antioxidant and anti-inflammatory. Artificial induction of HO-1 has demonstrated protective effects against ionizing radiation in multiple tissue types. We hypothesize that the cytoprotective effects of HO-1 are due to downstream biliverdin production from heme catabolism and activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway by the biliverdin reductase cell surface receptor. An alternative treatment could include supplementation with vitamin E, which attenuates radiation-induced cell death and scavenges reactive oxygen species.
Methods

Cell Culture
C2C12 cell stocks were maintained in their undifferentiated state with Dulbecco's Modified Eagles Medium (DMEM) and 10% fetal bovine serum (FBS) from HyClone, GE Healthcare. At passage 7, cells were seeded into the RCCS and control conditions at $2.5 \times 10^5$ cells/mL. Culture conditions were maintained at 37°C and 5% CO$_2$. Cell counting was performed with a Beckman Coulter ViCell, which uses a Trypan blue exclusion assay. The cells were seeded into DMEM 10% FBS. Every 3 days, the culture media was changed for fresh media. On day 6, the media was changed to DMEM 2% FBS to promote differentiation of the myocytes into myotubes. Experimental cultures were maintained for a total of 15 days. Experimental conditions were normal gravity, normal gravity with radiation, simulated microgravity, and simulated microgravity with radiation. Each condition was cultured in duplicate.

Microgravity Simulation
A Synthecon RCCS-4H with four 10 mL high aspect ratio vessels (HARVs) was used to generate simulated microgravity conditions [Figure 1A]. Cells were cultured on 5 mg/mL of HyClone HyQ Sphere Pplus 102-L microcarrier beads. Rotation of the culture vessels was maintained at 10-12 RPM to prevent settling and maintain cells in a constant state of free-fall. Microcarrier beads in Corning ultra-low attachment tissue culture flasks provide a normal-gravity control. Shear effects from media flow in the RCCS are accounted for by placing the control conditions on an orbital shaker plate at an equivalent RPM [Figure 1B].

Radiation Source
Gamma radiation was provided by two Spectrum Technologies cesium-137 source disks attached to the vessels for the duration of the culture [Figure 1C]. The radioactive source disks were approximately 10 µCi, calibrated to ± 5% according to the National Institute of Standards and Technology. Dose rate to the cells was 25-30 µSv/hr. Lead shielding with a thickness of 1.9 cm isolated control conditions from irradiated conditions, reducing dose rate to 0.5-1 µSv/hr [Figure 1D].

Figure 1: Model setup for microgravity and spaceflight radiation simulation. A – Rotary cell culture system. B – Normal gravity control. C – Cesium-137 source disks. D – Lead shielding.
Cumulative dose for the 15 day culture was approximately 10 mSv, equivalent to 24 days on the International Space Station.19

Spent Media Analysis
Amino acid concentrations in cell culture media from day 15 were determined with a Waters Alliance 2695 high performance liquid chromatography (HPLC) system. Analysis of alanine and the branch chain amino acids (BCAAs) leucine, isoleucine, and valine was conducted. Alanine is secreted into the bloodstream by muscle fibers while BCAAs are oxidized for nutrients.20,21 Lower alanine and BCAA consumption would indicate decreased metabolic activity, expected in simulated microgravity and irradiated conditions.

Fluorescent Imaging
Cell morphology, viability, lipid peroxidation, and DNA damage were determined via fluorescent imaging with a Zeiss confocal microscope. We expect decreased viability, increased lipid peroxidation from ROS, and double-strand DNA breaks in irradiated conditions. Simulated microgravity it expected to increase lipid peroxidation due to ROS. For live cell assays, the cells were rinsed with phosphate buffered saline (PBS) and incubated with the dyes for 30 minutes at 37°C before imaging. Live cell imaging for viability used 1 µM Hoechst 33342, 2.5 µM propidium iodide, and 3 µM Calcein-AM. Live cell imaging for lipid peroxidation used 1 µM Hoechst 33342, 50 nM MitoTracker CMX-Ros, and 20 µM dichloro-dihydrofluorescein (DCF).

For the fixed cell DNA damage H2AX assay, cells were first incubated in DMEM with 50 nM Mitotracker CMX-Ros for 30 minutes at 37°C. The media was removed and PBS containing 4% paraformaldehyde was added for 15 minutes at room temperature to fix the cells. The cells were rinsed once with PBS and then permeabilized with 2.5 µL/mL of Triton X-100 in PBS for 15 minutes at room temperature. The fixation buffer was removed, the cells rinsed again in PBS, and then blocked for one hour with 10 mg/mL of bovine serum albumin in PBS at room temperature to prevent non-specific binding of the primary antibody. After removing the blocking buffer, the 2 µg/mL of Anti-phospho-Histone H2AX (Ser139) primary antibody from Millipore, diluted in blocking buffer, was added for one hour at room temperature. The cells were then rinsed three times with PBS before adding 1 µg/mL of goat anti-mouse IgG (H+L) secondary antibody conjugated with Alexa Fluor 488, from Thermo Fisher Scientific. The secondary antibody was diluted in blocking buffer, along with Hoechst 33342, and incubated for one hour at room temperature. Finally, the cells were rinsed again three times with PBS and imaged.

