From the cover: Radishes grown at various pressures and oxygen partial pressure. From: “Radish (Raphanus sativa L. cv. Cherry Bomb II) Growth, Net Carbon Exchange Rate, and Transpiration at Decreased Atmospheric Pressure and/or Oxygen.” C.A. Wehkamp, et al., p. 3
GENERAL INFORMATION

*Gravitational and Space Biology* (ISSN 1089-988X) is a journal devoted to research in gravitational and space biology. It is published by the American Society for Gravitational and Space Biology, a non-profit organization whose members share a common goal of furthering the understanding of the biological effects of gravity and the use of the unique environment of spaceflight for biological research. *Gravitational and Space Biology* is overseen by a steering committee consisting of the Publications Committee, the Editor, the President, and the Secretary-Treasurer of the ASGSB.

The American Society for Gravitational and Space Biology was created in 1984 to provide an avenue for scientists interested in gravitational and space biology to share information and join together to speak with a united voice in support of this field of science. The biological effects of gravity have been acknowledged since Galileo’s time, but only since the 1970s has gravitational biology begun to attract attention. With the birth of the space age, the opportunity for experimentation over the full spectrum of gravity finally became a reality, and a new environment and research tool became available to probe biological phenomena and expand scientific knowledge. Space and spaceflight introduced new questions about space radiation and the physiological and psychological effects of the artificial environment of spacecraft.

The objectives of ASGSB are:

- To promote research, education, training, and development in the areas of gravitational and space biology and to apply the knowledge gained to a better understanding of the effect of gravity and space environmental factors on the flora and fauna of Earth.
- To disseminate information on gravitational and space biology research and the application of this research to the solution of terrestrial and space biological problems.
- To provide a forum for communication among professionals in academia, government, business, and other segments of society involved in gravitational and space biological research and application.
- To promote the study of concepts and the implementation of programs that can achieve these ends and further the advancement and welfare of humankind.

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Gravitational and Space Biology
Instructions to Authors

Brief Overview:

The journal of the American Society for Gravitational and Space Biology (ASGSB), Gravitational and Space Biology, publishes quality, peer reviewed manuscripts in several categories. Manuscripts should be self-contained, and all conclusions substantiated and supported by results in the form of figures and/or tables. Authors are held to standards of writing (American English) for clarity and material appropriate for the Gravitational and Space Biology (GSB) journal. Subject matter can include any topic within the following broad categories: the impact of gravity and changes in the gravity vector on biology, spaceflight research (ISS and Shuttle), satellite payloads, advanced life support, planetary and orbital analog research, suborbital research, parabolic flight, sounding rockets, high altitude balloons, astrobiology, plus hardware development, mechanobiology, and other disciplines exploring the interface of biology and engineering technology. Brief summaries of manuscript types and guidelines for each category are below; detailed instructions and templates follow.

I. Short Communications.

Short communications are submissions typically 2 - 3 formatted pages in length (1000 – 2000 words, excluding references). These submissions are to be comprised predominantly of preliminary data for a larger study or a brief report to support work of a larger nature. It may be beyond the scope of these submissions for further experimentation, but a reviewer may request additional explanation of the presented data and hold the authors to appropriate conclusions for those data.

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Methods papers are manuscripts typically 3 - 6 pages in length. These manuscripts are comprised of data and protocols that support flight experiments or ground control experiments, of protocols in support of fundamental studies exploring biological responses to altered gravitational environments, and to biological responses to space and planetary analogs. The manuscripts should contain sufficient detail to enable a reader to replicate the protocol. Reviewers should particularly address shortfalls of detail, validation of protocols, and inconsistencies in any aspect of presentation. Figures may include illustrations of procedures and set-up and should include data that verify the efficacy of the procedures.

III. Research papers.

Research papers are manuscripts of typically 8 - 15 pages in length and, although there is no strict limitation to size, a reviewer may address extremes of brevity or length as appropriate to conveying the information. These manuscripts present original research of interest to the gravitational and space biology community

IV. Review articles.

Review articles are typically 10 - 15 pages in length. These manuscripts are often solicited from symposium speakers at the annual ASGSB meeting, but they are not limited to those solicitations. Any author may approach the editorial board with a suggestion or request to submit a review article, to be peer-reviewed as any other paper. A review article will be judged principally for accuracy of information and citation and appropriate scope and relevance of the subject of the article.

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Format

The same basic format is used for each type of article. Consult a current issue of Gravitational and Space Biology, as well as the instructions below, for guidance on formatting, organizing, and preparing references, figures, tables, and legends. An article must have a brief abstract that summarizes the principal conclusions of the paper. Manuscripts are submitted electronically as single column, double spaced Word documents, and figures as separate, individual documents. Details are provided below.
Instructions to Authors

Arrangement

Arrange the manuscript in the following order, with all pages numbered consecutively in the footer of the lower right corner. The last name of the first author should precede each page number.

Cover page – In a separate page, include the title, suggested running head (not to exceed 60 characters, including spaces), the full names and affiliations of all authors, and detailed contact information for the Corresponding Author: name, address, e-mail, telephone number.

The remaining sections proceed without page breaks

Title – Use a descriptive title (not to exceed 200 characters, including spaces).

Authors – Provide the complete names and affiliations of all authors; indicate the corresponding author.

Abstract – Summarize the principal approach and conclusions of the paper. Abstracts are not to exceed 150 words in Short Communications and 250 words in all others.

Body of paper – For Research Papers, the body of the paper should be arranged into subsections for Introduction, Materials and Methods, Results, and Discussion. Review Papers should be organized in a manner appropriate to the subject. Methods papers should include a short Introduction and also a Discussion of the application addressing the significance of the method being described. The Short Communication papers are not required to contain subdivisions, other than a short abstract, but may be organized into subsections at the discretion of the authors.

References and Citations – Cite each reference in the text by author(s) name(s) and the publication date: Examples: Smith, 1989 (one author) Smith and Jones, 2001 (two authors) Smith et al., 2010 (more than two authors).

- Alphabetize the reference list by authors' last names.
- List only published or in-press articles. Unpublished results, including personal communications and submitted manuscripts, should be cited as such in the text.
- References formatted as follows: author(s): last name(s) comma followed by initial(s) and a period comma before next author; year of publication followed by a period; article title in sentence case, followed by a period; journal title (unabbreviated and italicized), followed by volume number, issue number in parenthesis (if applicable), a colon, and page numbers. Previous issues can be used as a guide, and an EndNote™ style template can be downloaded at the website. Two examples are provided below:
  
  Journal Article:
  
  Book:

Figures – Figures are submitted as separate graphic files. Resolution must be 300dpi

- Number Figures consecutively as they are used in the text.
- The first time a figure is discussed, refer to it actively rather than parenthetically.
- Provide enough information in the Figure Legend such that the reader can understand the figure without significant input from the text. For submission, provide Figure Legends at the end of the body of the manuscript, following the Reference section.
- Designate figure sections with letters and explain all symbols and abbreviations that are used in the figure.

Tables – Can be submitted as embedded in the text of formatted manuscript in an appropriate location, or subtended to the end of the manuscript.
Instructions to Authors

- Number Tables consecutively as they are used in the text.
- The first time a table is discussed, refer to it actively rather than parenthetically.
- Give each table a concise title, followed by a legend that makes the general meaning of the table comprehensible without reference to the text. For submission, provide Tables and Table Legends at the end of the body of the manuscript, following the Figure Legends.
- Tables should be constructed in Word or Excel with the general format:

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Atmospheric pressure relative to altitude.</th>
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<td>30 – 5</td>
<td>9000 – 27000</td>
</tr>
</tbody>
</table>


Abbreviations or Other Standards

- Do not use abbreviations other than those that are standard for international usage.
- Use SI units as far as possible.
- Use \( g \) (italicized) for unit gravity, to distinguish it from the standard abbreviation \( g \) (not italicized) for gram.
- Use spaceflight (one word) rather than space flight (two words).
- Any acronyms that are used in the manuscript must be defined at first mention.

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If reviewers recommend only minor textual changes, the editor may choose to make these changes and accept the manuscript essentially as submitted. The editor then sends the accepted manuscript to the journal’s publishing editor. Page proofs are provided to the authors for review prior to the journal going to press.

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Authorship of articles implies that an individual has made a substantial contribution to the article both in terms of the design of the study or collection/evaluation of data and with regard to the intellectual content of the manuscript.

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institutional and national guide for the care and use of laboratory animals was followed. For research using Recombinant DNA, physical and biological containment must conform to National Institutes of Health guidelines or those of a corresponding agency.

**Published Statement of Informed Consent:**

The general requirements for informed consent conform to guidelines and requirements outlined by the National Science Foundation [http://www.nsf.gov/bfa/dias/policy/docs/45cfr690.pdf](http://www.nsf.gov/bfa/dias/policy/docs/45cfr690.pdf) and Health and Human services [http://answers.hhs.gov/ohrp/categories/1566](http://answers.hhs.gov/ohrp/categories/1566). No investigator may involve a human being as a subject in research covered by this policy unless the investigator has obtained the legally effective informed consent of the subject or the subject's legally authorized representative. An investigator shall seek such consent only under circumstances that provide the prospective subject or the representative sufficient opportunity to consider whether or not to participate and that minimize the possibility of coercion or undue influence. The information that is given to the subject or the representative shall be in language understandable to the subject or the representative. No informed consent, whether oral or written, may include any exculpatory language through which the subject or the representative is made to waive or appear to waive any of the subject's legal rights, or releases or appears to release the investigator, the sponsor, the institution or its agents from liability for negligence.

**Basic elements of informed consent.**

In seeking informed consent the following information shall be provided to each subject:

- A statement that the study involves research, an explanation of the purposes of the research and the expected duration of the subject's participation, a description of the procedures to be followed, and identification of any procedures which are experimental;
- A description of any reasonably foreseeable risks or discomforts to the subject;
- A description of any benefits to the subject or to others which may reasonably be expected from the research;
- A disclosure of appropriate alternative procedures or courses of treatment, if any, that might be advantageous to the subject;
- A statement describing the extent, if any, to which confidentiality of records identifying the subject will be maintained;
- For research involving more than minimal risk, an explanation as to whether any compensation and an explanation as to whether any medical treatments are available if injury occurs and, if so, what they consist of, or where further information may be obtained;
- An explanation of whom to contact for answers to pertinent questions about the research and research subjects' rights, and whom to contact in the event of a research-related injury to the subject; and
- A statement that participation is voluntary, refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.
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Radish (*Raphanus sativa* L. cv. Cherry Bomb II) Growth, Net Carbon Exchange Rate, and Transpiration at Decreased Atmospheric Pressure and / or Oxygen

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**ABSTRACT**

To simplify engineering requirements for plant growth structures on the Moon or Mars, lower pressures are desirable to reduce mass and decrease atmospheric leakage. In order to establish the effect of reduced pressure and reduced oxygen on carbon assimilation, dark period respiration, transpiration, and plant growth, radishes (*Raphanus sativa* L. cv. Cherry Bomb II) were grown at 98 (ambient pressure), 66 (2/3 atm), 33 (1/3 atm), and 10 (1/10 atm) kPa total pressures with oxygen partial pressures of 20, 14, 7, and 2 kPa for 21 days. All plants were grown in rockwool using recirculating nutrient film technique hydroponics. Analysis of growth showed no significant difference among the 98, 66, 33, and 10 kPa total pressure environments when the oxygen partial pressure was ≥ 7 kPa, but a significant reduction was observed when the oxygen partial pressure was dropped to 2 kPa, regardless of the total pressure. Net carbon exchange rate (NCER) and transpiration showed a similar pattern, with no significant effect with pressure treatments. Only the reduced oxygen partial pressure treatment of 2 kPa resulted in significant reductions in NCER and transpiration. Results indicate that pressure has little effect on radish productivity as long as oxygen levels are maintained at or above 7 kPa.

**INTRODUCTION**

Interest in manned space exploration has furthered research into human life support technologies. As mission durations increase, the ability to re-supply from Earth diminishes (Simpson and Young, 1998; Wheeler et al., 2001). It is likely that advanced life support systems for long-term space exploration and habitation will be comprised of some combination of physical-chemical and bioregenerative systems, and that plants (for food and oxygen production and carbon dioxide removal) will be an integral part of those systems (Wheeler et al., 2001; Ferl et al., 2002b; Spanarkel and Drew, 2002). The utilization of low pressure plant growth facilities would lessen force on the structure, decrease the requirement for start-up consumables (e.g., pressurizing gas), and reduce the amount of atmospheric leakage from the structure (Corey et al., 1996; Rygalov et al., 2001, 2002; Spanarkel and Drew, 2002). Past studies of pressure effects...
on plants have typically used small to moderate scale growth chambers that allowed short-term experiments of small numbers of compact crops (Musgrave et al., 1988; Corey et al., 1996; Daunicht and Brinkjans, 1996; Iwabuchi et al., 1996; Massimino and André, 1999; Ferl et al., 2002a; Goto et al., 2002; Spanarkel and Drew, 2002; Richards et al., 2006; He et al., 2009). Many of these pioneer systems were built from chambers originally constructed for an alternate use, and often lacked the appropriate degree of environmental control (e.g., temperature, humidity, CO₂ partial pressure, O₂ partial pressure) (Daunicht and Brinkjans, 1996; Massimino and André, 1999). Additionally, deficiencies in available instrumentation and control systems limited the ability to produce consistent and reliable results (Daunicht and Brinkjans, 1996; Spanarkel and Drew, 2002).

The effects of reduced pressure and oxygen partial pressure on long-term plant growth and development have not been fully characterized. Due to increased diffusion coefficients at low atmospheric pressures, it has been hypothesized that there will be increased gas exchange between the plants and their environment thereby increasing photosynthesis by elevating the availability of carbon dioxide in the mesophyll (Gale, 1973; Rygalov et al., 2002). Past experiments have examined a variety of species including wheat (Massimino and André, 1999), tomato (Rule and Staby, 1981; Daunicht and Brinkjans, 1996), lettuce (Corey et al., 1996; Spanarkel and Drew, 2002; He et al., 2003; He et al., 2006, 2007), spinach (Iwabuchi et al., 1995), rice (Goto et al., 2002), and radish (Levine et al., 2008). But the results of these studies are difficult to compare if both total pressure and O₂ partial pressure (pO₂) changed (Richards et al., 2006; He et al., 2007, 2009) and if total exposure times varied (Iwabuchi and Kurata, 2003). Thus, depending on the study, the effects of pressure on plant growth have varied, but it is apparent that plants can withstand some degree of atmospheric alteration and that some acclimation likely occurs (Goto et al., 2002; Paul et al., 2004; Richards et al., 2006).

Many plant studies have shown the importance of atmospheric oxygen in seed development (Quebedeaux and Hardy, 1973, 1975; Musgrave and Strain, 1988; Kuang et al., 1998; Wehkamp et al., 2007) and during seed germination and early seedling establishment (Bewley and Black, 1994). As well, many plant species can tolerate some level of decreased oxygen partial pressure (hypoxia), such as deep in root tissue or during periods of high rates of cellular metabolism (Geigenberger, 2003; Bailey-Serres and Chang, 2005). Oxygen partial pressures are known to affect plant growth and development and the plant’s responses are dependent on the cell or tissue types, developmental stage, genotype, severity and duration of hypoxia, light and temperature conditions (Fukao and Bailey-Serres, 2004). Low oxygen levels reduce respiration by limiting adenosine triphosphate (ATP) production by oxidative phosphorylation (Geigenberger, 2003; Fukao and Bailey-Serres, 2004; Bailey-Serres and Chang, 2005), and in C3 plants low oxygen can increase net carbon exchange rates (NCER) and reduce photorespiration by reducing the oxygenase activity of RuBisCO (Warburg effect). Recent studies with lettuce grown under different combinations of pressure and oxygen have shown that pressures down to 25 kPa do not adversely affect gas exchange or vegetative growth (He et al., 2006, 2007, 2009).

To study the effects of reduced pressure and oxygen partial pressure on the growth and development on plants further, the following study was conducted with radish plants, an edible root vegetable, which have storage organs (hypocotyls) partially below ground and hence might be especially sensitive to reduced pO₂ levels. In this experiment, all other environmental parameters were maintained at stable setpoints and the radish plants were grown from young seedlings in order to ascertain the effects of reduced total pressure and/or oxygen partial pressure throughout plant development. Radish was chosen as the test crop due to its rapid growth, high harvest index, desirable nutritional characteristics, and its common inclusion as a candidate crop for space-based life support systems (Salisbury and Clark, 1996).

MATERIALS AND METHODS

Hypobaric Chambers

Each of the four fully automated (Argus Controls, White Rock, British Columbia, Canada)
hypobaric chambers used in this investigation measured 1.0 x 1.8 x 2.5 meters (WxHxD), with a total volume of approximately 4500 liters and an internal plant growing area of 1.5 m² (Figure 1). All internal surfaces were made of 316 stainless steel, with the exception of the heat exchangers, which were made of brass with a Heresite baked enamel coating, and the glass roof panels (20.5 mm 2 layer laminate).

Figure 1. One of five hypobaric chambers in the Controlled Environment Systems Research Facility (CESRF) at the University of Guelph (left). Visible are the main door and closing mechanisms, lighting canopy, and nutrient system. Also shown is a 21 day old radish crop prior to harvest (right).

Control of temperature and vapour pressure deficit (VPD - the difference between the amount of moisture in the air and how much moisture the air can hold when it is saturated at a given temperature) was performed by recirculating chamber air with a variable speed blower through chilled water (5°C) and hot water (55°C) heat exchange coils located at the rear of each chamber. The cold exchange coil was used to control the VPD, while the hot exchange coil was used to reheat the cooled air to regulate the final desired temperature. In order to maintain adequate airflow through the plant canopy at all pressures, the blower speed control was coupled to monitored pressure values. Chamber temperature and relative humidity were measured using two combination T/RH sensors (Model 4139: Honeywell Inc., Mississauga, ON, Canada) per chamber. Temperature control on the hot and cold heat exchange coils utilized Argus TN2 temperature sensors (4 per chamber), as did the nutrient film technique (NFT) media temperature probes (2 per chamber). Control of temperature averaged +/- 0.5°C over the course of the experiments at all pressures. VPD remained within 0.05 kPa of setpoint for the duration of the
experiments, although there were slight fluctuations during transitions between day and night due to changes from the lamp heat load. As VPD is coupled to temperature, the changes were greater in the low pressure (33 and 10 kPa) treatments. Relative humidity was controlled within +/- 2.5% of setpoint.

Evapotranspiration was measured by collecting atmospheric condensate from the heat exchange coils using a tipping bucket (Model: WS-7048U, La Crosse Technology, La Cross, WI, USA). The resolution obtained using this method was approximately 4.2 mL. Condensate values were converted to liters of evapotranspiration per m² per day and corrected for direct evaporation using condensate production rates collected when seedlings were small and transpiration was negligible. This provided an estimate of actual plant canopy transpiration.

Pressure control utilized a large vacuum pump (Model NC0070.ABMG.000F, Busch Vacuum, Mississauga, ON, Canada), capable of reducing ambient pressure to less than 1 kPa. Chamber pressure was measured using a pressure sensor (MKS Baratron Type 627B, MKS Instruments, Ottawa, ON, Canada) and was continuously monitored by the control system. When the pressure was above the setpoint, the control system opened a solenoid valve (Model SS-9254-C-SI, Swagelok, Sarnia, ON, Canada) connected to the low pressure system. The available control range was +/- 0.1 kPa, however a larger range was used to minimize potential volume losses during heating/cooling cycles, which alter chamber pressure and could signal a requirement for air removal.

The carbon dioxide/oxygen sampling system was based on repressurization of hypobaric chamber air. Air was continuously removed by a vacuum pump (Model UN820.3 FTP, KNF Neuberger Inc., Trenton, NJ, USA) and repressurized in a sampling loop controlled by a non-bleed precision pressure regulator (Model 35503020, Parker Hannifin Instrumentation Products Division, Cleveland, OH, USA) and needle valve (Model H-300-SS-L-R-1/4-A: HAMLET Valves and Fittings, Mississauga, ON, Canada) for precise control. A pressure gauge (Model 25-210-3psi: Noshok, Berea, OH, USA) pH levels were maintained through manual adjustment daily to +/- 0.1 pH units. Acid, base, was used to monitor and manually set the sampling stream to 1.4 kPa at chamber pressures from 90 to 10 kPa. Ambient pressure treatments did not require pressurization for sampling. Prior to introduction of the gas stream to the CO₂/O₂ analyzer (Model 200: California Analytical Instruments, Inc., Orange, CA, USA for 0-6000 μmol mol⁻¹, LI-COR LI-820, Lincoln, NE, USA for 0-20,000 μmol mol⁻¹), the air stream was chilled to remove water. Condensate and the sampled air stream were returned to the chamber to ensure full system gas loop closure.

Chamber gas composition was controlled by analyzer feedback to the control system that operated separate mass flow controllers (Model 810S: Sierra Instruments, Inc., Monterey, CA, USA) for pure oxygen, carbon dioxide, and nitrogen gases. Pure gases were supplied by external K-size cylinders (BOC Gas Supply, Ltd., Guelph, ON, Canada). The available carbon dioxide control range was between 0 and 20,000 μmol mol⁻¹, while oxygen could be controlled between 0 and 100%. Nitrogen was used to make up the balance of the gas composition.

Chambers were outfitted with six 1000 Watt HPS lamps (P.L. Light Systems Inc., Beamsville, ON, Canada). The externally mounted lighting canopy was cooled with a chilled water heat exchanger coupled to a blower that circulated chilled air across the chamber glass roof panels. Two LI-190SA PAR sensors (LI-COR Inc., Lincoln, NE, USA) continuously monitored irradiation from the light source.

