GENERAL INFORMATION

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Proceedings of the 20\textsuperscript{th} Annual Meeting of the American Society for Gravitational and Space Biology

Featuring:

Symposium I: Model Organisms for Exploration Biology

Symposium II: Pharmacological Countermeasures to Physiological Changes Induced in Space Flight

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Symposium I: Model Organisms for Exploration Biology

Nancy Searby, Editor
THE YEASTS SACCHAROMYCES CEREVISIAE AND SCHIZOSACCHAROMYCES POMBE: MODELS FOR CELL BIOLOGY RESEARCH

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ABSTRACT

Yeast species provide excellent models for fundamental biological research. In this review, I will describe characteristics of the two most common laboratory systems: the fission yeast Schizosaccharomyces pombe, and the budding yeast Saccharomyces cerevisiae. They have substantial similarities that make them powerful as research tools, and also striking biological differences that make them complementary experimental models. Each provides unique tools for understanding environmental effects on cellular systems.

INTRODUCTION

Yeast is a general term, covering a wide range of very different single-celled fungi. In the molecular biology laboratory, two species are commonly employed as models for biomedical research: the budding or brewer’s yeast Saccharomyces cerevisiae, and the fission yeast Schizosaccharomyces pombe. Although they share the designation “yeast”, these two species are evolutionarily very distinct from one another, with estimates of up to 1000 million years (MY) since they diverged from a common ancestor (Heckman et al., 2001; Hedges, 2002). Although single cells, they are true eukaryotes, and share fundamental cellular processes with metazoan systems. Each offers unique tools to the cell biologist, providing complementary approaches and insights into functions of larger eukaryotes (e.g., (Forsburg, 1999; Forsburg and Nurse, 1991; Lew et al., 1997; MacNeill and Nurse, 1997; Russell and Nurse, 1986)). Both yeasts are harmless, tractable genetic systems, easily manipulated in the laboratory using superb molecular tools (Forsburg, 2001).

HISTORY AND CHARACTERISTICS

S. cerevisiae was adopted as a model system for laboratory study in the 1930s, as investigators developed genetic tools to understand its life cycle and sexual differentiation (Hall and Linder, 1993). It provided an important tool to understand recombination and the transmission of genetic material, and launched into greater prominence with the molecular era in the 70s, when it became identified as a sort of eukaryotic E. coli. With potent genetic tools and a typical eukaryotic cell organization, budding yeast became a favorite system to tackle cell biology questions. It was the first eukaryote to be sequenced (Goffeau et al., 1996), which has sparked a whole new era developing genomics tools.

S. pombe lagged behind as an experimental system. It was isolated as late as the 1890s from East African millet beer (pombe means beer in Swahili), but limited genetic studies only began in the late 40s. It was picked up further in the 60s for studies of growth control, which were facilitated by its regular rod shaped morphology (Leupold, 1993; Mitchison, 1990). For some time, fission yeast was a model primarily for cell division and sexual differentiation (meiosis). At first, the molecular tools developed for S. cerevisiae were adapted to S. pombe, but were subsequently replaced with pombe-specific methods. In recent years, more investigators have chosen to work on S. pombe, and additional areas of research have opened up, although the community is still smaller than that working on budding yeast. The complete genome sequence was published in 2002 (Wood et al., 2002).

In the laboratory, both species are typically maintained as haploid cells. Cells are tolerant of cold and can be stored frozen at −70°C. Generation time varies with media and temperature, but is generally in the 2-4 hour range. In response to nutrient limitations, yeast cells exit the cell cycle and enter stationary phase; this a period of dormancy, more severe than the G0 phase in mammalian cells. A particular strength of both systems is that they also have a diploid sexual cycle: haploid cells of opposite mating types can mate, resulting in cell and nuclear fusion. Diploids can be maintained in the laboratory or induced to enter meiosis and sporulate. The four spores packaged in the yeast ascus are the fungal equivalent of human gametes. Thus, the entire life cycle of yeast cells provides a simple model for events occurring in human cells.