Western Blot
Protein analysis was accomplished using Western blot. Lower concentrations of the muscle proteins myosin and tropomyosin are expected in simulated microgravity and irradiated conditions. Intracellular proteins were extracted by lysing the cells while adhered to the microcarrier beads. Cells were rinsed with PBS and lysed using a urea based lysis buffer containing 8M urea, 300mM NaCl, 5mL/L Triton X-100, 50mM sodium phosphate dibasic, and 50mM Tris-HCl. A protease inhibitor cocktail consisting of 1mM PMSF and 0.1mg/mL of pepstatin, antipain, and leupeptin was added to the lysis buffer.

Lysate protein content was determined using a Pierce BCA protein assay kit and a Thermo Scientific NanoDrop. SDS PAGE was performed using pre-cast 4-20% Tris-Glycine gels from NuSep. 20 µg of each sample was loaded onto the gel along with 10 µg of actin.
and myosin standard from BioRad. After running at 150 V for one hour, the proteins were transferred to a PVDF membrane using a Pierce fast semi-dry blotter according to the manufacturer instructions. Primary antibodies MF-20 and CH-1, which have activity against myosin and tropomyosin, respectively, were purchased from the Developmental Studies Hybridoma Bank. Antibody concentration was 0.35 µg/mL. Antibody incubation was performed according to published protocol22. The blot was developed with SuperSignal West Pico Chemiluminescent substrate from Thermo Scientific and imaged with a Thermo Scientific myECL imager.

Enzyme Linked Immunosorbent Assay
HO-1 was assayed with an IMMUNOSET HO-1 (mouse) ELISA development set from Enzo Life Sciences. From the cell lysate, 20 µg of total protein, as determined by BCA assay, was loaded into each well. The plate was developed with 1-Step Turbo TMB-ELISA substrate from Thermo Scientific and imaged with a SpectraMax M2 plate reader.

Results & Discussion
Spent Media Analysis
Slight decreases in alanine concentration for radiation exposed conditions were observed [Figure 2]. Alanine is not present in DMEM and is released by muscle fibers into the media as part of the alanine cycle20. Reduced alanine secretion indicates fewer muscle fibers and/or lower metabolic activity, expected for cells under increased stress or atrophic conditions. Reduced alanine in radiation exposed conditions was in line with expectations. On the other hand, alanine concentrations in simulated microgravity vs. normal gravity controls did not follow the expected trend. Cells cultured in simulated microgravity had higher extracellular alanine concentrations than the normal gravity controls. Increased sample size is necessary to determine if these results are statistically significant.

![HPLC – Extracellular Alanine, n=2](image)

Figure 2: Alanine concentrations in spent media harvested on day 15. NG = normal gravity. MG = microgravity. Error bars are high/low for n=2.

No significant differences were observed in the concentration of BCAAs. BCAAs are oxidized during muscle fiber activity21. We expected to observe decreased BCAA consumption in conditions exposed to simulated microgravity. Similar BCAA consumption across all conditions may be due to insufficient sample size and/or culture time.

Fluorescent Imaging
Fluorescent imaging for cell viability did not reveal significant differences between conditions. Differentiation of myocytes into multi-nucleated myotubes was confirmed for all conditions, demonstrating that simulated microgravity does not prohibit formation of new muscle fibers. Lipid peroxidation was observed in both normal gravity controls and simulated microgravity conditions, as indicated by DCF [Figures 3-4]. Radiation provided by the cesium-137 sources induced minor double-strand DNA breaks, as visualized by H2AX phosphorylation23 [Figures 5-6]. Stronger radiation sources are being investigated to provide more significant damage.
Figure 3: Normal gravity control stained for nuclei with Hoechst (blue), muscle fibers with MitoTracker (red), and lipid peroxidation with DCF (green).

Figure 4: Simulated microgravity control stained for nuclei with Hoechst (blue), muscle fibers with MitoTracker (red), and lipid peroxidation with DCF (green).

Figure 5: No-radiation control under normal gravity stained for nuclei with Hoechst (blue), muscle fibers with MitoTracker (red), and H2AX phosphorylation (green, not present).