The nutrient solution delivery system utilized a nutrient film technique design. Water was stored in a 200 liter temperature controlled external stainless steel tank. A pump (Model PKG-UOG: International Pump Technology Inc., Fergus, ON, Canada) provided sufficient pressure for in-tank circulation through a sensor loop and chamber trough delivery. Return of water from the chamber was by gravity. All external storage tanks for stock nutrients, acid, and base were maintained at chamber pressure through a series of pressure compensation lines. Electrical conductivity (EC) sensors (Argus Control Systems, Inc., White Rock, BC, Canada) were used to measure nutrient concentration and EC control with a setpoint of 1200 μS was +/- 10 μS. and nutrient stock solutions were added using gravity feed from stainless steel reservoirs
controlled with air actuated valves (Model SS-92S4-C-S1, Swagelok, Sarnia, ON, Canada).

**Assessment of Growth and Productivity**

Plant growth and productivity measurements for the series of 21-day pressure studies with radish included dry mass of roots (swollen hypocotyls) and leaves as well as leaf area (LA). Values were obtained using an electronic balance (Sartorius, Gottingen, Germany) and a leaf area meter (LI-3000, LI-COR Inc., Lincoln, NE, USA). Data were collected on a per plant basis for the entire chamber and harvest index (HI) and specific leaf area (SLA) calculated from the resultant data. No attempts were made to retrieve fibrous roots from the rockwool media, but previous hydroponic studies have shown these comprise only about 5% of the total plant biomass (Mackowiak et al., 1994).

**Carbon Assimilation and Dark Respiration**

Whole stand NCER measurements were calculated from the slope of the daily carbon dioxide injection profile. Dark period respiration rates were calculated from the slope of the carbon dioxide evolved over the night period. As it was impossible to access the plants during the experiment, the photosynthesis, respiration, and transpiration data could not be reported on a dry weight or leaf area basis and was therefore expressed on the available growing area. Hence these rates are closely correlated with changing canopy cover (and light interception) as the plants grow. The growth period examined was from 3-17 days after planting (DAP). Carbon assimilation and dark respiration data for 18-21 DAP were not available due to subsequent tests to determine light and carbon dioxide compensation points, which are not reported here.

**Plant Material**

Radish (*Raphanus sativa* L. cv. Cherry Bomb II) was grown from seed in 1.4 x 0.4 m stainless steel troughs with stainless steel covers to minimize evaporation and algal growth on the growth medium. Each cover had 4 cm diameter holes placed at 9-10 cm centers within the rows and between troughs. There were five troughs per chamber and 24 plants per trough for a total of 120 plants per chamber. Rockwool slabs (Grodan, Hedehusene, Denmark) were used as the growth medium and a channel was cut from the underside of the slab to facilitate nutrient solution flow. Prior to planting, the rockwool was rinsed twice with deionized water to remove any fabrication residues or particulates. Nutrient solution was a modified, half-strength Hoagland’s solution (Wheeler et al., 1999) and the solution was maintained at an electrical conductivity of 1200 µS cm⁻¹ using concentrated stock solutions. pH was manually adjusted daily to 5.8 with 0.5 M nitric acid (HNO₃) or 0.5 M potassium hydroxide (KOH). Prior to the experiment, the nutrient reservoirs, feed lines, and troughs were rinsed with >10 ppm aqueous ozone, and the nutrient stock solutions were autoclaved to reduce microbial contamination for a concurrent bacterial study not reported here.

Radish seeds were planted three per position and allowed to germinate under ambient pressure (98 kPa) for 72 hours. Seedlings were then thinned to one per position and the defined pressure/oxygen treatment imposed. All plants were harvested at 21 days after planting (DAP).

**Experimental Conditions**

Temperature was isothermal and held at 22ºC with a 16/8 day/night photoperiod. A light intensity of 300 µmol m⁻² s⁻¹ PPF at the hydroponic trough level was provided by dimming the lamps with neutral density screening. Carbon dioxide and the VPD were maintained at partial pressures of 0.12 kPa (equivalent to 1200 µmol mol⁻¹ at ambient pressure) and 0.9 kPa (equivalent to 65% RH at 22ºC), respectively. Treatments included four total pressures (10, 33, 66, or 98 kPa) and four oxygen partial pressures (2, 7, 14, or 20 kPa) with 98/20 kPa combination acting as the ambient control. The average atmospheric pressure in Guelph, Ontario, Canada (334 meters above sea level) averages approximately 98 kPa.

**Statistical Analysis**

Experimental design for the 21-day plant tests was a randomized block design with each of four chambers being considered a replicate and each treatment was replicated four times. Replication was achieved over time and replicates were cycled through the four chambers to minimize chamber effects.
Regression analysis was performed in S-PLUS version 7.0/8.0 for Windows (Insightful Corporation, Seattle, WA, USA) and ANOVA analysis was performed using SAS version 9.1.3 for Windows (SAS Institute Inc., Cary, NC, USA).

RESULTS

Growth

Visually, there was little difference observed in the quality of the radish plants harvested from the control and the reduced pressure treatments (Figure 2) and there was no significant difference in leaf dry mass, root dry mass, specific leaf area (SLA), or harvest index (HI) across the different pressures when the oxygen partial pressure was at 7, 14, or 20 kPa (Table 1). There was, however, a visual decrease in plant size with plants grown at 2 kPa of oxygen being clearly stressed and stunted. Similarly, there were significant decreases leaf and root dry mass, SLA, and HI at an oxygen partial pressure of 2 kPa regardless of the total pressure (Table 1).

Figure 2. Visual analysis of radish (*Raphanus sativa* L. cv. Cherry Bomb II) grown at ambient, 66, 33, or 10 kPa total pressures (from left to right) and oxygen partial pressures (from top to bottom). Pictures were taken during harvest which was 21 days after planting.
Table 1. The effect of atmospheric pressure (10-98 kPa) and oxygen partial pressure (2-20 kPa) on the growth of radish (*Raphanus sativa* L. cv. Cherry Bomb II) over a 21-day period. The partial pressure of carbon dioxide was maintained at 120 Pa.

<table>
<thead>
<tr>
<th>Oxygen (kPa)</th>
<th>Pressure (kPa)</th>
<th>Leaf dry mass (g plant⁻¹)</th>
<th>Root dry mass (g plant⁻¹)</th>
<th>Specific Leaf area (cm² g⁻¹)</th>
<th>Harvest Index (%)</th>
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<tr>
<td>7</td>
<td>98</td>
<td>0.596¹ (0.0080)a²</td>
<td>1.19 (0.019)a</td>
<td>286.9 (1.64)a</td>
<td>65.80 (0.253)a</td>
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<td>14</td>
<td>66</td>
<td>0.619 (0.0086)a</td>
<td>1.17 (0.018)a</td>
<td>255.5 (5.05)a</td>
<td>64.64 (0.296)a</td>
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<td>33</td>
<td>0.593 (0.0086)a</td>
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<td>64.79 (0.278)a</td>
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<td>98</td>
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<td>1.16 (0.017)a</td>
<td>284.2 (2.03)a</td>
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¹The means reflect the average of single plants harvested from four replications of the experiment. The standard errors of the means are contained in brackets.

²Means comparison performed with PROC Mixed lsmeans (p ≤0.05). Means with the same letter within the same column are not significantly different.

**Carbon Assimilation and Dark Respiration**

There was little change observed in whole canopy net carbon exchange rates over the range of pressures from 33-98 kPa and oxygen partial pressures from 7-20 kPa (Figure 3). At 10 kPa total pressure and 7 kPa of oxygen there was a slight but significant decrease in the rate of canopy carbon assimilation, and at 2 kPa oxygen there was a further decline (approximately 50%) (Figure 2, Table 1). Similarly, dark period canopy respiration showed no significant change over the entire 10-98 kPa range of pressures and oxygen levels down to 7 kPa. As with canopy carbon assimilation, respiration levels were decreased by approximately 50% at an oxygen level of 2kPa (Figure 3).

**Transpiration**

The response of canopy transpiration to reduced pressures and oxygen showed results similar to those observed with carbon assimilation. There was little effect in response to pressures from 33-98 kPa and oxygen partial pressures of 7-20 kPa, with all the rates near 4 L m⁻² day⁻¹ at 17 DAP (Figure 4). Transpiration at 10 kPa of total pressure and at oxygen partial pressures of 7 and 2 kPa were significantly lower than the other treatments, and barely measurable at the 10/2 kPa treatment level.

**DISCUSSION**

There have been few reports describing plant growth, NCER, and transpiration at reduced atmospheric pressure and reduced partial pressure of oxygen at a canopy scale. In this study, hypobaria (10, 33, or 66 kPa) had no significant effect on biomass productivity. Canopy photosynthesis, dark respiration, and transpiration were unaffected by pressure in all but the 10 kPa total pressure treatment. Similarly, oxygen partial pressures of 7 kPa or greater...
Figure 3. Whole canopy carbon assimilation and respiration rates (µmol m⁻² growing area s⁻¹) for radish (*Raphanus sativa* L. cv. Cherry Bomb II) grown with atmospheric pressures of 98, 66, 33, or 10 kPa and oxygen partial pressures of 2, 7, 14, or 10 kPa over a 21-day period. Data shown (3-17 DAP) represent the period from chamber closure until a series of secondary tests performed at 18-20 DAP. The pCO₂ was maintained at 120 Pa. The error bars represent the standard error of the means.
Figure 4. Transpiration (liters m$^{-2}$ growing area day$^{-1}$) for radish (*Raphanus sativa* L. cv. Cherry Bomb II) grown at atmospheric pressures of 98, 66, 33, or 10 kPa and oxygen partial pressures of 2, 7, 14, 20 kPa. The pCO$_2$ was maintained at 120 Pa. Data shown (3-17 DAP) represent the period from closure until a series of secondary tests were performed at 18-20 DAP. Error bars represent the standard error of the means. Evaporation values (total evapotranspiration at 3 DAP) were subtracted from total daily evapotranspiration to provide an estimate of transpiration.
(7, 14, or 20 kPa) showed no significant effect on reported parameters. Only when the partial pressure of oxygen was reduced to 2 kPa was growth significantly reduced. These results agreed with previously published findings that demonstrate that plants can be grown for extended periods under hypobaric conditions with no detrimental effect on net productivity (Dixon et al., 2005; He et al., 2007).

Growth

Across all treatments from 10-98 kPa of pressure and 7-20 kPa of oxygen, radish growth parameters showed only minor decreases, which were similar to that observed by Levine et al. (2008), but these differences were not statistically significant. Hypobaric studies with lettuce (Spanarkel and Drew, 2002; Dixon et al., 2005; He et al., 2006, 2007, 2009) and spinach (Iwabuchi et al., 1995) also suggested that long-term growth at reduced pressure was comparable to ambient levels. Thicker leaves, as indicated by the decrease in SLA, were observed at reduced pressures. From this observation, one might expect a reduction in growth as the thicker leaves reduce the LA available to capture incident light. At an oxygen partial pressure of 2 kPa, the suppression of radish growth was most significant and would not be suitable in life support conditions where the crops would be the main source of nourishment for the crew and act as the air regeneration system. Although the low oxygen should have favored photosynthesis through the inhibition of the oxygenase activity of RuBisCO (i.e., photorespiration), the elevated CO\textsubscript{2} partial pressure (0.12 kPa) across all treatments should have eliminated photorespiration. It is likely the 2 kPa O\textsubscript{2} limited essential aerobic metabolism in the roots and throughout the plants during the dark cycles, resulting in decreased growth, and the adverse effects of the low oxygen partial pressures on radish are consistent with studies of lettuce, which showed reduced growth at 6 kPa O\textsubscript{2}, irrespective of total pressure (He et al., 2007).

Carbon Assimilation and Dark Respiration

We detected no significant differences in the rate of carbon assimilation at reduced atmospheric pressure when oxygen was kept at or above 7 kPa and total pressure was at or above 33 kPa. At 10 kPa total pressure and 7 kPa oxygen however, carbon assimilation was reduced when compared to the higher oxygen and pressure treatments, which is consistent with gas exchange measurements with lettuce under hypoxic condition and saturating CO\textsubscript{2} (He et al., 2007, 2009). This contrasted with the growth results, which showed no significant difference in dry mass accumulation in this treatment compared to the others. With all other variables (O\textsubscript{2}, VPD, T) being equal within each pressure series, this response is likely due to hypobaria alone. The plant response to hypobaria is complex, as demonstrated by the altered regulation of more than 200 genes (Paul et al., 2004). It is probable that long-term changes and acclimation are occurring, as noted by the decreased variation in pressure treatments observed by Richards et al. (2006) after only 16 hours of acclimation. The higher pressure treatment (33, 66, or 98 kPa) results were contrary to previous short-term studies in wheat (Massimino and André, 1999) and tomato (Rule and Staby, 1981), but were consistent with the long-term results with spinach (Iwabuchi et al., 1995) and lettuce (He et al., 2007). Although enhanced photosynthesis at lower pressures was expected due to the increased diffusion coefficients at low atmospheric pressures (Gale, 1973; Rygalov et al., 2002), it is not clear whether the observed lack of improvement was due to physical, biochemical, or combined adaptations, i.e., plant acclimation.

Enhanced photosynthesis in response to low pressure environments previously observed by others was likely due to the inhibition of the oxygenase activity of RuBisCO, often seen when plants are subjected to reduced oxygen partial pressures (Warburg effect), particularly if the pCO\textsubscript{2} dropped with pressure, and as a secondary effect of decreasing the atmospheric pressure (Zelitch, 1983; Musgrave and Strain, 1988). Our studies used enriched carbon dioxide levels (0.12 kPa) across all treatments, which likely suppressed photorespiration even at the highest oxygen partial pressure used in our tests (Maleszewski et al., 1988; Drake et al., 1996).

In this study with radish, canopy dark respiration was unaffected by either hypobaria or hypoxia at oxygen partial pressures as low as 7 kPa. This differed from the results of He et al. (2009) who found a reduction in dark respiration in lettuce with the atmospheric pressure reduced.
to 25 kPa in lettuce. When coupled with the decreased overall NCER (Figure 3) and no observable difference in vegetative growth (Table 1) between this and the other treatments, it is likely there was an increase in overall net carbon uptake induced by the reduced pressure. Gene expression in *Arabidopsis* has been shown to be different between hypoxia and with hypobaria (Paul et al., 2004), suggesting that the observed decrease in dark respiration was not a result of the lower oxygen partial pressure but is in fact unique to hypobaria.

Because the chambers could not be opened or accessed without violating total pressure and gas partial pressure control, all gas exchange measurements could only be monitored for the entire canopy. The reduced growth and reduced canopy cover at the lowest oxygen partial pressure would have resulted in less light interception, which would clearly affect NCER. This makes it difficult to assess whether there were direct effects of the reduced pO2 on a per unit leaf area basis. This might be addressed in future studies by having real-time measurements of canopy cover and normalizing the data for an actual canopy cover. Yet this would still be complicated by overlapping leaves within the canopy.

**Transpiration**

Given that the VPD for any given temperature is not affected by pressure one might conclude that transpiration would remain stable, but enhanced transpiration at reduced atmospheric pressure has been postulated due to the corresponding decreased aerodynamic resistance and increased diffusion coefficients (Gale, 1973; Iwabuchi and Kurata, 2003). However, as with the carbon exchange rates in this study, little effect was noted in transpiration among treatments from 33-98 kPa total pressure and 7-20 kPa of oxygen. The transpiration trends observed here are similar to the results of Iwabuchi and Kurata (2003), who noted no difference in the transpiration of spinach after acclimation by the plants to a low pressure environment; yet the findings contrast with those of Richards et al. (2006) where transpiration of *Arabidopsis* increased as much as of 50% at reduced pressures. Terashima et al. (1995) suggested that increased transpiration would be observed at reduced pressures even if the VPD remained constant (as in this study) due to the flux driven by the increased diffusion coefficient. Iwabuchi et al. (1995) explained their lack of increased transpiration rates as a secondary response in which the increased diffusion coefficient initially accelerated transpiration, which lowered the leaf temperatures. The plants apparently acclimated to the pressure environment and maintained similar transpiration rates. Migge et al. (1999) suggested that the thicker leaves used to dissipate radiant energy would result in decreased water loss. Our results suggest that radishes under the conditions of this study may have acclimated to their pressure environments and used an adaptive response to maintain constant transpiration rates, similar to the results with spinach by Iwabuchi and Kurata (2003). Modifications in stomatal aperture, pore length or numbers, leaf thickness, and leaf temperature may have all contributed to the transpiration rates observed.

This study demonstrated the ability of radish plants to withstand the effects of reduced pressures from seedling to harvest. Contrary to some previous observations, NCER and transpiration with radish in our studies were not greatly enhanced and dark respiration was not suppressed by reduced pressure (Corey et al., 1996; Daunicht and Brinkjans, 1996; Massimino and André, 1999; Musgrave et al., 1988; Spanarkel and Drew, 2002). Clearly, results may vary among species and there might be a number of complex factors involved that cannot be assessed using past methods of excised plant tissues, or through short-term investigation using plants first established at ambient conditions, as the short-term gains in photosynthetic capacity can be offset by adaptive measures in the long-term (Usuda, 2006). These results represent a comprehensive analysis of plant growth from seedlings under hypobaric and reduced oxygen partial pressure with respect to the range of atmospheric alteration and control. Further research into the biochemical and morphological adaptations created by reduced atmospheric pressure is warranted and consideration should be given to the age and stage of development of the plants studied.
ACKNOWLEDGEMENTS
The authors would like to thank the National Science and Research Council of Canada for their support of our current research program, Argus Control Systems Ltd. for their technical assistance, and NASA and Dynamac Inc. for their collaboration in experimental design. We also would like to acknowledge the Canadian Foundation for Innovation, the Ontario Ministry of Agriculture and Food, the Ontario Centres of Excellence - Earth and Environmental Technologies (OCE - ETech), the Canadian Space Agency, and the University of Guelph for their contributions in the establishment and continuing support of the Controlled Environment Systems Research Facility.

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Levine, L.H., Bisbee, P.A., Richards, J.T.,


Spaceflight Reduces Foreign Protein Expression in Tissue-engineered Skeletal Muscle

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ABSTRACT

Skeletal muscle atrophy is a well-known effect of spaceflight in both humans and animals. Although the mechanisms of this effect is multifactorial, it has previously been demonstrated in an in vitro model using tissue-engineered skeletal muscle (Bioartificial Muscles, BAMs) that spaceflight can cause skeletal muscle atrophy at the tissue level through down-regulation of total endogenous protein synthesis rates. In this study, BAMs were engineered from avian skeletal muscle cells genetically engineered to secrete recombinant human growth hormone (rhGH) and flown aboard the space shuttle on a 9-day mission to determine the effects of spaceflight on the synthesis of a foreign protein whose gene is controlled by a constitutively expressed retroviral promoter. Spaceflight did not significantly affect the rate of BAM glucose metabolism, but compared to ground-control BAMs, space-flown BAMs secreted 47.5% less rhGH (p<0.05). Based on this study, it appears that spaceflight has a direct negative effect on the secretion of a foreign protein in an in vitro tissue-engineered skeletal muscle model.

INTRODUCTION

Spaceflight-induced skeletal muscle atrophy has been well-documented in both humans and animals (Fitts et al., 2001; Ohira et al., 1992). Various mechanisms have been proposed, including mechanical unloading, humoral factors, direct tissue effect, and disuse.

Space-flown experiments using tissue-engineered skeletal muscle constructs have demonstrated that spaceflight can directly induce skeletal muscle atrophy in an in vitro model, resulting in a decrease in myofiber cross-sectional area and protein synthesis (Vandenburgh et al., 1999).

BioArtificial Muscles (BAMs) are three-dimensional tissue-engineered skeletal muscle constructs that have been used as an in vitro model for studying skeletal muscle physiology (Powell et al., 2002). BAMs have many in vivo characteristics and can be maintained in vitro for extended periods of time, making them an ideal model system for in vitro skeletal muscle studies (Chromiak et al., 1998).

Among the many potential strategies for preventing spaceflight-induced muscle atrophy,
various pharmacologic interventions have been proposed (Vandenburgh et al., 1999). Anabolic factors such as growth hormone and insulin-like growth factor 1 (IGF-1) have been demonstrated to have myogenic effects both in vivo and in vitro (Sacheck et al., 2004; Shansky et al., 2006). BAMs have been proposed as a potential platform for the delivery of therapeutic proteins to treat a range of conditions (Lu et al., 2002; Thorrez et al., 2006). It may be possible to implant BAMs made from autologous cells that have been genetically engineered to secrete myogenic proteins as a potential long-term countermeasure strategy for attenuation of spaceflight-induced muscle atrophy.

However, the effects of spaceflight on the expression of foreign genes or proteins in genetically engineered muscle cells are unknown. BAMs are an ideal model for studying the direct effects of spaceflight on the various stages of gene and protein expression of both native and foreign genes and proteins.

In this experiment, BAMs made from embryonic avian skeletal muscle cells genetically engineered to secrete human growth hormone were flown on the space shuttle to determine the effects of spaceflight on the expression of a foreign protein. The results of this experiment may have potential implications for both the application of gene therapy technologies in space as well as the use of genetically engineered cells for the study of the effect of spaceflight on cellular and tissue physiology.

MATERIALS AND METHODS

Cell Culture and Tissue Engineering Techniques

Cell isolation

Embryonic avian skeletal muscle cells were isolated from 12-13 day in ovo pectoralis muscle (SPAFAS, Preston, CT) using standard dissection techniques (Vandenburgh et al., 1988). The cells were cultured at 37°C and humidified with 5% CO₂ in growth medium (85/10/5) consisting of bicarbonate-buffered Eagle's Basal Medium (1.0 mg glucose/L) (GIBCO-BRL, Gaithersburg, MD) containing 10% horse serum (Sigma Chemical Co., St. Louis, MO), 5% chicken embryo extract and 50 U/ml penicillin G (Sigma). The avian muscle cells were then stably transduced with the recombinant human growth hormone (rhGH) gene with the MFG-hGH retroviral vector as described previously (Vandenburgh et al., 1998).

