Using Combined Microgravity and Radiation to Simulate Skeletal Muscle Damage in Space

Lori Caldwell, Charles Harding, Eryn Hansen, Elizabeth Vargis
Utah State University Department of Biological Engineering

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. Developing countermeasures for atrophy in spaceflight will require extensive screening of 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 under real microgravity conditions is not feasible. While traditional ground-based atrophy studies using the rodent hind-limb unloading model are effective at inducing physiological changes similar to spaceflight, they are not suited for first round screening of novel therapeutics due to resource and regulatory challenges. Additionally, these ground-based studies do not account for the high levels of ionizing radiation experienced in spaceflight that may increase atrophy. Here, we present a protocol to combine both microgravity and radiation using an in vitro ground-based model of microgravity with a rotary cell culture system combined with Utah State University’s Space Survivability Test Chamber (SSTC).

To model the levels of ionizing radiation received by astronauts in space, murine C2C12 cells were cultured in standard tissue culture well plates and exposed to Strontium 90 in both their undifferentiated and differentiated states using the SSTC. We hypothesize that cells will show a significant loss of viability when exposed to a dosage of approximately 4.0 Gy, the LD50 for most human tissue. Changes after exposure to radiation were indicated by cell viability counts and morphology characterization.

To model the microgravity conditions on the International Space Station, a custom rotary cell culture system was designed to be compatible with the SSTC. We hypothesize that combining both microgravity and radiation will lead to a greater biosimilarity for ground-based atrophy models.

Contrary to expectations, undifferentiated cells exposed to radiation did not experience a significant loss in viability withstanding doses up to 38 Gy. Irradiated differentiated cells followed a lethal dosage curve as expected to reach 50% viability at approximately 4.0 Gy. Ground-based simulation of microgravity and radiation will provide a valuable platform for drug discovery and an understanding of the multiple mechanisms underlying muscular atrophy.

Introduction
Astronauts experience significant muscle loss in spaceflight despite the current International Space Station exercise program. Physiological changes associated with atrophy include loss of muscle mass, reduced power, lower endurance, and atypical reflex responses. NASA flight protocol requires crew to exercise for missions lasting over 10 days; however, reduction in muscle mass and strength has been observed in as little as 5 days. Disuse, reduced protein synthesis, and reduced motor neuron activity all contribute to loss of muscle tissue and strength in spaceflight. The degree of atrophy is dependent on anatomical region and duration of exposure to the microgravity environment. Short duration missions can result in a 10-
20% loss of muscle mass, where losses in long duration missions can range from 30-50%\textsuperscript{5,6}. Atrophy is more severe for muscles responsible for maintaining posture under normal gravity, with the intrinsic back muscles losing 10.3% volume compared to 3.9% for calf muscles after 8 days of spaceflight\textsuperscript{5,7}. Atrophy affects cardiac muscle in addition to skeletal muscle, with a decrease in left ventricular mass of 12% following 10 days of spaceflight\textsuperscript{8}. Preventing muscle atrophy would preserve the strength and endurance of astronauts and help enable longer duration space travel and exploration.

In vitro simulation of microgravity can be conducted with rotary culture systems and 3D random positioning machines or clinostats\textsuperscript{9,10}. In vitro microgravity simulation has the benefits of lower costs and fewer regulatory concerns compared to animal models. For use in drug development, the quantity of test compound required to elicit a response is lower for small in vitro systems, conserving therapeutics that may be difficult to produce at early stages of development.

An additional risk factor in spaceflight is the elevated levels of ionizing radiation, which increase the risk of developing cancer\textsuperscript{11}. Radiation levels increase past the protection of the Earth’s magnetic field and present a significant concern for human spaceflight beyond low Earth orbit. Damage from radiation can occur both directly via DNA alterations and indirectly via the generation of reactive oxygen species\textsuperscript{12}. Ionizing radiation rapidly generates reactive oxygen species that can disrupt multiple intracellular systems for several days post-irradiation\textsuperscript{13}. In vitro simulation of radiation exposure can be conducted with Utah State University’s Space Survivability Test Chamber (SSTC), a beta radiation source\textsuperscript{14}.

Here, we propose a ground-based protocol to mimic the effects of microgravity and radiation with regard to cell viability and morphology changes. This model provides a means of screening anti-atrophy therapeutics prior to testing in vivo. We expect the model will be valuable for expediting the search for pharmaceuticals that enable long-duration spaceflight missions.

**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\textsuperscript{9}. Microgravity is simulated by the rotational motion of the vessel maintaining cells at their terminal settling velocity, similar to what astronauts experience in orbit around Earth. The settling velocity is described by the equation $v_s = \frac{2g(\rho_a-\rho)R^2}{9\mu}$, where $\rho_a$ is the density of the cell aggregate, $\rho$ is the density of the culture media, $R_a$ is the radius of the cell aggregate, and $\mu$ is the fluid viscosity\textsuperscript{15}. As described by Stoke’s Law, the force of gravity $F_g$ approaches zero at the terminal settling velocity due to the similar density of the cell aggregate and the surrounding fluid; $F_g = (\rho_a - \rho)g\frac{4}{3}\pi R^3$.