Figure 6: Radiation exposed condition under normal gravity stained for nuclei with Hoechst (blue), muscle fibers with MitoTracker (red), and H2AX phosphorylation (green, see inset for detail).
Enzyme Linked Immunosorbent Assay for Heme Oxygenase-1

HO-1 is upregulated during periods of oxidative stress to catabolize heme into biliverdin, a natural antioxidant\(^2\). Due to oxidative stress induced by radiation and the microgravity environment, we expected to observe an increase in HO-1 for radiation-exposed conditions as well as cell cultured in simulated microgravity. However, decreased HO-1 in cell lysate was observed for radiation-exposed conditions [Figure 7]. The large error bars preclude drawing conclusions regarding HO-1 in simulated microgravity vs. normal-gravity controls until more data is generated. To maximize protein concentration, undiluted lysate was used for the assay. Error may be introduced due to the high viscosity of the lysate, resulting in inconsistent pipetting.

![Intracellular HO-1, n=2](image)

**Figure 7:** HO-1 ELISA with cell lysate containing 20 µg total protein. NG = normal gravity. MG = microgravity. Error bars are high/low for n=2.

**Proposed Atrophy and Radiation Countermeasures**

Successful development of a ground-based model for microgravity-induced muscle atrophy and spaceflight radiation would provide a low-cost platform for testing anti-atrophy and radioprotective drugs. We have identified promising therapeutic compounds to counter cellular damage from spaceflight conditions. A major contributor to muscular atrophy is the increased production of tissue-damaging reactive oxygen species following immobilization or disuse, including disuse stemming from the microgravity conditions of spaceflight\(^25\)–\(^27\). Hydrogen peroxide (H\(_2\)O\(_2\)) released from murine mitochondria nearly doubles after two weeks of hind limb suspension, which in addition to its inherent cytotoxic effects stimulates the release of calpain and caspase-3, proteases that degrade myofilaments\(^28\). Exposure to ionizing radiation also generates reactive oxygen species via radiolysis of water\(^3\). Scavenging these reactive oxygen species would limit calpain and caspase-3 activation and protect cells from damage.

**Mesobiliverdin-IX\(\alpha\)**

To combat the oxidative stress associated with disuse atrophy of skeletal muscle, we propose mesobiliverdin-IX\(\alpha\), a microbial-sourced analog of biliverdin-IX\(\alpha\) with antioxidant and anti-inflammatory properties. Biliverdin-IX\(\alpha\) cannot be economically extracted from mammalian cells due to rapid conversion to bilirubin by biliverdin reductase. However, we have isolated gram quantities of mesobiliverdin-IX\(\alpha\) from cyanobacteria bile pigments using a scalable process. Mesobiliverdin-IX\(\alpha\) was found to behave similarly to biliverdin-IX\(\alpha\), including serving as a substrate biliverdin reductase in human cells\(^29\). Cytoprotective effects of mesobiliverdin-IX\(\alpha\) and biliverdin analogs have been demonstrated with pancreatic islet cells, vascular endothelial cells, and arterial smooth muscle cells\(^29\)–\(^31\).

Our preliminary data indicates increased protein production in both normal gravity and simulated microgravity conditions for cells supplemented with 10 μM of mesobiliverdin-IX\(\alpha\), as determined by Western blot [Figure 8]. The normal gravity control in this example performed worse than
expected, likely due to mechanical stress from a rocker plate. Current controls are cultured on an orbital shaker with a fluid motion more similar to that of the RCCS.

The direct antioxidant properties of bilirubin and biliverdin-IXα in cell-free systems have been established\(^1\). We hypothesize that mesobiliverdin-IXα will exhibit indirect cytoprotection by moderating expression of heme oxygenase-1, an inducible enzyme which combats oxidative stress in part by degrading heme into biliverdin-IXα\(^1\). Elucidating the influence of mesobiliverdin-IXα on HO-1 is critical due to the numerous downstream effects of HO-1 activation. Increasing HO-1 expression with hemin supplementation attenuates immobilization-induced skeletal muscle atrophy, potentially by preventing protein degradation from oxidative stress\(^3\).

However, not all of the products of heme degradation are beneficial. In addition to biliverdin-IXα, heme degradation also results in the production of carbon monoxide (CO) and iron (Fe\(^{2+}\)) [Figure 9]. Contrary to the antioxidant properties of biliverdin-IXα, free Fe\(^{2+}\) can react with \(H_2O_2\) to produce a hydroxyl radical\(^2\). Furthermore, CO inhibits CCAAT-enhancer-binding protein δ from binding to the MyoD promoter [Figure 9], thereby reducing the differentiation of myoblasts into contractile myotubes\(^3\). This side effect of the HO-1 oxidative stress response mechanism is especially detrimental in spaceflight, where significant muscle loss is experienced despite the current International Space Station exercise program\(^3\).