Retroviral transduction of avian muscle cells

Avian muscle cells were transduced with a replication deficient retroviral vector containing cDNA encoding the rhGH gene under the control of the constitutively expressed long terminal repeat (LTR) promoter. The MFG-hGH packaging cells were thawed and incubated at 37°C in Dulbecco's modified Eagle's medium (DMEM; Life Sciences Technologies, Grand Island, NY) containing 10% calf serum and 50 U/ml penicillin G. The virus-containing medium (VCM) was collected when the cells were confluent, pooled, and filtered through a 0.45 µm filter. The VCM was aliquotted and stored at -80°C until ready for use. VCM was thawed and fed to the avian muscle cells for 4 hrs every 24 hrs for a total of 3 incubations. On the day after the final incubation, the cells were harvested with 0.05% (wt/vol) trypsin (Sigma), 0.02% (wt/vol) EDTA in Ca²⁺Mg²⁺-free Earle's balanced salt solution (EBSS), and pelleted by centrifugation at 1,200 rpm for 4 min. The cells were replated and harvested one additional time in order to grow enough cells for tissue-engineering BAMs.

Tissue engineering

Tissue engineering techniques for BAMs formed from avian myoblasts were similar to those described previously (Shansky et al., 2006). Briefly, 5x10⁶ cells were suspended in chilled 85/10/5 growth medium containing 1.6 mg/ml type I rat tail collagen (Collaborative Biomedical Products, Bedford, MA) neutralized with 10% (vol/vol) 0.1 N NaOH. Six parts of this cell suspension were mixed with 1 part ice-cold (vol/vol) Matrigel™ Basement Membrane Matrix (BD Biosciences, Bedford, MA) and immediately cast into molds (0.4 ml/mold) made from silicone rubber tubing (4.8 mm i.d.) cut lengthwise into 30-mm lengths with a 3-mm x 4-mm piece of Velcro™ at each end. The molds containing the cell suspension were incubated at 37°C for 5 hrs before being fed with 85/10/5 growth medium. The cell suspensions form into BAMs, as seen in
Figure 1. Bioartificial muscle (BAM) morphology. A) BAMs (arrow) were tissue-engineered from 5x10^6 primary avian myoblasts isolated from 12-13 day in ovo pectoralis muscles. The cells were suspended in 6 parts type I rat tail collagen: 1 part Matrigel™ and cast into silicone rubber molds with Velcro™ attachments. B) The BAMs were fixed and immunocytochemically stained for sarcomeric tropomyosin showing aligned multinucleated muscle fibers (arrows). Bars in panels A and B equal 10 mm and 750 µm, respectively.

Figure 1a, by attaching to the two Velcro™ ends and become suspended as it lifts off the underlying tubing. The BAMs were maintained in the 37°C incubator and fed 85/10/5 growth medium every 3-4 days until ready for use. As seen in Figure 1b, the myoblasts fuse into parallel arrays of myofibers aligned in the direction of tension between the two Velcro™ ends.

Hardware and Spaceflight

The flight samples were maintained in the Dual Materials Dispersion Apparatus (DMDA, Instrumentation Technology Associates, Inc., Exton, PA). Each well allowed for a total volume of 305 µl and was maintained sealed at 20°C for the duration of the flight. The flight experiment was carried out at 20°C because of the requirements of other experiments with which the hardware was being shared. The ground control samples were maintained in sealed microcentrifuge tubes containing 305 µl at either 20°C or 37°C. 26-day-old BAMs were each cut into 10 equal sections and then randomly divided into three groups. Each group had 4 BAM sections and was maintained either at 37°C on the ground, 20°C on the ground, or 20°C in flight. All samples were loaded steriley in Exton, PA 5 days before launch with the flight samples being transported to Kennedy Space Center while the ground control samples were transported to and maintained in the laboratory in Providence, RI. Appropriate temperature controls were maintained during the transporting of all samples. The flight samples were flown for 9 days on Mission STS-95 aboard the Space Shuttle Discovery and recovered within 24 hrs of landing. Upon sample retrieval, flight and ground samples were processed simultaneously. Aliquots of media samples were stored at -20°C. BAMs were rinsed in chilled EBSS and either stored at -20°C or fixed as detailed below. All flight and ground tissue and media samples were analyzed simultaneously in the same facility after no more than two weeks of storage.
Analytical Methods

Medium glucose

15 µl aliquots were taken from 20ºC and 37ºC ground control samples at 4 time points after initial pre-flight loading until final post-flight sample recovery. The 20ºC flight samples were retrieved after landing. All aliquots were stored at -20ºC until ready for analysis. Samples were thawed and glucose concentration was measured with a Beckman glucose analyzer (Beckman Coulter Inc., Fullerton, CA). At each time point, there were four ground samples and three flight samples.

Total protein & DNA

The BAMs were removed from the chambers, rinsed in chilled EBSS, placed in individual microcentrifuge tubes, and stored at -20ºC for post-flight processing. The BAMs were later thawed and sonicated in 135 µl ice-cold sucrose buffer (0.25 M sucrose, 0.02 M KCl, pH 6.8). 50 µl aliquots were removed from each sonicate for DNA and total noncollagenous protein content determination, as described previously (Chromiak et al., 1998). Two ground and two flight samples were analyzed for total protein and DNA.

Human growth hormone

Media samples were collected after landing and stored at -20ºC until ready for rhGH assaying. Total rhGH concentrations in the samples were determined by an rhGH radioimmunoassay (Diagnostic Systems Laboratories Inc., Webster, TX). This assay is accurate and linear from 0.20 ng/ml to 40 ng/ml, as confirmed with each assay using the kit supplied controls. The samples were processed per kit instructions. Four ground and three flight samples were analyzed.

Immunocytochemical staining

Prior to sectioning, whole BAMs were fixed with 1:4 DMSO:methanol and stained immunologically with an antibody against sarcomeric tropomyosin (Sigma, Cat. #T-9283) followed by an avidin-biotinylated secondary antibody coupled to horseradish peroxidase (Vectastain, Vector Laboratories, Burlingame, CA). This was developed with 3,3’-diaminobenzidine (DAB), forming a brown precipitate. The stained cells were viewed as a whole mount with a Zeiss microscope (total magnification 100X to 1000X).

Statistical analysis

Calculation of means and standard deviation were made with SIGMASTAT software (Jandel Scientific, San Rafael, CA). Group means of measurements at different time points within groups, and those comparing measurements between groups, were compared using paired and unpaired t tests, respectively. ANOVA was used to compare differences among multiple time points within each group. p<0.05 was accepted as being statistically significant.

RESULTS

Glucose Utilization (20ºC vs 37ºC; Flight vs. Ground)

Avian skeletal muscle myoblasts were stably transduced with a retroviral vector to secrete assayable levels of rhGH and then tissue engineered into BAMs. Individual sections of BAMs were either flown at 20ºC for 9 days in spaceflight or maintained in appropriate ground controls at 20ºC or 37ºC. The rates of BAM metabolism in ground control samples at the two temperatures were determined from sample aliquots collected at 4 time points throughout the duration of the experiment. Figure 2a demonstrates that the rate of glucose utilization was linear from 5 days pre-launch to 10 days post-launch at both temperatures in ground controls. Significant glucose utilization was also observed between the final two time points, 5 and 10 days post-launch (p<0.05), indicating that the BAMs were both viable and metabolically active up to the end of the experiment. However, glucose utilization was significantly lower at 20ºC (136.6. ± 37.5 µg/BAM) compared to 37ºC (804.2 ± 212.5 µg/BAM) over 15 days. The differences between the two groups were statistically significant at each time point measured (p<0.005).
As seen in Figure 2b, compared to ground controls, there was no statistical difference in the rate of glucose utilization in the spaceflight samples at 20°C.

**Protein and DNA Content**

BAM total noncollagenous protein and DNA content were assayed using samples collected and processed post-flight. Figures 3a and 3b demonstrate that, compared to time-matched ground controls at 20°C, noncollagenous protein and DNA measurements were reduced in flight samples by 40.6% and 18.7%, respectively. However, these decreases were not statistically significant, likely due to the small sample size of two samples per group. The two groups had similar protein/DNA ratios, as shown in Figure 3c.

**Recombinant Human Growth Hormone Secretion**

The total amount of rhGH secreted by the BAMs was determined by assaying samples collected post-flight. A reduction in temperature from 37°C to 20°C in ground controls resulted in a 41.6% decrease in the total amount of rhGH secreted (p<0.001), with each BAM section secreting an average of 4.46 ± 0.10 ng/10^6 cells/day and 2.60 ± 0.45 ng/10^6 cells/day, respectively. Figure 4 shows that compared to ground controls at 20°C, there was a 47.5% decrease in the total amount of rhGH secreted in the spaceflight group (p<0.05), from 19.53 ± 0.34 ng/BAM to 10.26 ± 4.40 ng/BAM, indicating that spaceflight had a direct effect on the ability of the BAMs to secrete rhGH. The average rhGH secretion rate of the spaceflight BAMs was 1.37 ± 0.59 ng/10^6 cells/day.

**DISCUSSION**

Using tissue-engineered skeletal muscle constructs genetically engineered to secrete rhGH, we have demonstrated in an in vitro model system that spaceflight appears to have a direct effect on the secretion of a foreign protein. To the best of our knowledge, this is the first, or one of the first, uses of genetically engineered animal cells in a spaceflight experiment.

This experiment did have limitations, owing largely to limitations of the hardware used. The Dual Materials Dispersion Apparatus (DMDA) that was used was shared with other flight experiments requiring that the temperature be maintained at 20°C for the duration of the experiment. Although this was not an ideal temperature for a tissue culture experiment, we clearly demonstrated that there was ongoing glucose metabolism and rhGH secretion at the
A lower temperature. And despite the decrease in overall metabolism at 20ºC, we were still able to demonstrate a significant difference in the amount of rhGH secreted in spaceflight compared to temperature-matched ground controls. The tissue wells available to us were also of limited volume. But again, despite the small volume of culture medium used and the lack of a circulating tissue culture system, we were able to demonstrate ongoing tissue metabolism until the end of the experiment. The small volume also required that we use sections of BAMs that were no longer under tension.

Previous experiments using tissue-engineered BAMs demonstrated for the first time in an *in vitro* model system that although spaceflight does not affect tissue metabolism, the rate of synthesis of endogenous noncollagenous protein is significantly decreased without a concomitant increase in protein degradation (Vandenburgh et al., 1999). Although it is reasonable to suspect that a down-regulation in protein synthesis may also be the primary mechanism for the decrease in rhGH secretion in this experiment, due to the above-mentioned limitations of this study, such a conclusion cannot be made. Given that the rhGH gene was under the control of the constitutively expressed LTR promoter, a gene-specific down-regulation at the transcriptional level is possible, but unlikely. A generalized down-regulation in transcription is possible. Cell death, protein degradation, and alterations in protein trafficking and secretion were also not specifically studied and thus cannot be excluded as having a role in the decrease in rhGH secretion. However, previous experiments have not shown these mechanisms to be significantly altered by spaceflight in BAMs (Vandenburgh et al., 1999).

Although there was a small decrease in DNA content in the flight group, the difference was not statistically significant. While the lack of statistical significance may be largely due to the small sample size of two, it may also be that the difference is truly small or insignificant suggesting that cell death or down-regulation in cellular replication is not a major contributor to the decrease in rhGH secretion.

Although numerous human and animal studies have demonstrated the effects of spaceflight on skeletal muscle atrophy, the exact mechanisms leading to these effects are still unclear. The use of an *in vitro* system eliminates potential indirect effects on skeletal muscle, such as muscle disuse, humoral factors, and neuronal effects. Based on

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**Figure 3.** Total BAM noncollagenous protein and DNA content after spaceflight. BAM tissues were collected and processed post-flight from both flight and ground control groups at 20ºC. Total noncollagenous protein and DNA content were measured, and the protein/DNA ratio was calculated. Bars are mean ± SD of 2 samples per group, compared by unpaired *t* test at *p*<0.05. There was no significant difference between the flight and ground control BAMs.
previous spaceflight experiments, there appears to be a direct effect of spaceflight on skeletal muscle tissue leading to skeletal muscle atrophy (Vandenburgh et al., 1999).

The mechanisms by which the spaceflight environment directly affects these changes at the tissue level are unknown, although changes in muscle tension may have a putative role. The documented disruption of the eukaryotic cell cytoskeleton during spaceflight (Lorenzi & Perbal, 1990) and the dependence of ribosomal activity on the cytoskeleton (Hesketh, 1994) make an attractive working hypothesis for the effects of spaceflight on skeletal muscle protein synthesis rates. The use of genetically engineered cells transduced with genes under the control of various promoters may be an ideal method for confirming the hypothesis and elucidating the molecular mechanisms by which skeletal muscle tissues “sense” the spaceflight environment.

While the value of an in vitro model for studying the effects of spaceflight on muscle atrophy has previously been demonstrated, this experiment additionally demonstrates the potential utility of using genetically engineered cells and tissue-engineered constructs to investigate mechanisms by which the spaceflight environment directly affects changes at the tissue, cellular, and/or molecular level. Furthermore, if gene therapy technologies are to ever be considered as countermeasure therapy for long duration spaceflight (Vandenburgh et al., 1998), the effects of spaceflight on the expression of foreign genes and proteins must be understood. This experiment is the first demonstration of this potential effect and emphasizes the importance of further research in this area. Future in vitro spaceflight experiments using genetically modified, tissue-engineered skeletal muscle constructs are warranted as our understanding of the mechanisms of spaceflight-induced skeletal muscle atrophy on astronauts is still evolving.

ACKNOWLEDGEMENTS

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REFERENCES


Modeled Microgravity Conditions Suppress Innate Macrophage and Lymphocytic Responses to Common Mitogens and *Mycobacterium tuberculosis* Infection

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**ABSTRACT**

Immune dysregulation during and post-spaceflight is well documented. It is essential to understand the consequences of microgravity-induced immune dysfunction in host control of common infectious agents. *Mycobacterium tuberculosis* (MTB) is a facultative intracellular pathogen affecting over a third of the world’s population. Immune control of MTB requires specific immune functions known to be dysregulated during spaceflight. While not feasible to infect animals during actual flight, it is possible to establish *in vitro* conditions of modeled microgravity using a high-aspect rotating wall vessel (HARV). Mouse splenocytes were examined for activity against Bacillus Calmette-Guérin (BCG) and compared to mitogenic mediators, Concanavalin A (ConA) and lipopolysaccharide (LPS). Splenocytes responses to ConA were completely suppressed and lower production of TNF-α and IL-6 were observed with BCG in modeled microgravity conditions. However, splenocytes in modeled microgravity stimulated with LPS displayed increased levels of IL-6 and IL-10. Additionally, high-aspect rotating wall vessel (HARV)-cultured macrophages demonstrated reduced control of MTB growth, suggesting that microgravity conditions may generate an immune environment more conducive to propagation of intracellular MTB.

**Key words**: Bacillus Calmette-Guérin (BCG); *Mycobacterium tuberculosis* (MTB); Microgravity Simulation

**INTRODUCTION**

The immune dysregulation occurring during and post spaceflight poses significant challenges to human health during prolonged space explorations (Crucian and Sams, 2009; Gueguinou et al., 2009). Studies from isolated leukocytes (humans and rodents) post spaceflight indicate a wide range of effects, evidenced by decreased T-cell response to mitogen stimulation (Cogoli, 1996; Crucian et al., 2000; Gridley et al., 2009), natural killer cell activity (Konstantinova et al., 1995; Meshkov and Rykova, 1995), and response to pathogenic stimuli (tetanus toxoid and *borrelia burgdorferi*) (Cooper et al., 2001). The suppressed
cellular activity is supported by changes in cytokine production that indicate an imbalance between the pro-inflammatory and anti-inflammatory systems (Crucian et al., 2008; Fitzgerald et al., 2009). Thus, spaceflight poses unique, if yet indefinable, immune response changes that signify impending consequences to host protection against disease. As evidenced during the early Apollo missions, there was a high incidence of in-flight infections prior to the development of the preflight stabilization program (Hawkins and Zieglschmid, 1975; Taylor, 1993). Even with effective pre-screening, each traveler carries a cache of bacterial and viral “hitchhikers” that under appropriate circumstances may lead to complications in health status, and possibly affect other passengers. Using a modeled microgravity culture system as a parameter to examine effects of mitogenic and specific immune function offers a useful venue to assess potential consequences of prolonged space travel and gain insight into the associated long-term risks due to microgravity-induced immune suppression.

*Mycobacterium tuberculosis* (MTB) is an intracellular pathogen and is transmissible by inhalation of aerosolized droplets created during forcible exhalation events from infected individuals. The World Health Organization (WHO) estimates that a third of the world’s population is currently infected with MTB, with 1.4 million TB-related deaths worldwide occurring annually (WHO, 2009). Tuberculosis is primarily a disease of the lung, with disease progression largely due to host immune responses directed at controlling organism growth. The majority of immune competent individuals exposed to MTB remains “latently” infected for life and exhibit strong delayed type hypersensitivity (DTH) responses to tuberculosis antigens. However, changes in immune status, as those observed during microgravity-induced immune suppression, may result in conditions that render individuals susceptible to reactivation events. This has been observed in latent viruses such as herpes, cytomegalovirus, and Epstein-Barr viruses (Mehta et al., 2007; Stowe et al., 2011; Stowe et al., 2001).

At this time, it is impractical to conduct experiments in space using animal models of virulent tuberculosis infection. Therefore, we report *in vitro* experiments using modeled microgravity conditions by culturing cells in high aspect rotation wall vessels (HARV). Experiments addressed alterations in immune response that are known to affect control of MTB infection; namely, lymphocyte response activation, macrophage function, and synthesis of cytokines. These observations in the modeled microgravity cultures were consistent with previous findings in microgravity and with other investigators’ findings. Our results showed that the decreased responses were not only evident in lymphocytes, but also in decreased mycobactericidal effects in macrophages.

**MATERIALS AND METHODS**

**Mouse Splenocyte Stimulation**

Splenocytes were isolated from 10-12 week old female BALB/c mice (Harlan Laboratories) as previously reported (Hwang and Actor, 2009). Briefly, whole spleens were minced, red blood cells lysed, and cells dispersed using glass slides. The splenic leukocyte fraction was cultured at 1x10⁶ cells/mL in medium: Dulbecco’s Eagle’s Modified Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma), 2.2 g/L sodium bicarbonate (Sigma), 50 mg/L HEPES (Sigma), 50 mg/L L-arginine (Sigma), 100 ug/mL Penicillin G (Sigma), and 50 ug/mL Gentamycin (Sigma), and 2-Mercaptoethanol (Gibco). Splenocytes were cultured in media only or stimulated with 2 ug/mL ConA (Sigma), 200 ng/mL lipopolysaccharide (LPS, serotype O111:B4, Sigma), or *Mycobacterium bovis* Bacillus Calmette Guerin Pasteur strain (BCG, TMC 1011, ATCC) at a multiplicity of infection (MOI) of 10:1. Concentration of mitogen and MOI of BCG were determined from our previously published experimental protocols (Hwang et al., 2009; Hwang et al., 2007). Splenocytes were transferred to T-10 flasks for normal gravity conditions and into a 10 mL high aspect rotating vessels (HARV, Synthecon) at 18 rpm for simulated microgravity conditions and cultured at 37°C with 5% CO₂ (Unsworth and Lelkes, 1998). Supernatants were collected at 48 hrs and analyzed by enzyme linked immunosorbent assay (ELISA). Live and dead cells visualized by staining with 0.4% Trypan Blue (Sigma) at cell:dye ratio of 1:1, and subsequently counted using a hemocytometer.
Macrophage Infection with MTB

U937 human-derived monocytic cells (CRL 1593.2, ATCC) were cultured at 1x10^6 cells/mL in DMEM with 5% FBS (fetal bovine serum), sodium bicarbonate, HEPES, and L-arginine. Cells were infected with *Mycobacterium tuberculosis* (MTB; Erdman strain) (TMC 107, ATCC) at MOI 10:1 for 4 hrs at 37 °C with 5% CO₂ on a horizontal shaker. Infected cells were washed after 4 hrs with 1xPBS and re-suspended in new DMEM media and distributed into HARVs or T-flasks. Infected cells were lysed with 0.05% SDS in 1xPBS at 37°C for 5 minutes and neutralized with equal volume of 15% bovine serum albumin (BSA, Sigma); lysates were serially diluted in 1xPBS and plated onto 7H11 Agar plates (Remel). Plates were incubated for 3-4 weeks at 37°C and colonies enumerated.

ELISA

Supernatants were analyzed for TNF-α, IL-6, IL-1β, IL-12p40, IL-10, IFN-γ, and IL-2 using DuoSet ELISA kits (R&D Systems) as previously described (Welsh et al., 2008). Limit of detection was between 16-32 pg/mL.

Statistics

Experiments were conducted in duplicate, and assayed in triplicate. Data shown represents one experiment. Statistical analysis was conducted using TwoWay ANOVA followed by post-hoc T-test. Significance was considered if \( p \leq 0.05 \).

RESULTS

**Mouse splenocytes stimulated in modeled microgravity demonstrate differential cytokine production compared to normal gravity controls.**

Multiple investigations previously reported a general decrease in immune function caused by microgravity conditions (Cogoli, 1993; Cogoli, 1996; Sundaresan and Pellis, 2009). Using modeled microgravity conditions, directed changes in leukocyte responses were investigated. Total mouse splenocytes were cultured in HARVs or T-flasks and stimulated with a T-cell mitogen, ConA, an endotoxin, LPS, or the live vaccine BCG. Un-stimulated splenocytes cultured in HARVs and T-flasks were examined to allow for comparative analysis of secreted cytokines in response to stimulation.

The mechanism of ConA stimulation acts as the first signal of T-cell activation, involving antigen presentation molecules and CD3 (Cogoli, 1993), and bypasses the secondary signal requirement. Under modeled microgravity conditions, mouse splenocyte production of T-cell cytokines, IFN-γ (15 +/- 5 pg/mL) and IL-2 (18 +/- 21 pg/mL), in response to ConA was nearly completely suppressed as compared to normal gravity controls (1399 +/- 412 pg/mL, 391 +/- 28 pg/mL, respectively) (Figure 1A).