Both cell types have highly organized internal structures with the membrane-delimited compartments typical of eukaryotic cells, including a nucleus, mitochondria, Golgi and other structures. S. cerevisiae grows by budding, which requires highly targeted cell growth and mechanisms for spatial coordination with nuclear division (Pruyne et al., 2004). S. pombe is a linear, rod shaped cell that grows at its ends and divides by medial fission. This likewise requires significant positional awareness (Hayles and Nurse, 2001). The maintenance of cellular organization can be perturbed with drugs or mutants, and provides an interesting problem for effects of microgravity.
GENOME STUDIES AND SPECIES CONSERVATION

Comparison of the genomes reveals significant differences between the species (Goffeau et al., 1996; Wood et al., 2002; Wood et al., 2001). Although both have a haploid genome with over 12 megabases (Mb) of DNA, haploid \textit{S. cerevisiae} has 16 chromosomes while \textit{S. pombe} has only 3. There is no synteny (conserved gene order), not surprising given their long period of divergence. \textit{S. cerevisiae} has about 5800 likely protein-encoding genes. A significant fraction of these are paralogues (genes of related sequence that are not exact functional homologues). Evidence suggests that the budding yeast genome has undergone several large scale duplications through evolution, and following gene loss, the remaining duplicates may have diverged in expression or function (Dietrich et al., 2004; Kellis et al., 2004; Wolfe and Shields, 1997). Thus, it has proven a good model for genome evolution. In contrast, the fission yeast, with a similar total genome size has about 4800 genes, with no evidence for large scale duplication (Wood et al., 2002). Genome organization also differs: over 40% of fission yeast genes have introns (non-coding sequences that interrupt the gene), while a scant 5% of budding yeast genes are interrupted.

\textit{S. pombe} has proportionally more genes conserved in metazoans than does \textit{S. cerevisiae}, although each yeast species shares genes with metazoans that the other yeast lacks (Aravind et al., 2000; Wood et al., 2002; Wood et al., 2001). Interestingly, these often fall into functional groups (Aravind et al., 2000). For example, budding yeast has lost many of the genes associated with the signalosome, a proteosome-related complex required for diverse signaling pathways that is present in fission yeast (Aravind et al., 2000). \textit{S. cerevisiae} lacks a number of genes required for the spliceosome, that is responsible for removing introns: again, these are genes that are preserved in \textit{S. pombe} (Aravind et al., 2000). The proteins required for RNA interference (Dcr1, Ago1, Rdpl; (Hall et al., 2002; Schramke and Allshire, 2003; Volpe et al., 2002) are absent from budding yeast, but present in fission yeast.

Finally, a significant number of chromosome associated proteins are absent in budding yeast but shared between fission yeast and metazoa, including the conserved Cln4/SuVar3-9 histone methyltransferase (Bannister et al., 2001; Nakayama et al., 2001), and the Swi6 and Chp2 HP1-heterochromatin proteins (Eissenberg and Elgin, 2000; Ekwall et al., 1995; Halverson et al., 2000; Thon and Verhein-Hansen, 2000), telomere proteins Taz1/TRF2 (Cooper et al., 1997; Nimmro et al., 1998) and Pot1 (Baumann and Cech, 2001) and the centromere associated CENP-B proteins (Baum and Clarke, 2000; Irelan et al., 2001; Nakagawa et al., 2002). This difference extends beyond gene sequences to chromosomal elements. Typically, chromosome structures in budding yeast are very small and streamlined, in contrast to fission yeast and metazoan which have large, degenerate, and diffuse structures. For example, while the budding yeast origin of replication is a 100bp element with a short, highly conserved consensus sequence, the fission yeast origin is 10 times larger (rev. in (Clyne and Kelly, 1995; Newlon and Theis, 1993; Zhu et al., 1994)). There is no consensus sequence in the fission yeast origin, and no single mutation abolishes its function (Clyne and Kelly, 1995; Dubey et al., 1996; Kim and Huberman, 1998). Importantly, a large, diffuse structure appears to be characteristic of metazoan replicators as well (rev. in Gilbert, 2001), suggesting the fission yeast origin is particularly appropriate as a model for function of higher eukaryotic origins. Similarly, the centromeres of the two species are very different in size and structure (reviewed in Clarke, 1998; Hegemann and Fleig, 1993; Sullivan et al., 2001). In budding yeast, the centromere is a small element (about 100 bp) with a discrete consensus sequence. In contrast, the fission yeast centromere is a large, degenerate element of between 40-100 kilobases (kb) with numerous simple repeats, very similar to the centromere structure reported for \textit{Drosophila} (reviewed in Clarke, 1998; Hegemann and Fleig, 1993; Sullivan et al., 2001). For this reason, \textit{S. pombe} has become a particularly powerful system for understanding chromosome dynamics.

\textit{S. cerevisiae} proves to be a better model for other cellular functions. For example, \textit{S. cerevisiae} has been used to study the peroxisome, a metabolic organelle that functions in oxidation of fatty acids and oxidative stress (Brown and Baker, 2003; Lazarow, 2003); in contrast, \textit{S. pombe} lacks many of the conserved proteins involved in peroxisomal biogenesis and function ((Wood et al., 2002), and V. Wood, pers. comm.).