**Figure 9: Heme catabolism pathway with proposed mesobiliverdin route of efficacy**

Mesobiliverdin-IXα may provide the cytoprotective effects of HO-1 without induction of MyoD inhibition and Fe\(^{2+}\) generation. Activation of cell-surface biliverdin reductase (BVR) has important downstream consequences, inducing Akt via intracellular phosphorylation of BVR, which binds to the p85α subunit of PI3K\(^3\). PI3K activation suppresses phosphatase and tensin homolog (PTEN), a negative regulator of muscle growth\(^3\). Furthermore, Akt regulated protein synthesis pathways are inhibited during periods of inactivity\(^3\). Activation of the PI3K-Akt pathway also limits cellular apoptosis following irradiation\(^3,3\). Since mesobiliverdin-IXα serves as a substrate for human BVR\(^2\), we hypothesize that supplementation with mesobiliverdin-IXα will provide the benefits of increased PI3K and Akt expression. The prospective action
of mesobiliverdin-IXα through the BVR—PI3K—Akt pathway is encouraging. Activating Akt directly causes feedback inhibition of PI3K, associated with cardiac dilation and death in the long-term\textsuperscript{40,41}. Since mesobiliverdin-IXα’s action on Akt would be indirect, we do not anticipate the aforementioned complications.

It will be critical to determine whether or not mesobiliverdin-IXα inhibits HO-1 via a feedback mechanism. The breakdown of heme is important, as excess heme produces inflammation and apoptosis in a variety of cell types\textsuperscript{42,43}. If, upon mesobiliverdin-IXα supplementation, HO-1 induction is lessened in response to oxidative stress but not in response to free heme, MyoD-inhibiting CO and generation of hydroxyl radicals from Fe\textsuperscript{2+} will be limited while both the direct antioxidant properties of mesobiliverdin-IXα and downstream BVR—PI3K—Akt pathways are maintained.

**Vitamin E**
Another promising atrophy and radiation countermeasure is vitamin E. The antioxidant effects of vitamin E scavenge free radicals and reduce expression of calpains and caspase-3, -9, and -12, reducing muscular atrophy due to immobilization and unloading\textsuperscript{44,45}. In addition to preventing muscle loss, vitamin E also preserves bone mass and strength following unloading\textsuperscript{46}. Several forms of vitamin E have demonstrated radioprotective effects, including α-tocopherol, δ-tocotrienol, and γ-tocotrienol\textsuperscript{16–18}. Unlike mesobiliverdin-IXα, vitamin E inhibits the PI3K-Akt pathway, which may promote apoptosis of tumor cells\textsuperscript{47,48}. The effects of combined mesobiliverdin-IXα and vitamin E supplementation will also be investigated.

**Conclusions**
The RCCS successfully cultures and permits differentiation of multi-nucleated myotubes, basic components of muscle tissue. Irradiation for 15 days with 20 μCi of cesium-137 produces minimal double-stranded DNA breaks. Exposure to radiation resulted in decreased concentrations of alanine and HO-1, though more data must be generated to determine statistical significance. Stronger radiation sources are being investigated to provide a dose equivalent to a journey to Mars within a two week culture period.

The initial data generated since January 2015 are insufficient to determine whether or not the combination of a RCCS and radiation source provide a platform that generates conditions mimicking spaceflight. To conclusively demonstrate similarity of the model to spaceflight, muscle protein concentration must be lower and markers of oxidative stress must be higher in microgravity and irradiated conditions. Increased expression of the atrophy markers Atrogen-1, MuRF1, and Nedd4 in simulated microgravity would provide evidence of the model’s effectiveness\textsuperscript{49}. Analysis of these markers with qRT-PCR is ongoing. Culture on flat membranes instead of microcarrier beads may improve fluorescent imaging results. The Z-stacks required for 3D bead clusters can take more than one hour per image, leaving ample time for cells to undergo changes when performing live cell imaging. In the event the *in vitro* model does not demonstrate similarity to spaceflight conditions, the proposed therapeutic compounds will be tested using the rodent hindlimb unloading model, a well-established method for inducing muscular atrophy\textsuperscript{50}.

Completion of an *in vitro* ground-based model for spaceflight atrophy and radiation
injury would provide a valuable platform for testing countermeasures necessary to ensure astronaut health and fitness during long-term spaceflight. Radiation doses can be tuned to simulate a variety of mission circumstances from short-term low Earth orbit to multi-year expeditions on Mars. The metrics presented herein will also elucidate the mechanisms of action for atrophy and radiation countermeasures, providing a foundation for design and selection of novel therapeutics.

References