Lipopolysaccharide (LPS) stimulates through toll-like receptor 4 (TLR-4) in conjunction with co-receptor CD14. Both are expressed on the antigen-presenting cells, macrophages, dendritic cells, and B-cells (Miyake, 2004). Addition of LPS stimulated production of TNF-α, IL-6, IL-12p40, and IL-10. While HARV-cultured splenocytes did not show a differential response to LPS in production of TNF-α and IL-12p40, increased production of IL-6 (173 +/- 29 pg/mL) and IL-10 (748 +/- 41 pg/mL) was observed when compared to flask cultured controls (583 +/- 25 pg/mL, 573 +/- 54 pg/mL, respectively) (Figure 1B).

Bacillus Calmette Guérin (BCG) represents a common complex antigenic agent given as a childhood Tuberculosis vaccine to the majority of the world’s population (Behr, 2002; Oettinger et al., 1999). Live BCG, an intracellular bacteria, is phagocytosed by macrophages and dendritic host cells (antigen presenting cells). Under normal circumstances, BCG infection promotes production of TNF-α, IL-6, and IL-12p40. BCG-stimulated splenocytes cultured in modeled microgravity conditions demonstrated a significant decrease in production of TNF-α (24 +/- 15 pg/mL) compared to normal gravity controls (192 +/- 13 pg/mL, as well as IL-6 (3 +/- 2 pg/mL). However, no changes in levels of IL-12p40 were evident (Figure 1C).

Live and dead cell counts were performed for all samples collected for cytokine production. No differences in cell viability were observed, for cells cultured in T-flasks or in HARVs, whether grown under non-stimulating or mitogenic stimulation (Figure 2).
Figure 1. Altered cytokine production from stimulated mouse splenocytes cultured in modeled microgravity conditions. Splenocytes grown under flask (1G, open bars) or modeled microgravity (closed bars) were stimulated with ConA (A), LPS (B), or BCG (C) for 48 hrs, and supernatants analyzed for IFN-γ, IL-2, TNF-α, IL-6, IL-1β, IL-12p40, and IL-10. Media controls served as baseline for un-stimulated cells. Horizontal bar represents background levels for non-stimulated controls, with no statistical difference between normal gravity or modeled microgravity background levels. Data visualized as average and standard deviation error. *** = p≤0.001
**Modeled microgravity conditions suppress macrophage control of MTB bacterial growth.**

The decreased pro-inflammatory cytokine production by total splenocytes cultured in the HARV suggests potentially suppressed leukocyte activation. These events indicate a decreased macrophage ability to control mycobacterial infection. To investigate this further, non-activated U937 human monocytic cells were infected with MTB (Erdman strain). The MTB infected cells were subjected to simulated microgravity and examined for bacterial growth through 7 days.

Infection under 1G conditions (the flask control) demonstrated MTB growth with a classic lag phase during organism acclimation to the intracellular environment. However, growth of infected cells in HARV conditions demonstrated increased expansion of the intracellular bacteria population at days 1 and 3 post-infection ($p \leq 0.05$). By day 7 post-infection, intracellular MTB levels are similar under both simulated microgravity and normal gravity conditions (Figure 3).

Under these growth conditions, only modest levels of cytokines were produced (data not shown). While a slight increase in production of pro-inflammatory cytokines (TNF-$\alpha$, IL-1$\beta$, IL-6) was evident in HARV cultured cells stimulated under these conditions, this was not significant.

**DISCUSSION**

These experiments provide insight into the effect of modeled microgravity on the response of leukocytes to varying stimulation parameters. In modeled microgravity conditions, total splenocyte populations demonstrated decreased pro-inflammatory responses when stimulated with T-cell and antigen-presenting cell mitogens (ConA and LPS, respectively), as well as with a complex vaccine antigen (BCG). Overall, these findings suggest an impaired control of the growth of intracellular pathogens. Human monocytes infected with MTB were less capable of controlling intracellular organism growth in HARV culturing conditions. The cumulative data...
strongly suggest an overall suppression of leukocytes in modeled microgravity.

The decrease in T-cell activation/function is well documented. In vitro experiments using similar modeled microgravity devices demonstrated common trends (Cogoli and Tschopp, 1985; Cogoli et al., 1980). It is compelling that the decrease in T-cell activation caused by modeled microgravity occurs regardless of the stimulation parameters. Responses to PHA (Hales et al., 2002), PMA/Ionomycin, anti-CD3/CD28 (Gridley et al., 2009), or ConA (Cogoli, 1996) were all similarly diminished in microgravity conditions. Decreased production of IFN-γ was previously reported (Gridley et al., 2009; Hales et al., 2002). Cytokine production in humans post-flight also showed a decrease in IFN-γ production in response to mitogen stimulation of CD4+ cells only: whereas IL-2 macrophages (M/M) serve as both host cell and stimulators of T-cell activity. Previously published studies on microgravity and M/M production from all T-cell sets (CD3+, CD4+, and CD8+) are equally suppressed (Crucian et al., 2000). Although modeled microgravity using HARV devices does not take into account the other environmental variables during spaceflight, most notably stress (Sonnenfeld, 1999), T-cell responses stimulated by mitogens (ConA) are reduced in both modeled microgravity and post-flight analyses. This suggests that the dramatic decrease in IFN-γ production could be directed primarily towards CD4+, further implicating that modeled microgravity conditions decrease an important leukocyte that is critical in generating adaptive immune responses.

Adaptive immunity requires participation of antigen presenting cells, which are critical in promoting antigen specific T-cell activation. In the context of MTB infection and the mechanism of BCG vaccine efficacy, monocytes/macrophages (M/M) serve as both host cell and stimulators of T-cell activity. Previously published studies on microgravity and M/M
(Crucian et al., 2011) and showed a decrease in protein kinase C (PKC) translocation that is critical in monocyte differentiation (Hatton et al., 2002) compared to samples taken pre-spaceflight. However, in studies using mouse splenocytes, stimulation of cultured cells in HARV devices with LPS resulted in increased production of IL-6 and IL-10, compared to ground-based controls (Baqai et al., 2009). Thus, there is evidence for species specific changes induced by microgravity in response to LPS mitogenic stimulation. In response to bacterial agents BCG and MTB, the mouse splenocytes and human monocytic cell line demonstrated similar decreases in cytokine production levels of TNF-α and IL-6 and control of intracellular MTB proliferation. These data suggest that modeled microgravity alters M/M ability to respond to BCG and MTB, indicating that an environmental condition of spaceflight can influence host immune response to allow latent infections and/or opportunistic pathogens to flourish.

U937 is a human monocytic cell line that can be differentiated into macrophage lineage using factors that include phorbol myristate acetate (PMA) (Escobar-Alvarez et al., 2010), retinoid acid, vitamin D, and IFN-γ (Kikuchi et al., 1996). However, upon activation, the non-adherent U937 cell line becomes adherent, making culturing in HARV devices complicated without the requirement for attachment to microbeads. Adherent cells establish strong cell-cell contacts, which may be impeded under microgravity conditions, thus leading to relative immune dysfunction (Sonnenfeld and Miller, 1993). However, there is no indication that the observed immune changes measured here during modeled microgravity, or spaceflight, are directly due to cell-cell contact issues (Buravkova et al., 2005).

In vitro studies on immune cell function under modeled microgravity is sometimes inconsistent with data of immune cell functions in vivo during and post spaceflight (Sonnenfeld and Miller, 1993), suggesting effects of other factors specific to spaceflight that could play a role, such as stress (Sonnenfeld, 1999). However, studies with short-term/long-term spaceflight crew members show consistent increase in circulating granulocytes and decreased lymphocytes, decreased responses towards mitogen stimulation, and a significant decrease in production of IL-2 from CD4+ cells (Crucian et al., 2008; Konstantinova et al., 1993; Taylor, 1993). In addition, in vitro studies conducted in space labs found that cellular responses to ConA were also similarly suppressed, as observed in modeled microgravity culturing conditions at 1g (Cogoli, 1993; Cogoli, 1996; Gmunder et al., 1988). Spaceflight may bring numerous factors that could affect immune function that cannot be modeled at ground-based labs, but it is clear that modeled microgravity remains a main factor in affecting immune status. Understanding the specific alterations that microgravity induces in leukocyte populations will generate a foundation of knowledge towards achieving the goal of manned space planetary exploration.

In conclusion, this report confirms and extends the baseline findings of depressed immune function under modeled microgravity conditions, and demonstrates that modifications in the activation state of lymphocytic cells may be reflected in phagocytic cells. The latter leads to decreased capability to resist intracellular pathogens. The results herein show that phagocytic cells removed from the activation paradigm in vivo respond to the modeled microgravity environment by displaying decreased bacteriocidal activity during the early phases of exposure to bacteria (mycobacteria). Thus, the evidence suggests that maintaining host immunity in microgravity may require strategies that affect multiple members of various phenotypic cell populations that comprise the immune system.

REFERENCES


Research Article

Microgravity-Induced Fiber Type Shift in Human Skeletal Muscle

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ABSTRACT

Prolonged microgravity exposure alters human skeletal muscle by markedly reducing size, function, and metabolic capacity. Preserving skeletal muscle health presents a major challenge to space exploration beyond low Earth orbit. Humans express three distinct pure myosin heavy chain (MHC) muscle fiber types (slow → fast: MHC I, IIa, and IIx), along with hybrids (MHC I/IIa, IIa/IIx, and I/IIa/IIx). After reviewing current research, this paper presents evidence for a “slow to fast” microgravity-induced skeletal muscle fiber type shift in humans. Spaceflight and bed rest induce decreased MHC I fiber proportion while increasing fast hybrid types (particularly MHC IIa/IIx fibers). This alteration in muscle cell phenotype negatively impacts performance and induces undesirable metabolic adaptations. While exercise has been postulated to minimize the negative effects of microgravity on human muscle, past spaceflight countermeasures have insufficiently prevented fiber type shifts in humans. However, a new high-intensity, low volume resistance and aerobic exercise regimen has recently been implemented aboard the International Space Station (ISS). This paper aims to reveal that 1) a slow to fast microgravity-induced fiber type shift occurs in humans and 2) the new high-intensity, low volume exercise countermeasures program onboard the ISS has promise to mitigate this fiber type transition and preserve skeletal muscle health.

INTRODUCTION

Consistent residency aboard the International Space Station (ISS) places humans in position to explore the Moon, Mars, and beyond. Human physiological limitations present clear obstacles to long-duration space missions as microgravity exposure deleteriously affects many organ systems, including skeletal muscle. Spaceflight induces quantitative and qualitative modifications to skeletal muscle by markedly decreasing size, strength, and endurance (Fitts et al., 2000). Despite exercise countermeasures, muscle mass has been shown to decrease from -13% to -17% during long-duration spaceflight (Gopalakrishnan et al., 2010; LeBlanc et al., 2000; Trappe et al., 2009). Significant decrements in muscle size can impair substrate utilization and insulin sensitivity, as the largest metabolic reservoir in the human body is skeletal muscle. Furthermore, long-mission studies conducted aboard the ISS, Skylab, and Mir have shown significant decreases (-20-35%) in muscle performance (Lambertz et al., 2001; Rummel et al., 1975; Trappe et al., 2009). This magnitude of reduction in muscle size and performance not only impairs astronauts upon return to Earth, but may also inhibit their ability to complete essential mission tasks, extravehicular activities (EVA), and emergency egress.

Researchers suggest chronic unloading (i.e. spaceflight and bed rest) alters mammalian muscle

Key words: Myosin Heavy Chain; Exercise Countermeasures; Unloading; Bed Rest; Spaceflight

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fiber phenotype (Fitts et al., 2000; Pette, 2002). A slow- to fast-twitch transition characterizes this “microgravity-induced fiber type shift.” Given that muscle fiber types exhibit a wide range of functional and metabolic characteristics (Pette and Staron, 1997), this shift likely contributes to reduced muscle performance and undesirable metabolic adaptations during spaceflight. This paper aims to outline newly compiled evidence supporting the microgravity-induced fiber type shift in humans and overview the new high-intensity, low volume resistance and aerobic exercise countermeasures program recently implemented aboard the ISS. This new exercise prescription is based upon 15 years of ground-based research that titrated the optimal dose, intensity, and balance of aerobic and resistance exercise to protect skeletal muscle health (Bell et al., 2000; Putman et al., 2004; Schulze et al., 2002; Trappe et al., 2007).

MICROGRAVITY-INDUCED FIBER TYPE SHIFT

Myosin heavy chain (MHC) protein composition determines mammalian skeletal muscle fiber classifications. Humans express three distinct fiber types (MHC I, IIa, and IIx) along with hybrids containing more than one phenotype (MHC I/IIa, IIa/IIx, and I/IIa/IIx). MHC I are slow-oxidative fibers (slow isoform contractile proteins, high mitochondrial density), MHC IIa are fast-oxidative fibers (fast contractile velocity, relatively fatigue resistance), and MHC IIx are fast-glycolytic fibers (fastest contractile proteins, low mitochondrial volume) (Spangenburg and Booth, 2003). Figure 1 shows the human skeletal muscle fiber type continuum measured via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the current fiber typing “gold standard” (Pandorf et al., 2010). The MHC type and proportion expressed in skeletal muscle affects whole muscle performance (strength and endurance) and metabolic efficiency (ability to store and utilize energy).

Skeletal muscle is a dynamic tissue, continually adjusting to current conditions. Living and being active in a 1 g environment provides the “ideal phenotype” for human skeletal muscle, while removing gravity rapidly disrupts muscle homeostasis. Evidence suggests muscle fibers shift phenotype when exposed to certain chronic stimuli (Pette and Staron, 1997). To date, the most extreme example of a fiber type shift in humans was observed in spinal cord injured (SCI) patients that had been wheelchair bound for 3-15 years. The SCI patients expressed significantly less MHC I (-23%) and IIa (-20%) fibers and more IIx (+33%) fibers than ambulatory control subjects (Malisoux et al., 2007).

Research supporting a MHC fiber type shift during spaceflight in humans has been increasing since the mid-1990s (Edgerton and Roy, 1996; Zhou et al., 1995). Undoubtedly, rodent models show modifications in muscle phenotype following periods of unloading (i.e. hindlimb suspension), expressing a slow to fast fiber shift along with increased hybrid types (Fitts et al., 2000). These hybrid fibers are likely in transition from one phenotype to another (e.g., MHC I → I/IIa → IIa) (Pette, 2002). After several ISS missions and long-term bed rest experiments in the last decade, enough data now exists to draw conclusions on the presence of spaceflight related fiber type shifts in humans.

Figure 2 contains compiled data from our laboratory and others, lending support to the microgravity-induced fiber type shift paradigm in humans (Borina et al., 2010; Gallagher et al., 2005; Trappe et al., 2009; Trappe et al., 2007; Widrick et al., 1999; Zhou et al., 1995). Each of the studies report changes in fiber type from pre-
Figure 2. Changes in myosin heavy chain (MHC) fiber type during unloading (spaceflight or bed rest). The linear trend-line is based on mean fiber type % change from all studies and illustrates a slow to fast fiber type shift (Plotted using Microsoft Excel 2010, trend-line equation: y = 3.51x - 10.408). All studies fiber typed using SDS-PAGE. Bed rest data is from control subjects. Total Hybrids represent fibers with multiple MHC isoforms. VL, vastus lateralis. Sol, soleus. Gas, gastrocnemius.

to post-spaceflight (or bed rest) in men and women measured via SDS-PAGE. Unloading duration ranged from 11 to 177 days, with an average of ~81 days. The studies investigated one of three lower limb muscles: the vastus lateralis (VL), soleus (Sol), or gastrocnemius (Gas). MHC I (slow) fiber composition decreased and total hybrid fiber proportion increased in all studies by an average of -13% and +14%, respectively. While unloading duration probably dictates the transition magnitude, trends were similar regardless of duration, unloading mode, or the muscle studied. Furthermore, the trend-line compiled from mean percent changes of all studies clearly illustrates a slow to fast shift across the MHC fiber type continuum. Consistency in these human data supports previous speculations of a fiber type shift caused by unloading.

Skeletal muscle phenotype transitions likely stem from changes in transcriptional processes associated with MHC expression. Recent investigations from Dr. Kenneth Baldwin’s laboratory show MHC promoter elements regulate expression of MHC genes undergoing phenotypic remodeling in response to inactivity (Huey et al., 2003; McCall et al., 2009). While specific mechanisms responsible for MHC regulation during unloading remain under investigation, it is evident that the magnitude of fiber type shift affects astronaut physical performance (Trappe et al., 2009). The slow to fast shift explains, in part, the decrease in muscular endurance seen following spaceflight. To counteract microgravity-induced loss of slow fibers while maintaining muscular integrity across the fiber type spectrum, a new training protocol is underway onboard the ISS.

LONG-DURATION SPACEFLIGHT EXERCISE COUNTERMEASURES

Long-duration manned missions beyond low Earth orbit (LEO) remain a primary goal of the international space community. However, maintaining skeletal muscle health continues to be a major obstacle in human space exploration. Past exercise regimens onboard the ISS were varied among crewmembers, but generally included moderate intensity aerobic (~5 days/wk) and resistance exercise (3-6 days/wk) (Trappe et al., 2009). The guidelines prescribed exercise for up to 2.5 h/day for 6-7 days/wk (time included hardware setup, stowage, and personal hygiene)
utilizing a running treadmill, cycle ergometer, and resistance exercise device (Trappe et al., 2009). These previous exercise countermeasures failed to completely preserve skeletal muscle size and function, warranting modifications to long-duration mission exercise prescription and/or hardware.

For decades, ground-based exercise physiology studies have shown chronic high-intensity exercise promotes positive skeletal muscle adaptations (i.e. increases strength and endurance) and alters fiber type composition (Andersen and Henriksson, 1977; Baumann et al., 1987; Harridge et al., 1998; Parcell et al., 2005; Simoneau et al., 1985). Figure 3 illustrates fiber type changes (maintained MHC I, increased MHC IIa, decreased MHC IIx) following high-intensity and sprint cycle training in men and women ranging from 42 to 105 days in duration. These studies measured fiber type by SDS-PAGE or histochemical staining (standard technique of the 1970s and ‘80s). Hybrid fibers were not reported in these investigations. MHC I fiber percentage varied but was generally maintained (+1%), while MHC IIa composition increased (+6%) and MHC IIx composition decreased (-5%) on average. As opposed to spaceflight and bed rest, the trend-line compiled from these high-intensity/sprint cycling studies demonstrates a fast to relatively slower fiber type shift. Notably, Simoneau et al. (1985) showed MHC I fibers significantly increased (+6%), MHC IIa fibers were maintained, and MHC IIb (IIx) fibers significantly decreased (-6%) after 105 days of sprint cycling, suggesting lengthier training durations might induce increases in MHC I proportions as their transition may take longer to manifest. Additionally, resistance training has been shown to elicit overall fast to slow fiber type shifts (maintenance of MHC I, increase in MHC IIa, and decrease in MHC IIx) while decreasing hybrid types (Liu et al., 2003; Williamson et al., 2001). Data from Figures 2 and 3 suggest mitigation of the microgravity-induced slow to fast shift is possible by employing high-intensity exercise during spaceflight. The idea of high-intensity exercise preventing a shift in MHC phenotype during long-

Figure 3. Changes in myosin heavy chain (MHC) fiber type during high intensity exercise training. The linear trend-line is based on mean fiber type % change from all studies and illustrates a fast to slow fiber type shift (Plotted using Microsoft Excel 2010, trend-line equation: y = -2.86x + 6.32). *Fiber typing via ATPase histochemistry (IIb equivalent to IIx). VL, vastus lateralis.
duration unloading was recently shown with bed rest (60 day), which has served as a guide for moving the exercise countermeasure program forward (Trappe et al., 2007).

Past exercise countermeasures onboard the ISS have insufficiently prevented fiber type shifts in humans (as seen in Figure 2). Moving forward, two key changes to the exercise program for spaceflight have occurred. The first was placement of new hardware on the ISS that allows for greater loading and comfort for performing more robust exercise. Figure 4 shows images of these devices, which include the Advanced Resistance Exercise Device (ARED), Cycle Ergometer with Vibration Isolation and Stabilization System (CEVIS), and Combined Operational Load Bearing External Resistance Treadmill (COLBERT). Second, was the implementation of a new high-intensity, low volume resistance and aerobic exercise prescription for astronauts. The new regimen alternates days of high-intensity interval training with continuous aerobic exercise (opposed to predominately continuous aerobic exercise) and 3 days/wk of high-intensity resistance training (opposed to 3-6 days/wk at lower intensity) (NASA, 2011). Ongoing research is underway to investigate the validity of the new exercise program for protecting crewmembers’ skeletal muscle health after long duration stays on the ISS.

CONCLUSION

A substantial microgravity-induced fiber type shift would be detrimental to human health during long-duration spaceflight, increasing risk of crewmember injury and rendering essential mission tasks difficult to complete. Slow to fast fiber shifts alter skeletal muscle quality, affecting the entire body by decreasing physical performance (increasing fatigability) and negatively influencing muscle metabolism by modifying substrate utilization, insulin sensitivity, and myokine production (e.g., IL-6 and IL-18) (NASA, 2010; Plomgaard et al., 2005). Ground-based studies support newly employed high-intensity exercise countermeasures onboard the ISS, which aim to improve skeletal muscle health. Based on current data, we conclude that high-intensity, lower volume exercise will aid in maintaining MHC I, increasing MHC IIa, and decreasing fast MHC hybrid proportions during long-duration spaceflight. Both current astronauts and future space explorers will benefit from the ongoing exercise countermeasures research.

Figure 4. Images of astronauts exercising on equipment currently used aboard the ISS. A: Advanced Resistance Exercise Device (ARED), B: Cycle Ergometer with Vibration Isolation and Stabilization System (CEVIS), and C: Combined Operational Load Bearing External Resistance Treadmill (COLBERT). Images retrieved from http://www.nasa.gov.
conducted aboard the ISS. A greater understanding of optimal exercise paradigms for spaceflight can also be translated to the human based challenges of inactivity, aging, and disease on Earth.