The RCCS has been used to simulate microgravity in a variety of cell types, including lymphocytes, osteoblasts, and myoblasts\textsuperscript{15,16,18}. The C2C12 cell line used in the experiment presented herein 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 and mRNAs as human muscle tissue, making it a suitable analog for investigation of atrophic conditions\textsuperscript{19}.
Microcarriers are a commonly used substrate for suspension culture of adherent cells. The ease of scalability makes microcarriers an attractive substrate for producing large quantities of cells for therapeutic applications\textsuperscript{20,21}. A wide variety of surface chemistries are available from suppliers to tailor the beads for specific cell types and culture conditions\textsuperscript{20}. Successful culture of muscle cells on microcarriers has been reported with dextran, collagen, and polystyrene surfaces\textsuperscript{15,22,23}. While a more expensive substrate than alginate, the variety of commercially available bead types, ease of use and facile scalability make microcarriers an industry standard for suspension culture of adherent cells.

To calculate an appropriate experimental dosage, two conditions were considered: a 1-year Mars mission, and 10-year Mars colonist mission. Traveling outside the Earth’s geomagnetic sphere leaves astronauts susceptible to increased radiation exposure from galactic cosmic rays of $48.1 \pm 0.81$ $\mu$Gy/day\textsuperscript{24}. Assuming a 180-day one-way duration flight and a 365-day experimental stay on a round trip one-year exploration of Mars, an equivalent dosage of $0.66 \pm 0.12$ Gy is found. Under those same travel time assumptions for a 10-year Mars colonist, an equivalent dosage of $3.65 \pm 0.22$ Gy is found.

**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 11, cells were seeded into standard tissue culture flasks at $2.5 \times 10^5$ cells/mL. Culture conditions were maintained at $37^\circ C$ and 5% CO\textsubscript{2}. Cell counting was performed with a Trypan blue exclusion assay. 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 21 days. The 21 day culture time was selected to allow muscle cell differentiation prior to radiation exposure\textsuperscript{25}. Each condition was cultured in quadruplicate.

**Radiation Exposure**

Cultured C2C12 cells were trypsinized and centrifuged to form a cell pellet suspended in 200 $\mu$L of DMEM and 10% FBS in a PCR vial. These undifferentiated cell samples were contained in a sealed atmospheric pressure vessel held at $37^\circ C$ and placed in the SSTC for irradiation [Figure 1].

![Figure 1. Experimental set up for undifferentiated cells in a pressurized chamber.](image)
Figure 2. Radiation dose rate curve (kRad/hr) based on the source-to-sample distance (highlighted with green box) within the Space Survivability Test Chamber.

Sterilization of the aluminum vessel was achieved by autoclaving and performing all cell culture transfers in a laminar flow tissue culture hood. Undifferentiated cells were irradiated using a 21.5 cm source-to-sample distance to achieve a dose rate of 7 Gy/hr and accumulated dosages of 0.6, 7.2, 14.6, and 36.8 Gy [Figure 2]. Immediately following exposure, two of four samples were analyzed for cell viability using a Trypan blue exclusion assay. The remaining two samples were seeded into standard 25 cm² tissue culture flasks and grown for 7 days after which an identical viability assay was performed. Differentiated C2C12 cellular monolayers were grown in a 6-well tissue culture plate for 14 days then irradiated at atmospheric pressure conditions at the same dose rate to achieve accumulated doses of 0.5, 1.0, 2.0, and 4.0 Gy. Monolary samples were analyzed for viability using a Trypan blue exclusion assay.

RCCS Fabrication
Based on the Synthecon RCCS-4H, the mini-RCCS has six 4 mL culture vessels machined from polycarbonate. The endcaps of the vessels feature two O-rings to seal the cell culture area as well as syringe ports to facilitate media exchange and a breathable PDMS membrane for gas transport. The vessels attach to a motorized gear system capable of rotational speeds from 4 – 22 rpm [Figure 3].
**Results & Discussion**

Undifferentiated C2C12 cells exposed to radiation did not experience a significant decrease in viability at biologically relevant dosages [Figure 4]. This high tolerance for radiation exposure is likely due to protection from the combination of decreased exposed surface area and lower metabolic activity in this undifferentiated state. Cell viability improved in samples grown for 7 days when exposed to both 14.6 and 36.8 Gy.

![Figure 4. Percent viability of undifferentiated C2C12 cells exposed to radiation.](image)

Skeletal myotubes should appear striated and unidirectional and grow in a monolayer. Cell morphology was altered in all samples seeded post-irradiation with reduced levels of myotube formation in undifferentiated samples exposed to 7.2 and 14.6 Gy [Figure 5].

![Figure 5. Brightfield imaging of C2C12 cells exposed to radiation. Top row: Undifferentiated cells. Bottom row: Differentiated cells. Scale = 100 µm.](image)

Differentiated C2C12 monolayers exposed to beta radiation exhibited a marked decrease in viability, reaching the LD50 at approximately 4.8 Gy (estimated by linear interpolation). Significant decreases in viability occurred as radiation exposure was increased from 1.0 to 2.0 Gy and from 2.0 to 4.0 Gy with p-values less than 0.05 [Figure 6]. All sample viabilities differed significantly from the control and showed marked differences in cellular morphology. Notably, cells exposed to 1.0 and 2.0 Gy appeared to lose cell-to-cell contact inhibition and continued growing in additional planes of focus [Figure 5].

![Figure 6. Percent viability of differentiated C2C12 monolayers exposed to radiation.](image)

A differentiated monolayer of cells provides a better approximation of cellular damage in...
vitro compared to an undifferentiated cell pellet. Differentiated C2C12 cells on polystyrene microcarrier beads in the radiation compatible mini-RCCS will give a more realistic model of muscle atrophy due to both microgravity and radiation providing a better platform to test potential therapeutics.

References