Even where functions are superficially similar, study in the two yeasts provides unique and complementary information. For example, both species were studied intensely to determine mechanisms of cell division control (rev. in Forsburg and Nurse, 1991). These studies allowed identification and characterization of many genes that are required to regulate normal events in the cell cycle progression and respond to any defects. However, at first it appeared that they used a different regulatory logic: \textit{S. cerevisiae} regulates its cell cycle at the G1/S phase transition, when DNA replication occurs; in contrast, data suggested that \textit{S. pombe} primarily regulates the decision to initiate mitosis (chromosome segregation). Only by combining the data using a “compare and contrast” approach was it apparent that both species used the same regulatory molecules, and in fact, both control points were present in both species, with the balance shifting between them due to growth conditions. This resulted in a powerful experimental synergy that significantly expanded our understanding of cell cycle regulation and provided unique insights into mammalian cell function. Neither yeast was sufficient alone to explain cell cycle dynamics in all larger eukaryotes: both yeasts contributed essential information.
These cell cycle genes are conserved throughout metazoa, showing that they define fundamental pathways common to eukaryotes. Defects in their function are implicated in many forms of human cancer (reviewed in (Evan and Vousden, 2001; Fodde and Smits, 2002; Ford and Pardee, 1999; Maser and DePinho, 2002; Vessey et al., 2000; Wassmann and Benezra, 2001; Willers et al., 2002)). The significance of these studies in both yeasts was recognized by the 2001 Nobel prize that S. cerevisiae geneticist Lee Hartwell and S. pombe geneticist Paul Nurse shared with biochemist Tim Hunt.

**DAMAGE RESPONSE**

Further studies have examined the response of normal and mutant cells to specific genome insults caused by ionizing or ultraviolet radiation, alkylating agents, nucleotide starvation, or replication defects, thus providing a fingerprint of cellular responses that can be used to identify specific forms of genetic damage. These studies are well-developed and have provided significant insights into the maintenance of genome integrity. This wealth of information provides important tools to study the effects of space radiation on living systems, and moreover, provides a clear context to relate results to known effects on Earth.

When DNA is damaged or DNA synthesis is blocked, cells activate checkpoint pathways (rev. in Boddy and Russell, 2001; Carr, 2002; Caspari and Carr, 2002; Osborn et al., 2002). These pathways recognize a damage signal, transmit the signal and respond appropriately. They ensure that cells arrest S phase progression, protect replication structures, repair any defects, and finally restart the replication process and recover. Checkpoints also ensure that the cell chooses the correct mechanism of repair for the lesion it encounters. The response to DNA damage in the yeasts is mediated by kinase response pathways (reviewed in (Caspari and Carr, 2002; O’Connell et al., 2000; Rhind and Russell, 2000; Zhou and Elledge, 2000)). Recent data suggest that exposed single strand DNA (ssDNA) coated by replication protein A (RPA) as damaged regions are exposed or resected may be an activating signal for Rad3-family kinases (Zou and Elledge, 2003).

Although the checkpoint proteins are largely in common between S. pombe and S. cerevisiae, the details of their responses differ. Unusually, in S. cerevisiae the G2, or damage checkpoint operates by controlling the metaphase to anaphase transition, while in fission yeast and metazoans, it operates by controlling the Cdc2 kinase (reviewed in Nyberg et al., 2002). In addition, several of the checkpoint proteins are essential for viability in normal vegetative S. cerevisiae, due to an additional role in regulating nucleotide synthesis; while they are not essential in S. pombe (Liu et al., 2003; Zhao et al., 1998). These differences provide a further example of the complementary data obtained by studying both yeasts.

Many repair and replication genes interact with these checkpoint kinases providing the cell with multiple mechanisms to respond to genotoxic insults (rev in (Caspari and Carr, 2002; O’Connell et al., 2000; Rhind and Russell, 2000)). There are also differences in the repair of DNA damage. Fission yeast is significantly more resistant to UV or ionizing radiation than budding yeast, which suggests additional repair pathways are operating (discussed in (McCready et al., 2000; Murray et al., 1994)). It is known that fission yeast has an additional UV-repair system (the Uve1 endonuclease) not found in budding yeast (Yasui and McCready, 1998). Both species, however, show significantly more radio-resistance than metazoan cells. Mutants defective in various repair pathways can be used to categorize the damage that the cells have suffered.