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Bagley et al. – Microgravity-Induced Fiber Type Shift


Severity of Atherosclerosis in ApoE-/- Mice Following $^{56}$Fe Irradiation is Independent of Plasma Cholesterol Levels

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ABSTRACT

Epidemiological data from atomic bomb survivors, radiotherapy patients, and people with excessive occupational exposure show that radiation exposure increases the risk of myocardial infarction and stroke. Although the character of the radiation in interplanetary space is very different from that encountered on Earth, cosmic radiation might pose a similar risk for astronauts. We established previously that 5Gy 600 MeV iron ions ($^{56}$Fe), a particularly damaging component of cosmic radiation, exacerbates atherosclerosis in apolipoprotein-E deficient mice. Since the pathogenesis of atherosclerosis involves contributions from a number of risk factors, here we examine the relationship of $^{56}$Fe radiation to plasma cholesterol. In un-irradiated mice, the severity of disease, as measured by average aortic root lesion area, correlated with plasma cholesterol levels. In mice receiving 5Gy $^{56}$Fe, however, lesion size was independent of both total and LDL-cholesterol. This suggests that the effect of $^{56}$Fe may be dominant, overriding some other risk factors.

INTRODUCTION

Radiation is a well-established risk factor for atherosclerosis. For example, radiotherapy for cancer is often limited by the risk of cardiovascular disease. In fact, the benefit of radiotherapy for early breast cancer can be nearly offset by the increased risk of mortality from vascular disease (Early Breast Cancer Trialists' Collaborative Group, 2000). Similarly, after receiving radiation treatments, even relatively young head and neck cancer patients are at significantly elevated risk of stroke (Scott et al., 2009). Exposure to radiation from other terrestrial sources results in similar risk. Atomic bomb survivors had an increased incidence of both coronary artery disease and stroke. Even radiation technologists working before 1950 (when occupational exposure was higher) had increased mortality due to circulatory diseases (Hauptmann et al., 2003). Little is known about the molecular mechanism, however, whereby radiation exposure leads to atherosclerosis years later, so post-exposure therapies to mitigate cardiovascular risk

Key words: Heavy Ions; Radiation; Cosmic Radiation; Iron; Cardiovascular Disease; Stroke; Aorta; Atherosclerotic Plaques; Plasma Lipids; Cardiac Risk Factors

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are lacking. To date, the primary strategy to avoid risk has been to limit exposure.

Astronauts on missions beyond Earth orbit, without the benefit of protection from Earth’s magnetic field, will also be exposed to significant levels of radiation. The character of the radiation to which the astronauts will be exposed, however, is different from that of typical terrestrial forms in that it contains a substantial component of accelerated ions. These ions interact with both tissues and shielding differently than photons such as X-rays or γ-rays. An example is $^{56}$Fe, a small but particularly damaging component of galactic cosmic radiation. Recently, we demonstrated that $^{56}$Fe targeted to the aorta and carotid arteries exacerbates atherosclerosis in apolipoprotein-E deficient (apoE -/-) male mice at a dose 4-8 fold lower than that required for X-rays in the same model. Moreover, the plaques in irradiated mice were more advanced than those in control mice. Such advanced plaques are associated with an increased risk of rupture, which can precipitate myocardial infarction or stroke.

While it is possible to shield astronauts from this type of radiation, shielding will be problematic both because of weight constraints and the thickness of the shielding required. In addition, these ions can interact with shielding materials to produce secondary particles which can exacerbate the damage to tissue. Therefore, some radiation exposure will be inevitable. Since cardiovascular risk factors are often thought to be additive, one strategy to mitigate radiation-induced atherosclerosis might be to control other risk factors. For example, lowering plasma cholesterol might reduce the overall risk of cardiovascular disease and partially compensate for unavoidable radiation exposure.

MATERIALS AND METHODS

This question was addressed using apoE -/- mice, a well established atherosclerosis model. In this model, although atherosclerosis develops spontaneously, without the need for a special diet or other intervention, both X-rays (Stewart et al., 2006) and $^{56}$Fe (Yu et al., 2011) have been shown to increase the severity of disease. Ten-week old mice were anesthetized with intra-peritoneal injections of 0.15 mg/gm ketamine plus 0.015 mg/gm xylazine, immobilized in chambers developed for this purpose (Yu et al., 2011), and then irradiated with 5 Gy $^{56}$Fe at the NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory. Radiation exposure was limited to the upper aorta and carotid arteries by means of a collimator (supplied by NSRL). Control mice were anesthetized, but not loaded into chambers or irradiated. All mice were then shipped to the University of Alabama at Birmingham where they were maintained on a normal diet (Teklad 7917 NIH-31, Harlan Laboratories) for 12-14 weeks. The mice were then euthanized and dissected to assess development of atherosclerosis. Irradiated and control mice were alternated to control for any possible time effects during the two week period.

Figure 1. Correlation of total plasma cholesterol with aortic root lesion size. Cross sections of aortic root from un-irradiated control and irradiated (5Gy $^{56}$Fe) mice were stained with Oil red-O and counterstained with hematoxylin. Lesion areas were measured morphometrically under a Zeiss Axiostar Plus microscope using a 1-mm2 eye-piece grid (100×10,000 μm2) at 100× magnification. Average lesion area for each mouse was then plotted against plasma total cholesterol level. a) For un-irradiated mice, lesion area correlated with plasma cholesterol ($r = 0.593$, $p = 0.006$). For irradiated mice (b), however, this correlation was lost ($r = 0.0565$, $p = 0.808$).
RESULTS AND DISCUSSION

Atherosclerotic lesion size in the aortic root, an atherosclerosis-prone vascular area, was measured by sectioning the root and quantifying cross-sectional lesion area. All mice developed atherosclerotic plaques, regardless of radiation history, as is expected with this mouse model. The average plaque size, however, was significantly increased by radiation, consistent with earlier reports demonstrating a pro-atherogenic effect of $^{56}$Fe (Yu et al., 2011). Average lesion area in mice receiving 5Gy $^{56}$Fe was $23.32 \pm 2.64 \times 10^4 \ \mu m^2$ as compared to $12.36 \pm 2.07 \times 10^4 \ \mu m^2$ (mean $\pm$ SEM) for un-irradiated control mice ($p<0.05$ by Mann-Whitney test). As shown in Figure 1a, plaque size strongly correlated with plasma cholesterol levels for un-irradiated mice. For mice receiving 5Gy, however, lesion size was independent of plasma cholesterol (Figure 1b).

The LDL component of plasma cholesterol is considered a strong risk factor for atherosclerosis, while the HDL component is considered protective. Therefore, the relationship between LDL and HDL cholesterol levels with atherosclerotic plaque size was also examined. As shown in Figure 2, while atherosclerotic plaque size correlated strongly with LDL cholesterol levels in un-irradiated mice, the correlation was absent in mice receiving 5Gy $^{56}$Fe. In contrast, as shown in Figure 3, plasma HDL levels showed no correlation with severity of atherosclerotic

![Figure 2](image2.png)

Figure 2. Correlation of plasma LDL cholesterol with aortic root lesion size. Aortic roots of control and 5Gy mice were sectioned and stained as in Figure 1, and lesion size was plotted against plasma LDL cholesterol for each mouse. As with total cholesterol, there was a strong correlation with lesion area in un-irradiated control mice (a) ($r = 0.591, p = 0.006$), but not in irradiated (5Gy $^{56}$Fe) mice (b) ($r = 0.0206, p = 0.993$).

![Figure 3](image3.png)

Figure 3. Lack of correlation of plasma HDL cholesterol with aortic root lesion size. Aortic roots of control and 5Gy mice were sectioned and stained as in Figure 1, and lesion size was plotted against plasma HDL cholesterol for each mouse. HDL cholesterol, a negative risk factor for cardiovascular disease, did not correlate with atherosclerotic lesion size for either control (a) ($r = 0.0683, p = 0.775$) or irradiated mice (b) ($r = 0.166, p = 0.473$).
It should be noted that mice are relatively resistant to radiation. For example, 8 to 14 Gy of x-rays targeted to the major vessels have been shown to accelerate the development of atherosclerotic lesions in apoE -/- mice (Hoving et al., 2008; Stewart et al., 2006), yet epidemiologic studies indicate that radiation can be a risk factor for humans at doses as low as 1Gy (Hauptmann et al., 2003; Ivanov et al., 2001). Since there are no epidemiological data for heavy ions, it is difficult to predict what $^{56}$Fe radiation doses in mice correspond to pro-atherogenic doses in humans. If a similar ratio of susceptibility holds for $^{56}$Fe as for X-rays, however, 0.36-0.63 Gy in humans might pose a similar risk for humans as the 5Gy dose used for mice in this study.

Conventional wisdom holds that atherosclerotic risk factors are additive, and improvement in any one factor should reduce overall risk. For example, even modest reductions in cholesterol levels are thought to reduce the overall risk of clinically significant cardiovascular disease. In this study, the relationship between disease severity and naturally occurring variation in cholesterol levels within the mouse population was examined. Cholesterol levels were not manipulated to rigorously test the hypothesis that lowering cholesterol can protect against radiation-induced atherosclerosis. The results of this study suggest, however, that control of cholesterol may not be effective in ameliorating heavy ion radiation effects on plaque development. As this is a report of a single study using a single animal model with effects at a single radiation dose, however, a definitive answer will depend on further studies. In addition, the time course of loss of the correlation between cholesterol and plaque size is not known, since this is a report of a single time point. Whether other risk factors, such as tobacco use or lack of exercise correlate with severity of $^{56}$Fe radiation-induced atherosclerosis remains unknown. Future studies will be needed to address the possible independence of heavy ion radiation from these and other risk factors.

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Short Communication

Moderate Intrauterine Asphyxia Impairs Surface Righting in Neonatal Rats

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ABSTRACT

A lack of vestibular sensory experience during early life, including that normally produced by the Earth’s gravity, is associated with deficient neonatal responses to gravitational cues. Linking paradigms for studying the form and function of the developing vestibular system after microgravity exposure with biomedical models of inner ear dysfunction or gravistatic receptor deprivation on Earth can provide an important bridge in our understanding of the role of gravity in vestibular development. Intrauterine asphyxia is a major obstetric complication that confers risk for developing neonatal hearing impairment, possibly due to neuropathological damage to the developing hair cells of the inner ear. Vestibular function in asphyxiated infants has not been well examined but a handful of studies suggest potential vestibular deficits. Using a rat model of moderate intrauterine asphyxia, we provide evidence for impaired vestibular mediated adjustments of body position against the gravity vector.

INTRODUCTION

Pregnant rat dams flown on the shuttle during the early developmental period of their fetuses, vestibular apparatus and onset of vestibular function gave birth (after Recovery) to neonates with deficient behavioral adjustments to Earth gravity (Ronca and Alberts, 2000; Ronca et al., 2008). In the present study, we analyzed contact righting responses using a potential model of inner ear dysfunction in neonatal rats associated with a common obstetric complication. Our overall goal is to establish connections between spaceflight findings and with biomedical outcomes on Earth, thereby augmenting both types of investigation.

Intrauterine asphyxia is a condition of impaired fetal blood-gas exchange observed in 1.6 out of every 1,000 live full term births (Pierrat et al., 2005), and nearly 60% of preterm births (Vannucci et al., 1999). Intrauterine asphyxia is a component of several major obstetric complications, including infection (Eitzschig and Carmeliet, 2011), preeclampsia (Myatt and Webster, 2009), maternal diabetes (Van Lieshout and Voruganti, 2008), and intrauterine growth restriction (Marsal, 2009).

Intrauterine asphyxia has been associated with brain damage and subsequent motor, cognitive and behavioral impairment (Marlow and Budge, 2005). Even moderate asphyxia at levels often viewed in the delivery room as unremarkable, can
lead to long-term cognitive and behavioral disabilities (van Handel et al., 2007). However, these outcomes are unpredictable (Bjelke et al., 1991) and the precise causative factors unknown.

Notably, intrauterine asphyxia has been identified as a high risk factor for developing neonatal hearing loss (Borg, 1997; D’Souza et al., 1981; Eavey et al., 1995; Sanders et al., 1985). Pathological alterations of the inner ear and apoptosis in hair cells as well as impaired brainstem auditory evoked responses (Jiang et al., 2008) have been reported in infants exposed to intrauterine asphyxia (Koyama et al., 2005; Schmutzhard et al., 2009). While few studies have addressed vestibular dysfunction following intrauterine asphyxia, there appears to be a relationship between low Apgar score, sensorineural hearing loss, and reduced responses to caloric stimulation (Zagólski and Jurkiewicz, 2006).

In the present study, we tested the hypothesis that moderate intrauterine asphyxia impairs vestibular-mediated behavioral responses in rats. We utilized a translational preclinical rat model of global asphyxia established in our laboratory that provides normoxic, within-litter controls and with high clinical relevance to the third trimester of pregnancy in humans. On the first postnatal day, we analyzed surface righting, a species typical behavioral response to gravity in which rats placed in the supine position will rotate their bodies to prone.

METHODS

Subjects

Neonatal offspring derived from time-mated female Sprague-Dawley (SD) rats were used. During timed matings, vaginal cytology was examined daily until pregnancy was confirmed by presence of sperm (Gestational Day [G] 0). All experimental procedures adhered to the NRC Guide for the Care and Use of Laboratory Animals and were approved by the Wake Forest School of Medicine Animal Care and Use Committee.

Procedure

On G22, the expected day of parturition, each dam was administered under isoflurane anesthesia, a chemical transection of the spinal cord (vertebral level L1/L2). The uterus was externalized and the blood supply feeding one of the dam’s uterine horns was ligated for 15min to produce fetal asphyxia (APX). The other uterine horn remained undisturbed (NON).

Pup Delivery

Fetuses in each uterine horn were delivered by cesarean section, stroked to stimulate respiration, and the umbilical cord occluded (Figure 1) at 36.5°C.

Figure 1. Experimental protocol for within dam comparisons of perinatally asphyxiated (left) and non-asphyxiated (right) offspring. The target fetus in each uterine horn is shown (colorized, leftmost image). Fetuses are cesarean delivered (second image from the left), stroked to stimulate respiration (third image from the left), and temperature is maintained at 36.5°C (fourth image from the left).
Comparisons were made between cesarean delivered neonatal rats derived from the APX and NON conditions. Further comparisons were made with Vaginally (VG) born pups to control for the surgical cesarean delivery. Neonates in all three conditions were placed in a heated incubator maintained at 36.5°C for 90-120 min postpartum, then fostered to a non-manipulated dam.

To validate degree of asphyxia, rapid decapitation was performed in a subset of newborn pups and blood collected from the mixed venous/arterial pool using a clinical blood gas analyzer (ABL 5, Radiometer, Copenhagen, Denmark).

Whole brain lactate (WBL) was measured from snap frozen postpartum brains (Trinity Biotech, St. Louis, MO).

RESULTS

Validation of Asphyxia

We found clear evidence for asphyxia as measured by blood pH levels and whole brain lactate.

One-way Analysis of Variance revealed a main effect of condition on blood pH \( [F(2,12)=47.06; \ p<.0001] \) and WBL across conditions \( [F(2,12)=27.40; \ p<.0001] \). Newman Keuls posthoc tests revealed the following significance APX<NON=VG, \( p<0.05 \) for blood pH; APX>NON=VG, \( p<0.05 \) for WBL. Ns=5 pups/condition.

Surface Righting Responses

At 24 hr postpartum, righting responses were analyzed, illustrated in Figure 2. Statistical significance determined by chi-squared analysis.

On Postnatal day (P)1, neonates were placed in the supine position on a solid surface (Figure 2, upper left). Each neonate was held gently with the experimenter’s fingers around the pelvic girdle and head and then released.

Righting from supine to prone was assessed with ‘None’ reflecting no attempt to right (Figure 2, top image) and ‘Attempt/Success’ (Figure 2, lower three images).

APX failed to show righting movements significantly more frequently and attempted to right or achieved righting success significantly less frequently as compared to NON and VG.

Table 1. Blood pH and whole brain lactate (WBL) in neonatal rats delivered vaginally (VAG), or by cesarean section either without asphyxia (NON) or with asphyxia (APX).

<table>
<thead>
<tr>
<th>Birth Condition</th>
<th>pH</th>
<th>WBL (ug/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAG</td>
<td>&gt; 7.0</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>NON</td>
<td>&gt; 7.2</td>
<td>&lt; 25</td>
</tr>
<tr>
<td>APX</td>
<td>&lt; 6.9*</td>
<td>&gt; 55*</td>
</tr>
</tbody>
</table>
Table 2. Percent P1 pups making no attempt to right (None) and those either showing attempts to right or successful righting (Attempt/Success).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Righting</th>
<th>None</th>
<th>Attempt/Success</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAG</td>
<td></td>
<td>30.1</td>
<td>69.9</td>
</tr>
<tr>
<td>NON</td>
<td></td>
<td>30.4</td>
<td>69.6</td>
</tr>
<tr>
<td>APX</td>
<td></td>
<td>55.4*</td>
<td>38.7*</td>
</tr>
</tbody>
</table>

*p<.05

CONCLUSIONS

Past studies of sensory loss associated with perinatal asphyxia have focused on auditory impairments and identified inner ear pathology within the cochlea. The present findings provide new evidence for disrupted vestibular mediated responses in neonatal rats one day following moderate perinatal asphyxia. In this study, we were not able to rule out tactile or proprioceptive deficits associated with the placement of pups on their backs. We previously found that eliminating these sources of compensatory adjustment can be accomplished in rats that underwent gestation during spaceflight using a water immersion righting test (Ronca and Alberts, 2000). In the present study, the vestibulosensory specificity of the deficit is not yet known. By expanding the range of vestibular mediated tests and identifying inner ear damage in this model, we hope to create a bridge between findings derived from spaceflight and studies modeling biomedical concerns thereby advancing our understanding of how vestibular form and function are shaped by development on Earth and in space.

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REFERENCES


Increased Intrauterine Pressure (IUP) Magnitude during Labor at 2g Revealed By Telemetry in Freely Moving Pregnant Rats

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ABSTRACT

Past studies of pregnant rat dams flown onboard the NASA space shuttle from mid-gestation until 48-72 hrs prior to birth reported two times the number of labor contractions relative to ground controls. Conversely, pregnant rats exposed to 1.5 or 2g centrifugation during the same gestational period showed significantly fewer labor contractions relative to 1g controls. Here we test the hypothesis that the down-regulation of labor contractions at 2g is associated with intrauterine pressures of greater magnitude than those measured in 1g controls. Intrauterine pressure (IUP) associated with labor contractions was analyzed using telemetry in freely moving female rats exposed to 2g centrifugation. Female rats were bred at 1g and 2g (n=9 per condition). On gestation day 19 (G19) a pressure telemeter was implanted into one of that dams’ paired uterine horns to measure IUP before and during parturition. Dam behavior was simultaneously video recorded to quantify frequency of contractions and time of birth. Our results indicate that sustained gravitational loading during the latter half of pregnancy increases IUP during labor. These ground-based findings contribute to our overall understanding of how the complex process of mammalian reproduction may be altered in space.

INTRODUCTION

Powerful forces exerted on the fetus during labor initiate adaptive responses that help lay the foundation for the neonate’s successful transition to life outside of the womb. We previously reported that numbers of labor contractions in pregnant rat dams that had been launched on the space shuttle and exposed to microgravity (µg) at mid-gestation and returned to Earth 48-72 hrs prior to birth were doubled relative to 1g controls (Ronca and Alberts, 2000). Using centrifugation-induced hypergravity, female rats exposed during the same period of pregnancy to either 1.5 or 2g exhibited significantly reduced numbers of labor contractions relative to 1g controls (Ronca et al., 2002). Systematic variations in contraction frequency spanning µg and hypergravity (hg) occurred in the absence of altered timing or duration of birth, or reduced numbers of live neonates. These observations led us to speculate that the efficacy of uterine contractions systematically varies with gravity load. In other...
words, a greater complement of weak contractions (i.e., at $\mu g$) and fewer strong contractions (i.e., at $hg$) would be required to complete the delivery process.

In the present study, we tested the hypothesis that pregnant rat dams exposed to 2g exhibit fewer labor contractions characterized by higher IUP as compared to 1g controls. Unlike our past studies, nulliparous female rats and male breeders were adapted to 2g for one week followed by timed-breeding. Thus, female rats spent their entire pregnancies and underwent birth at 2g. We utilized a telemetric system for IUP measurement, established in our laboratory, permitting acquisition of IUP signals from freely moving pregnant dams during labor (Ronca et al., 2003).

METHODS

Subjects

Animal experimentation was conducted in accordance with the guidelines of the NASA and Wake Forest School of Medicine Animal Care and Use Committees and the NRC Guide for the Care and Use of Laboratory Animals (copyright 1996, National Academy of Science).

Eighteen female and six male Sprague-Dawley (SD) rats were used. Rats were housed in standard vivarium cages (47 X 26 X 21 cm) lined with cob bedding and maintained under standard colony conditions (12L:12D cycle [lights on 0600 h], 21° C ± 1° C, 30-50% humidity). Rat chow (Purina 5102, Richmond, IN) and water were available ad libitum. The centrifuge was stopped 1 hr daily at 0800 hr for husbandry and veterinary evaluations.

One-half (N=9 females; N=3 males) of the rats were housed on the 24 ft diameter centrifuge at the NASA Ames Research Center (ARC) Center for Gravitational Biology Research (CGBR) and exposed to continuous 2g centrifugation (2g group; 19.98 RPM). The remaining subjects were housed identically in the same room and did not undergo centrifugation (1g controls).

Procedure

After 1 week of adaptation to centrifugation, females were time-mated with male rats in the same gravity condition. Gestational day (G)1 was considered to be the first day that sperm was observed in a vaginal lavage.

Figure 1. Size Perspective. Data Sciences International (DSI) PA C-20 pressure telemeter shown adjacent to a newborn rat pup.
Sensor Implant

On G19, the centrifuge was stopped and a modified mouse blood pressure telemeter (Data Sciences International [DSI] PA C-20; Figure 1) configured to fit within a small saline filled balloon was surgically implanted within each dam’s uterus.

Under general isoflurane anesthesia (3% isoflurane in 100% O₂) administered through a rodent rebreathing apparatus, a small incision was made on the midline of the externalized, and two adjacent fetuses were dams’ abdomen. Following sensor implantation, the uterine incision was carefully sutured, the uterus gently re-inserted into the abdomen, and the external incision closed. Centrifugation was reinitiated post-operatively (on the same day), once the dams resumed ambulation, eating and drinking.

Data Analysis

On G20, dams in both gravity conditions were singly housed in specialized observation cages. Dams were video-recorded in real time for quantification of behavioral expressions of labor (Ronca et al., 1993; Ronca and Alberts, 2000). Time of birth was operationally defined as the moment that the first pup emerged from the birth canal, derived from video records. IUP was measured continuously for 1 hr prior to and 1 hr following the birth of the first pup. Contraction frequencies (illustrated in Figure 2) were quantified by trained observers during playback of the video records using a computerized scoring system. (Inter-rater reliabilities were calculated at $r > .96$).

RESULTS

We found that 2g exposure from conception until birth did not affect the timing of parturition but resulted in significantly fewer labor contractions (Figure 3).

These findings corroborate our past studies of reduced labor contraction frequencies in parturient rat dams with 2g exposure initiated at mid-gestation.

Coincident with reduced behavioral expressions of labor in 2g exposed dams, we observed significantly elevated pressure during the hour prior to birth and the hour following birth (Figure 4).

Concordant with past studies (Ronca et al., 2001), neonatal mortality was significantly greater in 2g vs 1g conditions (2g, 15% vs 1g, 2%).

Figure 2. Behavioral expressions of labor in parturient rats. Left: Lordosis contraction; Right: Vertical contraction.

Figure 3. Number (mean ± sem) of lordosis and vertical contractions during labor (1 hr prior to birth) after continuous 2g exposure beginning ≥1 week prior to conception (*p<0.05). Data analyzed from real-time video.
CONCLUSIONS

Fewer contractions and greater IUP magnitudes were observed during labor and birth at 2g. One possible interpretation of these findings is that the reduced contraction number may be supported by the increased IUP. Gravity-induced changes in myometrium and/or abdominal musculature are potential mechanisms underlying altered contraction number and strength.

Burden, Zary, and Alberts (1999) reported reduced connexin 43, the major gap junction protein in the myometrium of dams exposed to spaceflight from G11 to G20, two days prior to birth. Connexin 43 is involved in the synchronization and coordination of labor contractions (Burden et al., 1999); however, we previously found no differences in this protein in pregnant rats exposed to 2g from G11 to G20. Unfortunately, in the present study, we were not able to measure uterine connexin proteins on G22.

Collectively, these findings contribute to our understanding of the complex process of mammalian reproduction in space and potential role(s) for gravity in the birth process.

Understanding parturition and the role of uterine contractile forces in altered gravity are essential first steps toward establishing developmental and inter-generational studies of mammals on space platforms.

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A Perspective of Magnetic Levitation as an Earth-based Low Gravity Analogue: What It Is and What It Ain’t

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ABSTRACT

The Earth-based low gravity environments available by magnetic levitation are not as widely employed as one might expect. For the purpose of highlighting and explaining the capabilities of magnetic levitation, this perspective surveys the history, the physical concepts, and the state-of-the-art of the technique.

INTRODUCTION

The ability to magnetically levitate objects, Figure 1, is not a new phenomenon. In fact, Werner Braunbek reported the first experiments with graphite and bismuth using facilities located in Tübingen, Germany (Braunbek, 1939a,b). However, the magnetic levitation of organic materials, typically modeled by physicists as, to first approximation, spheres of H2O, did not occur until 1991, when Eric Beaugnon and Robert Tournier used the strong magnetic fields and the corresponding gradients available in Grenoble, France (Beaugnon and Tournier, 1991a,b). Within a decade of this report, as the required magnetic fields and gradients became readily available at magnetic field laboratories located around the world, the curiosity driven fun was performed by many researchers, including Michael Berry and Andre Geim* (Berry and Geim, 1997), who shared an Ig Nobel Award for Physics in 2000 for their work with levitating frogs (Ig, 2000). The levitation of a mouse by a group at the Jet Propulsion Laboratory in Pasadena, CA has rekindled interest in the near zero-g environment (Liu et al., 2010), but the corresponding low-g platforms that mimic lunar and martian conditions should not be forgotten (Valles et al., 2005).

The purpose of this paper is not to comprehensively describe the phenomenon and the field, since several detailed and accessible accounts have appeared (Berry and Geim, 1997; Valles et al., 1997; Geim, 1998; Brooks et al., 2000; Brooks and Cothern, 2001; Kitazawa et al., 2001; Simon et al., 2001; Nikolayev et al., 2011). Instead, our discussion focuses on aspects that may be of interest to the readers who might be pondering the possibilities of applying these techniques to their own research requiring a low gravity environment.

Key words: Magnetic Levitation; Low Gravity Simulators; Earth-based Low Gravity Analogue; Biomagnetism; Moses Effect

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* Andre Geim and Konstantin Novoselov shared the Nobel Prize for Physics in 2010 for their work on graphene (Nobel, 2010).
THE BASICS

Most introductory physics textbooks contain a discussion about the magnetic properties of materials being generally classified as either ferromagnetic, paramagnetic, or diamagnetic. For the case of diamagnetism, the application of an external magnetic field, $\mathbf{B}$, induces magnetic dipoles, $\mathbf{m}$, localized to the molecules but oriented in a direction opposite of the applied magnetic field. The number of magnetic dipoles per unit volume, $V$, is known at the magnetization, $M = \frac{\mathbf{m}}{V}$, which can be written as a linear function of $\mathbf{B}$ for describing paramagnetic and diamagnetic materials, i.e.

$$M = \chi_m \mu_0 \mathbf{B}, \quad (1)$$

where $\chi_m$ is the magnetic susceptibility, which is positive for paramagnetic and negative for diamagnetic materials, and $\mu_0$ is the permeability of a vacuum, a fundamental constant. Less commonly appearing in introductory physics textbooks is the expression for the force, $F_m$, that a dipole experiences from a gradient of the magnetic field, where

$$F_m = (\mathbf{m} \cdot \nabla) \mathbf{B}, \quad (2)$$

and the first terms show the magnetic dipole vector dot product with $\nabla$, the del operator. In the simplest approximation restricted to one dimension taken to be the $z$-axis, the substitution of the first equation into the second one yields

$$F_{m,z} = V \chi_m \mu_0 B_z \left( \frac{dB_z}{dz} \right). \quad (3)$$

In other words, the magnetic force on a diamagnetic material is proportional to the strength of the applied magnetic field times the gradient of the magnetic field, and these parameters can be controlled in the laboratory.

The general expression for a force is $F = m a = V \rho a$, where $m$ and $\rho$ are the mass and density, respectively. For the force due to gravity, $a = -g$, where the one-dimensional orientation of $g$ is taken to be oriented in the negative $z$-direction. The force from gravity can be balanced by the magnetic force, Equation 3, of equal magnitude but oppositely oriented, and the expression describing this situation is often quoted as the requirement for magnetic levitation, namely

$$B_z \left( \frac{dB_z}{dz} \right) = \left( \frac{\rho}{\chi_m} \right) g \mu_0. \quad (4)$$

It is important to stress that once the material is chosen, its properties give the ratio $\left( \frac{\rho}{\chi_m} \right)$, and the experiment only has to tune the magnetic field and its gradient to the appropriate value to generate the low-$g$ conditions. A striking demonstration of magnetic levitation conditions “at your fingertips” has been elaborately described (Geim et al., 1999; Simon et al., 2001). A non-levitation demonstration of the consequences of Equation 4 involves the deformation of water and is known as the “Moses Effect” (Kitazawa et al., 2001; Chen and Dahlberg, 2011).
BEYOND THE BASICS

The preceding section assumes, strictly speaking, the object being levitated is in a vacuum, whose magnetic susceptibility and density are zero. To include the magnetic susceptibility of the surrounding medium into the analysis, the $\chi_n$ term in Equations 3 and 4 should be replaced by $\Delta\chi_n = \chi_{n,\text{object}} - \chi_{n,\text{medium}}$. In addition, a force of buoyancy can be generated if the density of the medium is different than the density of the object. Including this possibility in the analysis leads to the $\rho$ term in Equation 4 being replaced by $\Delta\rho = \rho_{\text{object}} - \rho_{\text{medium}}$. In fact, these details form the basis of using magnetic levitation to measure the densities of solids and liquids (Mirica et al., 2009). Finally, the nature of the metastable levitation state (Berry and Geim, 1997; Simon et al., 2001) and the dynamics of the object in this low-$g$ environment (Brooks and Cothern, 2001; Nikolayev et al., 2011) are beyond the scope presented in this perspective.

WHAT IT IS AND WHAT IT AIN’T

Whenever discussing magnetic levitation with potential users of the technique, a standard set of questions arise, and we will briefly address the most common points in order to elucidate the advantages and disadvantages of the method. The following frequently asked questions and their answers are not meant to be an exhaustive discourse of all of the issues.

What are the mass and volume limits for a sample to be magnetically levitated? A striking feature of Equation 4 is that the volume of the object does not appear explicitly. Consequently, if the $B$, $dB/dz$ conditions can be established over a sufficiently large volume, then objects such as mice can be levitated (Liu et al., 2010), and magnets with a range of bore diameters have been used (Nikolayev et al., 2011). In other words, almost anything can be studied, and the aforementioned review articles provide an extensive discussion of the wide variety of materials that have been investigated.

How long can magnetic levitation be performed? Another striking aspect of the technique is that the low gravity environment can be established for hours to days in resistive (Brooks et al., 2000) and hybrid (Watanabe et al., 2003) magnets, or many days to weeks in room-temperature-bore, superconducting magnets (Valles et al., 2005; Liu et al., 2010). This time scale is a drastic improvement over the opportunities accessible on parabolic flights or drop towers. The only caveat is that magnetic levitation, by its nature, is a metastable situation, so long-term stability depends on a number of variables (Brooks and Cothern, 2001; Nikolayev et al., 2011).

Isn’t magnetic levitation the same as neutral buoyancy in a fluid? Generally speaking, the answer is No, but there are some subtle issues (Brooks and Cothern, 2001; Guevorkian and Valles, 2004; Nikolayev et al., 2011). During conditions of neutral buoyancy in water, the outer surface of a diver feels a net upward force of buoyancy while the internal organs still experience the Earth’s gravitational pull. Contrastingly, if the diamagnetism and density of a body are uniform throughout the sample, then each infinitesimally small volume element of the sample experiences a magnetic force that is equal to but oppositely oriented to the gravitational force (Valles et al., 1997; Guevorkian and Valles, 2006a,b; Nikolayev et al., 2011). Naturally, this ideal world may sound like it only exists in the minds of physicists who model an elephant as a sphere of water, but the approximation is not too bad, as variations of the values of diamagnetic properties of most organic materials is small. Unfortunately, not all material in living tissue is diamagnetic, and in fact, most molecules containing iron are paramagnetic or ferromagnetic. Consequently, differential forces can arise in these cases. To summarize, single-celled microorganisms can be studied in a magnetic levitated low gravity environment that is distinctly different from a neutrally buoyant setting. A similar statement can be made for the eukaryotic animals, but some consideration needs to be given to the differential forces that the internal organs will experience.

With a large gradient of the magnetic field being used, are magnetophoretic effects or other perturbations to biological processes present when studying living tissue? The answer to this question is Yes. In fact, large magnetic field gradients have been used to study the magnetophoretic effects on plants (Kuznetsov and Hasenstein, 1996; Galland and Pazur, 2005), but these effects are typically smaller than the ones
arising from magnetic orientation, which originates from the torque that a magnetic dipole senses in a homogeneous magnetic field (Guevorkian and Valles, 2006a,b). Consequences of these types of effects have been reported by several groups (Denegre et al., 1998; Stalcup et al., 1999; Valles, 2002; Ikehata et al., 2003; Paul et al., 2006; Coleman et al., 2007), and the effect is extensively employed by the MRI (magnetic resonance imaging) community investigating the structure of biomolecules (Bax and Grishaev, 2005).

Are there any subtle issues that one should consider when designing an experiment? Yes, there are some points that one should ponder prior to performing an experiment. For example, the container of some samples, such as liquid ones containing cells to be studied, might provide a surface tension that could dominate any magnetic field induced effects. In other words, the confining media should be “matched” to the sample in order to avoid this kind of secondary effect, which is one that is easy to overlook. An illustrative example is the egg, which is an arrangement where the yoke is held by albumen in such a way as to allow the magnetic field effects to be manifested (Denegre et al., 1998).

FUTURE PROSPECTS

From our viewpoint, a plethora of opportunities exist for using magnetic levitation facilities as Earth-based low gravity analogue platforms, and the curiosity to explore this region of parameter space will be driven by researchers working a variety of fields, including gravitational and space biology. We suggest that you give the technique a try, as every time an object is successfully levitated, the event externally provokes a smile and internally ignites profound thought.

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Double Containment Transfer Tool for Liquid Sample Manipulation on the International Space Station


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ABSTRACT

With the termination of flights of the U. S. space shuttle, which has historically returned life sciences and fluids materials to Earth for post-flight analysis, there is an immediate need for on-orbit analysis capabilities. In many life sciences, fluids, and human research projects it will be necessary to transfer fluids from an experiment facility to an analytical facility. This requirement is often in conflict with the NASA requirement that fluids classified as Toxicity Level-1 must be always doubly contained. In view of this need we have designed, fabricated, and tested prototypes of transfer tools capable of removing and doubly containing fluids without necessarily requiring a glovebox.

FEATURES

The Double Containment Transfer Tool transfers up to 1.0 mL and up to 5.0 mL of Tox-Level-1 fluids from and to appropriately equipped experimental devices on ISS. The capacity depends on the size of a sterile, off-the-shelf commercial syringe that is installed in the Double Containment Transfer Tool housing. The transfer tool accommodates commercially available syringe bodies and utilizes an off-the-shelf needleless luer fixture and septum. These elements are enclosed so as to prevent their contact with the spacecraft atmosphere. Various experimental cassettes, vessels, and sample carriers can be modified with female connectors so they can be sampled by or can receive fluids from the Transfer Tool.

INTRODUCTION

With the termination of flights of the U. S. space shuttle, there has been an abrupt reduction in experimental sample return capabilities from the International Space Station (ISS). There is an immediate need for an increase in on-orbit analysis capabilities and therefore for sample-transfer capabilities. We have therefore designed and fabricated prototypes of transfer tools capable of removing and doubly containing up to 1.0 mL and up to 5.0 mL of Tox-Level-1 fluids between devices on ISS. The Double Containment Transfer Tool accommodates commercially available syringe bodies and off-the-shelf needleless luer fixtures in isolation from the spacecraft atmosphere. Experimental vessels can be modified with female connectors so they can be sampled by or can receive fluids from the Transfer Tool. Prototypes have been subjected to leakage, pressure, vacuum, and performance testing, and prototypes are available for testing in experimenters’ laboratories.
Several detailed features have been incorporated in anticipation of safety certification requirements for crewed orbital flight. During transport a simple package will secure and protect the Transfer Tool during transport or storage. If the plunger is accidentally depressed the sample that is ejected will be contained within the Sealing Tube (see Figure 1). Two levels of containment are required through all phases of operations. Double containment between the Sealing Tube and the ISS transfer site is maintained through design of the target mating port. The target hardware is required to have a female port that contains a needleless septum. When not in use this septum remains protected with a tethered cap or plug. When a transfer is to be made the cover is removed from the female port and the tip of the tool is inserted. Once mated within the female port the Transfer Tool is rotated clockwise to lock the threaded male luer fitting on the end of the tool with the needleless septum within the female port. Upon internal luer engagement the syringe housing is pushed forward and twisted clockwise to engage the needleless septum and syringe housed within the tool. Once engaged, the double o-rings on the front of the tool provide and maintain a seal through the transfer process. Once the selected volume has been transferred by
pushing the plunger, the syringe housing is rotated counterclockwise within the sealing tube to disengage the internal luer seal within the tool. The sealing tube is then rotated counterclockwise within the mating female port to disengage the connection at the sample transfer site. Once disengaged, the Transfer Tool is removed from the female port, the end cap is placed over the end of the tool, and the cap reattached to the female port. Both end caps are tethered to their respective devices. An o-ring ring seal provides containment around the plunger, which has a cylindrical shaft. The “stock” syringe plunger provides the primary fluid containing seal, while the o-ring prevents leakage in the event of a plunger seal failure or fluid ejection into the Sealing Tube.

The appearance and main features of the Double Containment Transfer Tool are illustrated in Figure 1. Prototypes of this tool are available for ground-based laboratory testing in user applications.

TEST METHODS AND RESULTS

Extensive performance testing was conducted on the Double Containment Transfer Tool prototype. The battery of tests conducted on the initial prototype is summarized by the following categories:

**Test 1: Mate and De-Mate with needle-less septum port** (off-the-shelf Becton-Dickinson “BD Q-Syte Septum, 385100”).

**Result:** Mate and De-Mate connection performed as designed

**Test 2: Sample input/ withdrawal from experiment cassette**

**Result:** Sample of water containing green food coloring was successfully injected into a cassette (“Light Microscopy Module Dynamic Stage” (LMM-DS)) and was also withdrawn from the cassette as designed. The test procedure is seen in the Figure 2 photographs.

**Test 3: Containment testing**

**Result:** A leak path was found around End Cap fasteners. This leak was avoided by a modification that was successfully retested in a second prototype with a design modification to the seal.

**Test 4: Rapid depressurization/ re-pressurization**

**Result:** The Transfer Tool prototype was subjected to rapid depressurization and repressurization in both the filled and empty states in accordance with NASA Document SSP 5200-IDD-ERP Rev. H (NASA, 2009). In both the filled and empty states the Transfer Tool prototype experienced no leakage or structural failure.

**Test 5: Rapid temperature cycling**

**Result:** A water-filled Double Containment Transfer Tool prototype was temperature cycled between 71°C and -40°C with a soak period of 3 hours. During the cycling from 71°C to -40°C, the Protective End Cap softened and deformed around the end of the Double Containment Transfer Tool. This occurrence was remedied in the second prototype design with addition of a thicker Protective End Cap that features a pressure relieving breather.

**Test 6: Freeze/thaw cycling (empty and filled)**

**Result:** When tested in the empty state the Double Containment Transfer Tool prototype experienced no damage or failure from temperature cycling from 24°C to -40°C. In the filled state the prototype did produce a 10 μL drop of fluid within the Sealing Tube. This drop was the result of fluid expansion during freezing and the drop was completely contained within the Sealing Tube.

All testing was documented for the sponsor, and any design deficiencies found through the initial testing process were addressed with the fabrication and testing of a second generation prototype. Testing of the second generation Double Containment Transfer Tool proved the design updates to be successful and these results have been formally recorded as reports to the sponsor.

DISCUSSION

Fabrication and testing efforts to date are consistent with the Double Containment Transfer Tool becoming available for exercises in users’ laboratories in 2012 and prepared for flight certification in 2013. Figure 3 shows an example
of how the Double Containment Transfer Tool would find applications by transferring samples among specific life science facilities that might exist aboard ISS.

Figure 2. Left: a photograph of the Transfer Tool engaged to the Needle-less Septum Port prior to the sample input (see also image in Figure 3). Right: After being emptied into the cassette bag.

Figure 3. Example of an application of the Double Containment Transfer Tool: Transfer of biological suspensions between an experiment facility, such as a variable-g centrifuge with culture modules equipped with a needle-less septum port, and a receiving cassette for microscopy.
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Hypothesis

Yoga Therapy as a Complement to Astronaut Health and Emotional Fitness – Stress Reduction and Countermeasure Effectiveness Before, During, and in Post-Flight Rehabilitation: a Hypothesis

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ABSTRACT

Long duration spaceflight and exploration missions require increasing the effectiveness of countermeasures to the abnormal physiology that manifests itself in healthy astronauts living in space. The basic hypothesis proposed posits that yoga practices – breathing maneuvers, relaxation, meditation, and specific muscular movement – may serve this purpose by addressing as a whole, both the effects of microgravity and those of non-gravitational stressful conditions, before, during, and in post-flight rehabilitation. By providing self-directed stress relief tools the reduction in stress-related cortisol and catecholamine spikes observed throughout the space program may be expected to ameliorate microgravity-induced changes such as immuno-deficiency and regional loss in bone architecture, as well as accelerate re-adaptation of balance and coordination, bone, muscle, and cardiovascular systems to Earth’s gravity on return. Several hypotheses are presented based on the review of evidence from the scientific literature that defines physiological responses and relationships associated specifically with the practice of yoga as relevant to mission-related stress relief as well as post-flight orthostatic intolerance secondary to the central hypovolemia caused by microgravity. This hypothesis has far-reaching implications for the integration of yoga-based practices in complementing the effectiveness of current countermeasure approaches and provides direction for future research that might bridge the knowledge gap in the use of Yoga practices in the practice of space medicine.

INTRODUCTION

Astronauts living in the microgravity of space experience characteristic physiological changes that become progressively more pronounced with increasing mission duration (Vernikos, 1996). This abnormal physiology that manifests itself in healthy humans during their adaptation to the microgravity of space persists despite a variety of countermeasures (CMs). Increasing the effectiveness of current CM approaches is
therefore a matter of some importance since less than adequate practical CMs present a handicap for the emerging public space travel opportunities as well as in planning exploration missions.

Artificial gravity provided by an onboard centrifuge has been considered as a plausible option for greater CM effectiveness (Iwasa et al., 2010; Young et al., 2011). Limited studies using the head-down bed rest (HDBR) model to induce the effects of microgravity on the ground have supported the usefulness of centrifugation as a CM (Iwasaki et al., 2001). However, serious and stable commitment is needed to support the research required to validate its use (Iwasa et al., 2010; Young et al., 2011). In addition to microgravity, astronauts assigned to a space mission are exposed to conditions that would be considered highly stressful, such as the undesirable pathological consequences of excessive or sustained adreno-cortical and sympatho-adrenal responses (McPhee et al., 2012). In the case of cortisol (as well as other endocrine stress responding systems), excesses could exacerbate at the very least musculoskeletal, cardiovascular, gastro-intestinal, neuro-sensory, and cerebral structure and function known to be affected by microgravity. This physiological stress condition points to the importance of complementing current physical CMs (e.g., resistance exercise) to address non-gravitational aspects of spaceflight in support of overall health in space.