Probably the most threatening form of damage that a cell can suffer is double strand breaks (DSBs) in the genome. Therefore, checkpoint and repair pathways operate to minimize the conditions where DSBs can occur and maximize efficient repair. Ionizing radiation induces DSBs which also occurs following treatment with the alkylating agent MMS, radiomimetic drugs such as bleomycin, or collapse of unprotected replication forks (Kostrub et al., 1997; Memisoglu and Samson, 2000; Rhinds and Russell, 2001). DSBs are repaired by two broad pathways (reviewed in Haber, 2000; Krejci et al., 2003). The first is recombination, either error-free homologous recombination (HR) or error-generating single strand annealing (SSA). The second is non-homologous end joining (NHEJ), which is likely to generate errors, rearrangements and translocations. HR and NHEJ may be distinguished by the enzymes they employ. Mutants lacking these functions are not surprisingly very sensitive to ionizing radiation and other DSB related challenges.

UV radiation produces a variety of lesions including pyrimidine dimers and other photoproducts. These are generally repaired by photolyases (light-activated repair
enzymes), or by recombination and in some species, including fission yeast, there is an additional excision repair pathway termed UVDE (rev. in Yasui and McCready, 1998). Interestingly, recombination mutants in S. pombe are generally also sensitive to UV radiation, suggested a role for HR proteins in processing UV damage in this species (McCready et al., 2000). Alkylation agents such as MMS have a range of effects. They typically generate abnormal bases, which are substrates for repair by base or nucleotide excision repair (BER and NER). In addition, MMS damage ultimately results in DSBs (discussed in (Memisoglu and Samson, 2000)). DNA damage associated with UV and MMS often is associated with mutagenic repair, in which genetic information is lost or changed. The genetic requirements for these different repair pathways tend to merge as the pathways feed into common mechanisms of resolution, so that they share various components.

Damage sensitivity of different mutants, and the activation of different forms of repair can distinguish the nature of the damage suffered by cells. Overall viability in response to damage can be determined by cell viability. Because some repair mechanisms are mutagenic, rates of mutation provide an additional metric to determine the cellular response. If cells can be fixed in ethanol or formaldehyde, cytological methods that distinguish particular forms of damage can be employed. For example, in mammals, ATM/ATR-dependent phosphorylation of the H2AX histone variant marks regions of DSBs and undergoes ATM/ATR dependent phosphorylation; in fission yeast, the same role is served by phosphorylation of “regular” H2A (Burma et al., 2001; Nakamura et al., 2004; Redon et al., 2002; Shroff et al., 2004; Ward and Chen, 2001). Similarly, recruitment of repair proteins such as the recombination proteins Rad52 (SpRad22) or Rad51 (SpRhp51) can be used to generate a variety of DNA lesions that include single strand DNA and DSBs (e.g., (Caspari et al., 2002; Du et al., 2003; Grischchuk et al., 2004; Kim et al., 2000; Noguchi et al., 2003)).

Thus, analysis of the biological responses of wild type and mutant yeast cells to space travel is likely to provide significant insights into the sorts of damage suffered by living systems in space. Since the two species have different response pathways, they provide complementary information to one another.

CONCLUSION

The yeasts S. pombe and S. cerevisiae are the workhorses of modern cell biology. Their study has provided significant insights not only into cell cycle control and damage responses, but to every aspect of cell behavior from chromosome segregation to protein secretion. These two organisms provide sophisticated genetic and molecular tools, as well as genome-level strategies to examine gene regulation and cellular responses. Despite superficial similarities, these species are significantly diverged from one another. Studies over many years, particularly in the cell cycle field, has shown that there is a terrific synergy to examining phenomena in both species. If the goal is insight into the responses of metazoa, particularly human cells, long experience shows that these yeasts provide complementary information, which has led to important advances in our understanding of mechanisms of cell growth and regulation.

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INTRODUCTION

Although it is well known that radiation causes mutational damage, little is known about the biological effects of long-term exposure to radiation in space. Exposure to radiation can result in serious heritable defects in experimental animals, and in humans, susceptibility to cancer, radiation-sickness, and death at high dosages. It is possible to do ground controlled studies of different types of radiation on experimental animals and to physically measure radiation on the space station or on space probes. However, the actual biological affects of long-term exposure to the full range of space radiation have not been studied, and little information is available about the biological consequences of solar flares. Biological systems are not simply passive recording instruments. They respond differently under different conditions, and thus it is important to be able to collect data from a living animal. There are technical difficulties that restrict the placement of an experimental organism in a space environment for long periods of time, in a manner that allows for the recovery of genetic data. Use of the self-fertilizing hermaphroditic nematode, Caenorhabditis elegans offers potential for the design of a biological dosimeter. In this paper, we describe the advantages of this model system and review the literature of C. elegans in space.