Stress has always been considered of primary importance in spaceflight as reflected by significant elevations in the so-called ‘stress’ hormones during launch and after landing (Leach-Huntoon et al., 1994). However, even though astronauts are selected based on their resilience and coping skills and have access to professional counseling during the flight, we propose that pre-flight training needs to include learning skills in maintaining their stress response within physiological limits.

Yoga provides a variety of common sense, practical techniques that draw on nature. Several observations have been reported in clinical literature that describes application of Yoga in the effective treatment of a range of medical disorders (Gokal et al., 2007). Brownstein and Dembert (1989) described the use of yoga relaxation technique to effectively treat a USAF pilot suffering from essential hypertension. More recently, yoga therapy in conjunction with other alternative medicine approaches has been tested in long-term lymphoma survivors (Habermann et al., 2009). In addition, the first double blind NIH-funded clinical study showed Ayurvedic medicine was more effective in treating rheumatoid arthritis than methotrexate. A large-scale clinical trial is to follow (Furst et al., 2011).

Our hypothesis posits that the study and practice of Yoga breathing, stretching, relaxation techniques, and meditation will provide astronauts and other space travelers with self-administered approaches for relief from physiological stress associated with the adaptation to spaceflight, as well as complement the effectiveness of physical CMs. These could be useful for:

1) Stress relief before and during the flight,
2) Post-flight support and rehabilitation, and
3) Counteracting in-flight symptoms.

The purpose of this paper is to present numerous hypotheses regarding the potential application(s) of Yoga during spaceflight, and a review of data from the scientific literature that defines physiological responses and relationships associated with the practice of Yoga principles. Our primary objective is to provide direction for future research that might bridge the knowledge gap(s) in the use of Yoga practices, ultimately providing a basis for assisting astronauts with stress relief before and during flight, and post-flight orthostatic intolerance secondary to the central hypervolemia caused by exposure to microgravity.

**STRESS RELIEF BEFORE AND DURING FLIGHT**

Astronauts face a range of stressful situations throughout a mission. These include the intense physical, mental, and emotional preparation before flight and conditions unique to spaceflight such as extravehicular activity, a heavy work schedule, and hypergravity during launch and re-entry. Important stress-related conditions during spaceflight include (but are not limited to) confinement and a change in normal living conditions such as eating, sleeping, and personal hygiene; separation from family and familiar places; human factor issues resulting from
negative social interactions with multicultural crewmates; and communication issues with ground control. Unexpected risks from equipment failure or narrow encounters with space debris are some of the more likely acute stressful issues. These general conditions may bear some similarities, but collectively differ dramatically from other modern day expeditions on Earth such as living on an Antarctic base or on a submarine.

The history of evidence of stress during space missions, from the first Mercury mission to the current International Space Station (ISS) era, has overall supported outpouring of cortisol and catecholamines that might have been expected, during and after the hypergravity of launch and re-entry, the discomfort of space sickness early in flight, and the reimposition of gravity after landing (Leach, 1992; Leach-Huntoon et al., 1994).

Immediately on entry into orbit, the relative absence of gravity results in unloading of the body (Convertino, 2007). As a result, inflight urinary 17-OHcorticosteroids for example, were decreased throughout Gemini VII in both astronauts (Lutwak et al., 1969) and urinary cortisol was unchanged or increased in Skylab and the 2-11-day Shuttle missions, whereas plasma cortisol showed at the same time no change or a decrease, leading Gazenko et al. (1990) to conclude that microgravity did not produce stress continually. The increased ratio of epinephrine (Epi) to norepinephrine (NE) might further indicate instead that the stress was emotionally triggered (Gazenko et al., 1990).

Similarly, circulating NE levels and catecholamine excretion after prolonged spaceflight were indeed reduced (Davidova et al., 1989). Reductions in NE excretion in HDBR studies parallel the sustained inhibition of sympathoneural release, turnover and synthesis of (NE) without affecting adrenomedullary Epi secretion or renal dopamine production (Goldstein et al., 1995). Direct measurement of baseline sympathetic nerve activity (MSNA) during the 16-day Neurolab Shuttle mission was only mildly elevated (Ertl et al., 2002a). However, as expected, MSNA responses to the stress of lower body negative pressure (LBNP), to induce an orthostatic response in space, were increased, as was the circulating NE response (Ertl et al., 2002b).

Higher levels of cortisol were also occasionally seen as missions increased beyond 30 days in both Soviet and U.S. astronauts (Macho et al., 1991). Ground research studies supported Gazenko’s theory. Although plasma cortisol and excretion were consistently reported to be increased in male volunteers during horizontal (Leach et al., 1973) and subsequently in HDBR (Dallman et al., 1984), this was not the case when females were first similarly studied (Vernikos, et al., 1993). In concurrent 7-day HDBR studies, male and female subjects differed. No increase in plasma cortisol was evident in the females, where in fact a progressive decrease in the amplitude of their cortisol daily rhythm and the mean daily circulating levels of cortisol were consistent with their observed positive attitude in the study (Vernikos et al., 1993).

Anticipating that these differences were not due to gender but “emotionally triggered” as Gazenko et al. had suggested (1990), all subsequent studies with male volunteers in the same facility at the Ames Research Center no longer showed increased plasma cortisol during HDBR. This stress relief was achieved by pairing a first-time volunteer with one who had experienced bed rest before (Vernikos, unpublished observations).

Stress and its endocrine response such as cortisol, preflight and during spaceflight, contribute to physiological changes such as bone and muscle loss. Stress of all types, as well as microgravity, compromise the immune system (Convertino, 2007). Microgravity affects the system at all levels from micro-organism to the whole body (Pierson et al., 2007). Gravity plays a role on bacterial virulence in vitro, and on its resistance to antibiotics (Wilson et al., 2008). Antibody production in response to immunization in space in an amphibian test system produced fewer antibodies and of poor quality (Bascove et al., 2011). Lebsack and his colleagues (2010) found that spaceflight changes gene expression patterns in the thymus of mice that had spent 13 days in space. These changed genes primarily appear to affect signaling molecules involved in programmed cell death and the regulation of the stress response. Long believed that the immunosuppression in astronauts is entirely due to microgravity, this assumption was reversed when an astronaut came down with shingles the...
day before he was due to launch, suggesting it was preflight stress and not space that was responsible for the viral reactivation in this case (Stowe et al., 2011). Subsequent testing procedures were modified to take into account the possibility that preflight stress and certainly inflight stress underlies at least some of the immune suppression associated with spaceflight.

However, in the longer missions of up to six months on the ISS, even mild negative social stress could compromise the immune system of astronauts. A recent study (Chiang, et al., 2012) showed that relatively mild, daily negative social relational interactions can affect the immune system and did so through an inflammatory mechanism. Negative social events were related to higher IL-6 and sTNF αRII (a type II soluble receptor for TNFα) and greater sensitivity to stress. Social stressors, including negative social interactions, lead to increases in cortisol (Dickerson and Kemeny, 2004; Kiecolt-Glaser et al., 1997), and cortisol tends to have a suppressive effect on inflammatory processes. However, repeated exposure to negative social stress and cortisol may lead to resistance to the anti-inflammatory effects of cortisol (Miller et al., 2008; Stark et al., 2001). Negative social interactions also increase blood pressure and heart rate and overall sympathetic activity (Sgoifo et al., 1997). Sympathetic activity has been shown to be positively related to inflammation whereas the opposite is true for parasympathetic activity (Marsland et al., 2007).

Yoga practices have been found useful in reducing the immunodeficiency caused by examination stress (Gopal et al., 2011) or of psychological stress in general (Kulkarni and Bera, 2009). A Scandinavian study reported that the use of yoga improved the ability to cope with stress in the workplace and improved the sense of "well-being" (Hartfiel et al., 2011). People who practice Yoga regularly have lower inflammatory responses to stress with lower blood levels of interleukin-6, a component of the body’s inflammatory response that contributes to heart disease, stroke, arthritis, and type-2 diabetes (Kiecolt-Glaser et al., 2010). We hypothesize therefore that Yoga practice may represent an option for maintaining the immune system that astronauts may use as an adjunct to their countermeasure repertoire.

**Meditation**

For stress relief, meditation is a keystone of Yoga practice. Centers for Integrative Medicine that have meditation and mindfulness at their core include those led by Jeffrey Brantley, MD, at Duke University (McConigal, 2011) and John Kabatt-Zinn, using Mindfulness-Based Stress Reduction (MBSR) at the University of Massachusetts (Fisher, 2010). To better understand how mindfulness meditation reduces stress and promotes a feeling of physical and mental well being, subjects were studied with fMRI while meditating. Midline cortical structures, including the bilateral anterior insula, left ventral anterior cingulated cortex, right prefrontal cortex, and bilateral precuneus, showed decreased signal, whereas a signal increase was noted in the right posterior cingulated cortex, supporting the theory that positive outcomes are achieved through a process of reduced sense of identity (Ives-Deliperi et al., 2010). Meditation training has been gaining ground after studies evaluated its usefulness for stress reduction, anxiety, and burnout among Canadian and Spanish family physicians (Lee et al., 2008; Franco, 2010). In this regard, we hypothesize that meditation may be effective as a countermeasure against emotional stress in astronauts.

**Respiration Yoga Therapy Practice**

In yogic terms, microgravity can be considered a non-grounding environment, in contrast to Earth’s gravitational environment, which is considered a grounding environment. A variety of Yoga body postures or poses (called āsana_s) and Yoga breathing exercises (prānāyāma_s) combined with finger-thumb hand gestures (mudrā_s), are claimed to provide stress-relief and many other health benefits (Sarkar and Deepak, 2009, 2011). However, there is no scientific evidence to support or dismiss the notion that Yoga postures, breathing exercises, or hand gestures would provide the same effects with the upward shift of abdominal organs and blood volume induced by the microgravity environment of space, or that the reduced stress response associated with Yoga might provide protection against some of the deleterious physiological adaptations induced by living in the microgravity environment.
POST-FLIGHT SUPPORT AND REHABILITATION

On return from spaceflight, the body of an astronaut changes in an attempt to adapt to living without the load, downward pull, and directional information that gravity on Earth normally provides. During spaceflight, the demand on the body is not only to re-adapt to living in Earth’s 1g, but first, to withstand the stress of increased gravity or hypergravity during the re-entry. The immediate post-flight response is characterized by orthostatic hypotension (OH), the tendency to faint on standing up, muscle weakness with balance and coordination problems, all of which hamper mobility, especially critical in an emergency situation. It is followed by a long recovery process to restore muscle and bone strength and mass, balance and coordination, and a variety of metabolic changes to their Earth-adjusted norm. The duration of the process of recovery depends on the length of the flight as well as the efficiency and effectiveness of the rehabilitation measures, and compliance on the part of the astronauts. We hypothesize that Yoga breathing practices as well as yoga muscle and joint exercises performed by astronauts in space represent one approach that may prove effective in the restoration of normal function sooner during recovery on Earth without the risk of injury imposed by regular structured exercise.

Accelerating Constructive Rehabilitation, Balance, and Coordination

We further hypothesize that small isometric and resistive Yoga activities complementary to traditional exercise may be of particular benefit, specifically in accelerating rehabilitation post-flight when proper bone architecture recovery is so crucial (Vernikos and Schneider, 2010). In addition, Yoga practice during rehabilitation might also be of value in accelerating the restoration of balance and coordination, as has been demonstrated in stroke victims (Schmid et al., 2011). Balance and coordination are severely compromised by adaptation to microgravity. While in space, the balance system loses the up and down cues that gravity on Earth provides. It adapts by making the best use of the other senses (Young et al., 1984). On return to Earth, the re-introduction of gravity into the equation takes re-adaptation over a period of time. On landing, an astronaut may lose their sense of falling when tilted forward with their eyes closed, until using gravity as the prime reference for the vertical is re-established. After returning from space, astronauts find that even small head movements, such as inclining the head even slightly, may give an exaggerated sensation of tumbling forward. Leaning the head sideways feels like a significant lean (Clement et al., 2003). The link between Yoga practices that enhance the relationship of gravity, balance, and coordination on Earth may be expected to enhance rehabilitation following spaceflight.

Post-flight Orthostatic Hypotension (OH)

Orthostatic intolerance (OI) is characterized by low blood pressure and the inability to continue standing without feeling faint. OI is experienced by about half the male and most female astronauts on return to Earth from flights of any duration (Waters et al., 2002). Considerable research has demonstrated that the standard approach of salt solution drink before re-entry has been less than effective in ameliorating post-spaceflight OI (Vernikos and Convertino, 1994). It had been observed (Rickards et al., 2007a) that OI symptoms experienced in individuals during standing, e.g., light-headedness or dizziness, were associated with reduced cerebral blood flow. A more recent investigation that was conducted on 27 astronauts following their Space Shuttle missions lasting from 8 to 16 days (Blaber et al., 2011) revealed that OH and intolerance to standing after flight were also associated with reduced cerebral blood flow. On the other hand, a higher cerebral blood flow was characteristic of those astronauts who completed 10 minutes of standing without difficulty. It follows that the use of maneuvers that can increase cerebral blood flow might reduce the incidence of OI after return to Earth.

One of the maneuvers used by those practicing Yoga is slow, deep breathing. Deep inspirations lower the pressure (i.e., create a vacuum) inside the thorax (Moreno et al., 1967). The resulting negative intrathoracic pressure is transferred to the brain and the resulting elevation in pressure difference between the arterial circulation and the brain results in increased cerebral blood flow. This effect has been
eloquently demonstrated through experiments in both animals and humans in which the intrathoracic and intracranial pressures can be dramatically reduced by the application of resistance during inspiration (Convertino et al., 2011), resulting in significantly higher cerebral function (Rickards et al., 2007b). More importantly, the interaction between lower intrathoracic pressure and increased cerebral blood flow induced by slower and deeper breaths with the use of resistance inspiration has been shown to improve tolerance to standing and other conditions that can result in fainting (Convertino et al., 2011).

The benefit of slower and deeper breaths on cerebral blood flow and OI is clearly evident by the data presented in Figure 1 collected from the same individual under two conditions that produced different breathing patterns. In these experiments, the subject underwent exposure to lower body negative pressure (LBNP), a technique that has been used for decades in the space program to cause shifting of blood from the upper body to the lower extremities similar to that experienced when we stand.

The two panels in Figure 1A represent the breathing and cerebral blood flow responses to a central hypovolemia induced by LBNP. This level of LBNP caused this person to become presyncopal while breathing at 0.28 Hz without resistance during inspiration (16.5 breaths per minute; lower left panel), but was feeling fine at the same level of LBNP when breathing slower and deeper at 0.20 Hz through greater reduction in intrathoracic pressure caused by resistance inspiration (~11 breaths per minute; lower right panel). When the person was breathing slower at 0.20 Hz: 1) mean blood flow (velocity) in the middle cerebral artery (Mean MCAv) was greater (upper panels); 2) the magnitude of Mean MCAv oscillations was greater (upper panels) and associated with greater cerebral perfusion and no symptoms (Rickards et al., 2007b); and 3) respiratory rate (measured in the lower panel by end-tidal CO₂) is very regular (i.e., controlled) at 0.20 Hz, but irregular at 0.28 Hz. Although these responses are presented in a single individual for demonstration purposes, they reflect those of the general population (Rickards et al., 2007b).

Figure 1B shows a frequency analysis plot of the same data as in Figure 1A. If the breathing is controlled, the frequency of breathing is represented by a single “spike” as compared to multiple “spikes” if the breathing is not controlled. It is clear from these data that regular controlled slow deep breathing is associated with greater cerebral perfusion (greater magnitude and oscillations of blood flow), less hypotension, virtually no symptoms, and greater orthostatic tolerance. We hypothesize, therefore, that the results of these experiments support the notion that the practice of Yoga could also be effective in preventing post-flight OI.

Specifically, the Yogic respiration exercises as described in Sarkar and Deepak (2011) – Bellows, Victorious, Straw-like rolled tongue, and other Yoga Breathing exercises with inspiration resistance – all involve active inhalation that would decrease intra-thoracic pressure. The best-known of these is Resistive Breathing, called Ujjayi-Prānāyāma (U-P), or Victorious Breathing Exercise (VBE; the name “ujjayi” literally means victorious). U-P or VBE is performed by slowly breathing through a constricted larynx, as if breathing through a straw within the throat. Since resistive inspiration increases cerebral blood flow and improves OI, we hypothesize that U-P or VBE could similarly be effective in preventing post-spaceflight orthostatic hypotension in astronauts.

COUNTERACTING INFLIGHT SYMPTOMS

Deconditioning during spaceflight affects every organ and system in the body, evident initially with space sickness followed by metabolic and cardiovascular changes in organ mass and function (Vernikos, 1996). Current CM precautions include an array of traditional exercises and some nutritional approaches. The extent of inflight physiological changes progress in the presence of these CMs at a considerable rate. Any non-invasive intervention, such as those found in yogic practice, may complement and enhance the effectiveness of CMs currently prescribed.

Space Sickness in Flight

Space sickness that occurs in a large proportion of space travelers during the first two to four days of flight (and sometimes on return to Earth) has evaded reliable preventive treatment.
Figure 1. The two panels in Figure 1A represent the breathing and cerebral blood flow responses to a central hypovolemia induced by LBNP. Figure 1B shows a frequency analysis plot of the same data as in Figure 1A. If the breathing is controlled, the frequency of breathing is represented by a single “spike” as compared to multiple “spikes” if the breathing is not controlled. Figures 1A and 1B are adapted from Rickards et al., 2007b.
As with OI, nausea and motion sickness are also associated with a decrease in cerebral blood flow (Serrador et al., 2005). Since controlled breathing with slower and deeper inspiration is associated with increased cerebral blood flow, we hypothesize the possibility that Yoga may provide relief from the nausea associated with early exposure to microgravity, which would be particularly relevant to the commercial space travelers of tomorrow whose early missions would only last for a few hours or days, when space sickness could ruin the pleasurable experience of a lifetime.

We hypothesize that breathing designed to decrease intra-thoracic and intra-cranial pressure and thereby increase cerebral blood flow, provided by slow, deep inspirations, particularly with resistance inspiration, should adequately assist space travelers from fainting in response to standing on return from flight as well as from space sickness during the early portions of flight.

**In-flight Musculoskeletal Loss and Post-flight Rehabilitation**

More sophisticated techniques and longer duration exposures to space as on the ISS have drawn attention to the seriousness of the continuing rate of loss in bone and muscle in the microgravity of space despite rigorous countermeasures (Vernikos and Schneider, 2010). These have mainly focused on providing aerobic and resistive physical exercise according to regimes practiced on Earth’s gravity (1g) such as a single bout of intense exercise once a day. Extending the duration and intensity of the exercise has helped marginally.

Spaceflight differs from Earth in that the reduced loading on muscles and bones during physical exercise makes the CM less effective. Unlike Earth, where non-exercise activities continue in 1g when exercise stops, the astronaut returns to a relative unloading of microgravity in space. To bridge this gap in non-exercise activity, we hypothesize that Yoga practice that requires no setting up of complicated equipment could be useful in providing forces on the musculoskeletal system by stretching and flexing movements throughout the non-exercise day, and reaching muscles such as those that support the spine that evade traditional exercise.

Finally, we hypothesize that yogic activities involving frequent and repetitive ankle, toe, or other flexion may well contribute to increased blood flow to muscle and bone regions, as well as tendon and ligament stimulation, and muscle contractions in a way that could encourage healthy bone architecture, as has been suggested by Rittweger and Felsenberg (2009).

**CONCLUSIONS**

Thus, based on data from the scientific literature that links various postures, muscular movement, and breathing maneuvers used during performance of yogic activities, we hypothesize that the wealth of approaches in Yoga practice may provide options that can be added to an astronaut’s personal health kit. Breathing and mental practices may in themselves be used as powerful stress management and non-medicinal CM tools. Others may need validation in an actual or simulated spaceflight environment.

The benefits of regular Yoga practice continue to be documented. These activities will not cause pain or injury as long as they are learned and practiced under the charge of a medically trained Yoga Therapy practitioner. Learning, practice, and education are crucial to the effective and safe use of Yoga exercises, and should be started early in the pre-flight training period so that they become a habitual practice. Our hypothesis acknowledges that stress and the microgravity of space disturb the balance of the adrenocortical, autonomic, and immune systems resulting in a generalized inflammatory response. Our hypotheses also reflect the need for more evidence that yoga practices can be used to restore balance and thereby complement the effectiveness of current CMs used during spaceflight. In this we are in strong agreement with Streeter et al. (2012) whose recent hypothesis proposes parallel application of Yoga practices for the treatment of medical conditions exacerbated by stress. Regardless of the physiological benefits that such complementary Yoga approaches provide, the significance of further investigation into the potential psychosomatic benefits of the sense of controllability by the astronaut should not be undervalued.
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Educational Outreach

Butterflies and Spiders in Space: Space Life Science Investigations for the Classroom

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Abstract

Two experiments were conducted aboard the International Space Station (ISS) in 2008 and 2009 that engaged elementary and middle school teachers and students worldwide in authentic science investigations designed to increase student knowledge of, and interest in, biology and space life science studies and biomedical careers. In the first project, a pilot called Butterflies and Spiders in Space, 1,876 middle school students tested a protocol for comparing, at near-real time, the behaviors of orb-weaver spiders and painted lady butterflies living in microgravity (aboard ISS) to those of comparable subjects in students’ classrooms. Teachers reported that, as a result of project activities, 33% of their students designed additional experiments and 80% of students expressed interest in science careers. The second program, Butterflies in Space, enabled students to observe and investigate the life cycle and behaviors of painted lady butterflies living on ISS, and compare them to butterflies being studied in their own classes. Combining this near real-time experiment with hands-on explorations and web-based instructional strategies, Butterflies in Space reached more than 3,000 teachers, representing an estimated 180,000 students (grades 3-6) or more worldwide. It also received international coverage from a variety of media. Investigators at BioServe Space Technologies of the University of Colorado designed and built the chambers in which the spiders and butterflies were housed on ISS, and led technical and logistical operations for both programs. Baylor College of Medicine (BCM) educators and scientists developed the education framework and managed the web-based distribution of project data and teaching resources.

Introduction

The President’s Council of Advisors on Science and Technology recently highlighted national needs for preparing and inspiring the next generation of professionals in science, technology, engineering, and mathematics (STEM) fields (PCAST, 2010). Unfortunately, students’ STEM learning experiences often are limited to readings from textbooks or preparation for high-stakes standardized assessments. At the same time, a
growing body of research indicates that immersing students in authentic scientific investigations builds interest and proficiency (Minner et al., 2010; Geier et al., 2008).

In 2008 and 2009, Baylor College of Medicine (BCM) and BioServe Space Technologies at the University of Colorado collaborated on two educational programs designed to 1) engage students and their teachers in open-ended scientific investigations involving organisms living aboard the International Space Station (ISS); 2) increase students’ science knowledge, understanding of, and participation in the scientific process, and interest in science studies and careers; and 3) determine the educational value of space-based, investigatory educational programs. The first project, a pilot called Butterflies and Spiders in Space, featured two orb-weaver spiders and six painted lady butterfly larvae flown to the ISS via Space Shuttle Endeavor (STS-126). Twenty-five participating teachers and their students conducted classroom investigations to test a protocol for determining 1) the impact of microgravity on the butterflies’ life cycle (larva to adult butterfly) and 2) the ability of an orb-weaver spider to spin a web, feed, and remain healthy in space. Though limited in size, Butterflies and Spiders in Space demonstrated the feasibility and strong appeal of using “real-time” space life science as a focus for classroom experiments.

Building on the Butterflies and Spiders in Space experience, BCM and BioServe set out to refine and amplify the scope of the pilot project with another “butterfly investigation,” to be flown on Space Shuttle mission STS-129 (November 2009), with support from NASA and the National Space Biomedical Research Institute (NSBRI). The second, full-scale project, Butterflies in Space, followed a similar design as Butterflies and Spiders in Space. Its objective was two-fold: 1) increase students’ interest in, and understanding of space life science by engaging them in classroom experiments in which they collect and share data and investigate their own questions related to biological responses to the microgravity environment aboard the ISS; and 2) determine whether teachers, limited by rigid schedules and test-driven curricula, would use the content created for this project (and offered free of charge) with their students.

Both the pilot project and the large-scale Butterflies in Space program were designed to be easily replicable in any classroom. Images and video of the butterflies and spiders flown on ISS, as well as downloadable teacher guides and other resources, remain available on BCM’s BioEd Online (http://www.bioedonline.org/space/) and K8 Science (http://www.k8science.org/space/) websites for any teacher or class wishing to conduct the investigations.

Both projects used “life in space” as a topic to engage students in guided and open-ended science investigations that address National Science Education Standards for grades 5-8 related to scientific inquiry, structure and function in organisms and living systems, and Earth and space science (NRC, 1996). We focused primarily on elementary and middle school because early educational experiences are critical in establishing students’ interest and aptitude in science, and they contribute to later interest in other science courses and even science careers (Tai et al., 2006). However, Butterflies in Space was open to any interested teacher, and some high school teachers did participate with their students.

By serving to “publicize to the intramural and extramural communities, the availability of ISS as a research environment that can accommodate a variety of experimental approaches, and address a vast range of research questions,” the Butterflies and Spiders in Space pilot and the full-scale Butterflies in Space initiative also supported the Memorandum of Understanding between the NIH and NASA for Cooperation in Space-Related Health Research. The 2007 Memorandum sets forth a framework of cooperation between NIH and NASA and designates the US portion of ISS as a national laboratory focused on areas of research related to health and microgravity (NIAMSD, 2007).

**BUTTERFLIES AND SPIDERS IN SPACE (PILOT PROGRAM)**

The Butterflies and Spiders in Space pilot project launched on November 14, 2008, with six *Vanessa cardui* (painted lady) butterfly larvae and two orb weaver spiders of the family Araneidae transported aboard the Space Shuttle Endeavor.
Moreno et al. -- Butterflies and Spiders in Space

(STS-126) to the ISS. BCM developed a set of accompanying educational materials and activities to engage classes in ground-based experiments similar and concurrent to those being performed on ISS. (These resources, with video and images from the ISS, remain available, free of charge, on BioEd Online). The Commercial Generic Bioprocessing Apparatus growth habitats (approximately 18 x 13 x 9 cm in size), in which spiders and butterflies lived while on the ISS, were designed and built by BioServe to provide a suitable living environment, and to allow photography, at regular intervals, of the subjects’ life cycles and behaviors in microgravity (e.g., eating patterns, web spinning, flight, emergence from chrysalis). Specific project objectives were as follows:

1. Investigate the feasibility of transporting and maintaining painted lady butterflies and orb-weaver spiders in specialized chambers on the International Space Station.
2. Enable students to compare observations from the ISS-based experiments to their own data collected during ground-based control investigations in classrooms.
3. Obtain estimates of students’ and teachers’ interest in investigating space life science-related questions.
4. Develop, field test, and evaluate an educational package including educators’ guides and recommended materials, teachers’ tools, and supplies for real-time classroom support of the investigation.

Procedure. For geographic and logistical reasons, middle school teachers in Colorado and Texas were recruited to participate in the Butterflies and Spiders in Space project. A total of 25 teachers (representing 1,876 students) piloted BCM’s classroom protocol for growing and investigating butterflies and spiders similar to those being flown on ISS. (Painted lady butterflies and orb weaver spiders are appropriate classroom organisms because they are readily available from science suppliers and care is easily managed.) Prior to the start of the investigation, each participating school received a draft educational kit, consisting of educators’ guides, control experiment butterflies and spiders, and other necessary materials. Teachers were instructed on how to build inexpensive, easy-to-assemble habitats, and grow the organisms in their classrooms. Students prepared their habitats in advance, so that classroom pilot testing could be synchronized with the flight investigation.

Classes had access to space flight (from the ISS) and ground control images almost immediately after the spider and butterfly habitats were transferred to the ISS. Those images and other data from the Space Station were made available for students to view and download, in near real-time from BCM’s BioEd Online (www.bioedonline.org) and K8 Science (www.k8science.org) websites. Students conducted their own classroom-based experiments to compare the life cycles and activities of their Earth-bound animals with those of the butterflies and spiders in the “live” experiments conducted in microgravity aboard the ISS.

Because crew time is in high demand on the Space Shuttle and ISS, BioServe’s butterfly and spider habitats were set up to require very little intervention. Twice during the ISS mission, astronauts had to expose fresh food for the butterflies (the spider chambers were self-sustaining because they also contained fruit fly cultures). Through the use of different lighting, the habitats provided a day/night cycle, with dozens of images captured during each hour of “daylight.”

Outcomes. Unfortunately, the food for the pilot test butterfly larvae was defective, so the larvae failed to pupate. The same lack of development plagued the ground control larvae. The project team identified the problem (defective food) with the help of students who were conducting the investigation with a different batch of food. Those students’ larvae successfully pupated, and the problem was resolved prior to the full-scale, follow-up mission on STS-129 (described below).

The orb-weaver spiders, on the other hand, thrived for many weeks in space. Originally, the two spiders were carried in separate chambers (one as a “spare” in case the primary spider did not survive). But early in the mission, the “backup” spider found its way into the main spider’s chamber and begin to build its own webs. For a short time, it was unclear where the secondary spider had gone, and a comment by one ISS astronaut led to the mistaken belief that the
spider had escaped into the Space Station. This observation led to significant interest among various media outlets and prompted headlines such as, “Lost in space: Now spider goes missing on orbital station 200 miles above Earth” (Bates, 2008). Eventually, we learned that the second spider was simply momentarily out of view in a corner of the main chamber.

At the completion of the investigation, the 25 participating teachers were surveyed regarding the quality, usefulness, and overall appeal of the project and supporting materials. As can be seen in Table 1, teachers were highly enthusiastic about participating in similar programs, and they reported a high level of interest among their students. Teachers also reported that, as a result of project activities, 33% of their students designed additional experiments and 80% of students expressed interest in science careers. Further evidence of the project’s impact can be gleaned from student questions that arose during the investigation—and from the fact that these inquiries were appropriate and relevant. Typical questions, as reported by teachers, included: *How does space affect the angles at which a spider makes a web?* ... *the number of U-turns in a spider’s web?* ... *the positioning of the hub in a web?* ... *the position of the spider in its web?*

The spider portion of experiment provided opportunities for students to ask original scientific questions and focus on particular aspects of the spiders’ activities in microgravity. For example, a 7th grade student at Aurora Hills Middle School in Aurora, Colorado, presented the outcomes of her investigation as a poster at the American Arachnological Society’s 2009 Annual Meeting in Russellville, Arkansas. The poster, which received a special award, compared the web hub placement of a spider in microgravity to that of a control spider in the student’s classroom.

### Table 1. Pilot-test Teacher Survey Outcomes (N = 25 teachers)

<table>
<thead>
<tr>
<th>Question</th>
<th>Mean</th>
<th>StDev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Please rate your students’ interest in the project by checking the number that most closely represents your opinion. (1=Not Interested; 5=Very Interested)</td>
<td>4.7</td>
<td>0.52</td>
</tr>
<tr>
<td>Did this project support your curriculum objectives? (1=Not Supportive; 5=Very Supportive)</td>
<td>4.7</td>
<td>0.67</td>
</tr>
<tr>
<td>If there were an opportunity for you and your students to participate in an improved version of this project, would you? (1=Definitely Would Not; 5=Definitely Would)</td>
<td>4.8</td>
<td>0.42</td>
</tr>
<tr>
<td>How interested were your students in learning about the organisms used in this investigation? (1=Not Interested; 5=Very Interested)</td>
<td>4.7</td>
<td>0.48</td>
</tr>
<tr>
<td>How interested were your students in caring for the organisms used in this investigation? (1=Not Interested; 5=Very Interested)</td>
<td>4.6</td>
<td>0.52</td>
</tr>
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**BUTTERFLIES IN SPACE (FULL-SCALE PROJECT)**

The success of the Butterflies and Spiders in Space pilot in 2008 led to the implementation of a similar experiment the following year using painted lady butterfly larvae. Applying data gathered and lessons learned, BioServe refined the original technical model—most notably the procedures for feeding painted lady butterfly larvae and adults within their habitats. BCM and BioServe then set out to design and conduct a full-scale ISS investigation that involved students nationally, even globally, in a project that
combined the excitement of an authentic, real-time space-based experiment with hands-on classroom explorations and engaging web-based resources. While students collected and shared experiment data and observations related to their own inquiries about life on Earth and in microgravity, project managers sought to determine whether teachers, limited by rigid schedules and test-driven curricula, would use the content created for this project (and offered free of charge) with their students.

**Procedure.** Butterflies in Space followed a similar design as the Butterflies and Spiders in Space project. However, given the disappointing and incomplete outcomes of the pilot project’s butterfly component, we focused the second investigation solely on butterflies. In November 2009, the subjects of the Butterflies in Space project (*Vanessa cardui* butterfly larvae) were transported into space on Space Shuttle Atlantis (STS-129) and housed on the ISS in Commercial Generic Bioprocessing Apparatus habitats created by BioServe. (Prior to launch, ground-based butterflies living in the same habitats were consistently being cultivated through complete life cycles.) Butterfly habitats for classroom investigations featured an inexpensive clear plastic “clam-shell” box and plastic portion cups to provide water and food. The real-time investigation spanned November and December 2009.

While planning the Butterflies in Space project, BCM convened an experienced team of curriculum writers and evaluators to create a teacher guide, focused on grades 3-8, with curricular content that reinforced key science concepts related to space life science, microgravity, and spaceflight. The Butterflies in Space curriculum guide shown in Figure 1 was published in 2009, for teachers and students to use in conjunction with this experiment (Vogt et al., 2009). Along with a series of manuals created by BCM to facilitate successful classroom experiments (“Designing Your Investigation,” “Scientific Image Processing,” and “Naturalist Journals”) the free Butterflies in Space curriculum guide is available on the BCM websites noted earlier. It contains background science information on painted lady butterflies, instructions for creating an Earth-based habitat and caring for butterfly larvae and adults, and six guided inquiry lessons that provide a foundation for students’ own investigations. Since little is known about how or whether butterflies rely on gravity for growth and development, orientation, feeding, or wing expansion, this experiment enabled students to investigate unique questions and even contribute to general scientific knowledge.

BioServe handled all Butterflies in Space mission-related requirements, including payload integration, safety, mission planning, payload verification, and flight certification. BioServe also conducted crew training; supported the required NASA flight bench review; performed final payload integration, testing, and flight certification processes at a NASA facility; communicated regularly with science teams on payload status; and received payload at turnover from NASA after the orbiter landed. Still and video images of the butterflies were recorded at regular intervals and transmitted to BioServe’s servers, from where they were relayed to BCM for posting on project web pages. An example is provided in Figure 2.

Utilizing the Internet to disseminate educational content and products globally, immediately, and at relatively low cost, BCM was able to share Butterflies in Space with classrooms around the world via its BioEd Online and K8 Science websites (Moreno and Erdmann, 2010), various email lists of science educators, and social media sites, such as Facebook and Twitter. Classes conducting butterfly experiments had free access to near real-time photo and video archives of the butterflies on the ISS, which they could compare to the painted lady butterflies living in their own classrooms. In addition, BCM provided online streaming video demonstrations showing key portions of each student activity, with user tips and interactive vocabulary connections; a trouble-shooting section for common questions about procedures and outcomes of classroom activities; downloadalbe PDF files of teaching resources (e.g., lesson plans, handouts, activity worksheets, student reading and mathematics extension materials); references or links to other background or student materials that could be used to enhance or extend the activities; and a password-protected discussion forum for instructors.
Figure 1. Teacher and student guides for space-based investigations. BCM created and published a teacher guide and several supporting manuals that provide classroom lessons, procedural/technical recommendations, and background information for classes conducting Butterflies in Space investigations.
Figure 2. Space butterflies. Still shot from video of fully emerged Painted Lady butterflies and floating debris aboard the ISS. Via the BioEd Online and K8 Science websites, images and teaching materials from the Butterflies in Space project were disseminated to thousands of teachers and students globally.

Upon conclusion of the Butterflies in Space project, BCM emailed a link to a web-based survey to all registered participants. The survey asked whether registrants and their students had participated in the experiment. It also collected basic information about the kinds of activities carried out by students and teachers (e.g., raised butterflies, downloaded images, asked research questions, analyzed space images, entered butterfly projects in science fairs, participated in the Butterflies in Space poster competition held after data collection had ended). The survey also served as a formative assessment, because respondents provided suggestions about ways to improve the teaching materials and project delivery.

Outcomes. The STS-126 pilot investigation provided invaluable guidance for the design of the STS-129 Butterflies in Space project. In the second mission, all four (100%) of the butterfly larvae aboard the ISS successfully fed, pupated into chrysalises, and emerged as adults. Photo albums of the “space butterflies” at various life stages were archived and remain available on BioEd Online and K8 Science. By the end of December 2009, the project’s four painted lady butterflies flown to the International Space Station
had completed their normal lifespans, and data collection from space ended. The final two images from the ISS, taken 11 minutes apart on December 10, 2009, show that all movement by the adult butterflies had ceased.

As illustrated in Figure 3, Butterflies in Space engaged thousands of teachers and students around the US and the world. Many classes raised their own painted lady butterflies to compare with those on the ISS. Through December 2009, more than 3,000 teachers, representing approximately 180,000 students in all 50 states and more than 23 countries, had registered on BioEd Online to download the free Butterflies in Space teacher guide. Registering teachers indicated their intent to use the guide’s activities with their students, and also estimated the number of students who would be involved. Following the mission in November-December 2009, the total number of teachers who registered and downloaded the guide increased to 3,076. Butterflies in Space lessons and accompanying resources were developed primarily for US grades 3-6, but actual project participants represented a much wider range of grade levels. To date, an estimated 3,600 PDF copies of the teacher guide have been downloaded.

Figure 3. Distribution of teachers registered for Butterflies in Space. The Butterflies in Space project reached thousands of teachers and students in the US and globally.
Beyond its appeal to teachers and students, Butterflies in Space generated significant interest from US and international media outlets. Hundreds of websites covered the project, including those of Discovery News, Scientific American, Howard Hughes Medical Institute, Cornell University Department of Astronomy, Google News, NASA, India Times, The Register UK, USA Today, Yahoo News, and Space.com. TV stations and newspapers in many states also reported on the activity. Live streaming video of the ground-based larvae attracted more than 400,000 viewers from around the world, and several videos of the larvae and adult butterflies on the ISS have been viewed more than 100,000 times. Additional dissemination via Facebook and Twitter updates posted by NSBRI reached thousands of additional followers.

The project’s follow-up online survey produced 373 completed questionnaires, a response rate of 12%. Of the respondents, 72 percent stated that they had participated in the Butterflies in Space investigation by raising butterflies or downloading flight images. Registrants indicated that their students had participated in the investigation in the following ways.

- 67% of students downloaded images.
- 50% of students raised butterflies.
- 42% of students analyzed images from space.
- 33% of students asked research questions.
- 4% of students entered butterfly projects in a science fair.
- 3% of students entered the Butterflies in Space poster contest.

In addition, 39% of the survey respondents reported that their students participated in other ways, such as writing poetry, using measurement data for math problems in an Algebra class, planting a butterfly garden, and keeping butterfly journals. Teachers also made suggestions for improving future similar projects. The most common recommendations are listed below.

- Provide more advance notice before the experiment begins, so that teachers have enough time to plan activities and obtain materials.
- Offer additional teaching materials, such as data sheets, graph templates, related lessons, and links to supporting resources.
- Provide opportunities for students to communicate with each other about the project, or with astronauts or scientists.
- Provide quantitative data from experiments on the ISS, in addition to photographs.
- Send regular project updates and notices via email, in addition to posting them on the Web.
- Add lessons that link the project to other subject areas, such as mathematics.

A poster competition held at the conclusion of the Butterflies in Space project allowed students to submit, electronically, scientific posters reflecting their outcomes. A poster template was provided on the Butterflies in Space webpage, and individuals or groups of students submitted their investigations as posters. The winning upper division poster, created by students in grade seven, investigated microgravity’s effect on painted lady butterfly wingspans, and presented the conclusion that “the average butterfly wingspan on the ISS is larger than our test subjects on Earth.” The winning lower division poster asked, “What effect does gravity have on butterfly larvae frass?” The second grade students who created the poster predicted that frass would float in space, but would remain on the floor of the habitat in their classroom. The winning posters can be seen on BioEd Online.

DISCUSSION AND CONCLUSIONS

The Internet enabled BCM to promote Butterflies in Space, distribute supporting materials, communicate with participants, disseminate near real-time flight data, and rapidly engender worldwide participation among thousands of teachers and students, many of whom would not have been aware of the project or able to participate otherwise. Furthermore, although the flight investigation has concluded, the project images and data are permanently archived on the BioEd Online and K8 Science websites and available for further study. Teachers and students can construct habitats, obtain butterfly larvae, and repeat the ground investigation at any time.
Indeed, the investigation exemplified the value of the World Wide Web in communicating the availability of unique educational resources and enabling teachers and students to participate in authentic scientific research. Enlistment of the public to collect scientific data and contribute to science knowledge has been shown effective in a number of activities, such as species inventories (Bonney et al., 2009), mapping and monitoring of species distributions and migrations, and even hypothesis-driven research, such as the Evolution MegaLab study of shell polymorphisms in European banded snails (Silvertown, 2009). The Global Learning and Observations to Benefit the Environment (GLOBE) program, in particular, has successfully engaged students, teachers, and scientists worldwide in large-scale data collection and inquiry investigations related to environment and climate (Gazal et al., 2008). The 10-year evaluation of that program concluded that engaging students with subject matter content and data analysis contributed to the improvement of their science inquiry skills (Penuel et al., 2006). Butterflies in Space differed from these programs, however, because the project did not involve data collection in different locations over multiple years. In addition, students and teachers participating in Butterflies in Space generated their own hypotheses, and used observations from a common data set and their own ground-based controls as evidence, rather than contributing data related to an overarching question posed by project investigators. Nonetheless, it reinforced the view that “non-scientists” can participate in, and make relevant contributions to, scientific research, and that engagement in shorter duration, authentic experiments contributes to students’ interest and skills development in science.

Butterflies in Space also highlighted the growing importance of social media in science education. Facebook posts by NSBRI and BCM generated additional traffic back to BioEd Online, which on peak project days had more than 4,000 unique visitors. Project videos posted on the video hosting site, YouTube, attracted hundreds of thousands of views of the painted lady butterflies “in action” on ISS. The experiments were covered by general and science news organizations, and generated numerous pages of posts on discussion forums and blogs.

Further, Butterflies in Space demonstrated that teachers and schools will involve students in open-ended science experiments when the experience has merit, is available free of charge, and does not occupy excessive amounts of class time. Active engagement of students in scientific investigations has been found to be more effective in increasing conceptual understandings than are passive strategies that simply aim to prepare students to pass standardized assessments benchmarked to local or state standards (Minner et al., 2010). Thus, it is critical for science educators to continue to design and implement low-cost learning experiences that bring real-world science experiences to students of all ages.

Following the Butterflies in Space project, BCM and BioServe extended their educational collaborations to conduct two additional space-based missions featuring spiders and plants, respectively. Thousands more teachers and students participated in these projects, demonstrating both the validity and appeal of this approach. Refinements continued to be made to the protocol (for example, ISS habitats for a 2011 spider mission were remodeled to allow clearer photos), and new concepts were explored (Plants in Space, conducted in 2011, used a common classroom plant, *Brassica rapa*, to investigate and compare plant root growth in microgravity and on Earth). Given the inexpensive materials required for these experiments, the open-ended nature of student investigations, availability of free curricular guides and resources, and the ongoing interest in space-based content, this model is highly replicable and likely to appeal to students and teachers across wide segments of the K-12 population.

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