THE C. ELEGANS SYSTEM

C. elegans is a well-established animal model, which is easy to culture in a laboratory. Normally it is maintained on agar culture plates and fed a non-pathogenic bacteria. C. elegans is a self-fertilizing, effectively isogenic hermaphrodite which produces approximately 300 progeny from a single individual. Hermaphrodites have two X chromosomes while males, which are XO, are produced spontaneously as a result of X-chromosome loss or nondisjunction. Once mated by a male, a hermaphrodite produces out-cross progeny. This is a very useful feature for doing genetic crosses. The hermaphroditic life style is especially useful for maintaining animals over several generations, on for example, sustained missions. The complete cell lineage is known, including those cells genetically programmed to die (apoptosis). The worms are transparent at all developmental stages, making it possible to examine cell division and development in real time. C. elegans is a metazoan with a number of tissue types, such as nervous system, muscle, intestine and gonad. Many of the developmental and biochemical pathways are conserved with human. There is a large community of C. elegans researchers taking genetics-based approaches to understanding fully the biology of this organism. An introduction to the C. elegans system and literature has been reviewed in Riddle et al.(1997) and is available at a web site maintained by Leon Avery at http://elegans.swmed.edu/. At this site there is also information about up-coming meetings, abstracts, in-house publications, researchers’ contact information, and current methodologies.

EXPERIMENTAL RESOURCES

The genome has been completely sequenced and consists of approximately 20,000 predicted protein-encoding genes (Sequencing Consortium 1998) and at least 50% of C. elegans’ genes have human homologs (McKay, et al. 2004). A powerful approach for studying loss of gene function technology has recently been developed. Double-stranded RNA can be introduced be either injection into the worm or be feeding. The most widely used approach, developed by A. Fire’s laboratory, involves feeding worms bacteria, which are producing double-stranded RNA for the gene of interest. The ingestion of the dsRNA causes inhibition of gene expression (RNAi) (reviewed in Fire et al. 1999). Libraries containing bacterial strains for most of the predicted protein-encoding genes have been constructed, and the phenotype for each of the genes observed (Fraser et al. 2000; Kamath, et al. 2003). In addition, the targeted deletion of specific genes has been undertaken by the C. elegans Reverse Genetics Consortium (a collaboration of the R. Barstead laboratory in the USA, the D. Moerman laboratory in Canada and the Y. Mitani laboratory in Japan), and requests and information are available at http://www.celeeganskoconsortium.onr.org/. An alternate approach to obtaining gene deletions is the use of transposable element insertion and excision by L. Segalat’s laboratory in France. Mutant strains of the gene knockouts are made available by the Caenorhabditis Genetics Centre (CGC) (http://www.ebs.umn.edu/CGC/). The CGC maintains and provides thousands of strains generated by these and classical genetic approaches used by the hundreds of researchers that make up the C. elegans research community. In addition there are numerous rearrangements, duplications and deletion strains generated by the Genetic Toolkit Project, the cosmid transgenetic rescue project, and other researcher projects. A further aid to mapping mutants is the database descriptions of single nucleotide polymorphisms (SNP) between strains. Many of these SNP markers can be assayed by polymerase chain reactions (PCR) followed by...
analysis of repair genes would be of interest.  *C. elegans* has a large number of repair genes which function in highly conserved pathways, that is the protein sequences are conserved with both yeast and higher organisms, including man.  A review of the repair pathways (Rose, unpublished), and many other aspects of *C. elegans* biology, will be accessible in the upcoming worm book, *C. elegans* III which will be available on-line in 2005 at www.wormbase.org.  Genes involved in responding to radiation damage were first identified by Hartman and Herman (1982).  The review presents a summary of the genes for which mutant phenotypes have been described, including components of the pathways for nucleotide excision repair, mismatch repair, DNA damage checkpoint, non-homologous end joining, homologous recombination repair, and chromosomal structure surveillance. A broad perspective on genes involved in DNA repair has been gained using high throughput, genome-wide analysis of RNAi phenotypes (Piano et al., 2002; Kamath et al. 2003; Pothof et al., 2003; Lettre et al., 2004; vanHaaften et al. , and protein interactions (B) and protein interactions (Boulton et al. 2002; Li et al.).  As part of the interactome analysis, known proteins implicated in replication, nucleotide excision repair, mismatch repair, base excision repair, nonhomologous end joining, homologous recombination and checkpoint pathways were used in yeast 2-hybrid experiments to identify physical interactors in the predicted proteome (Boulton et al. 2002; Li et al. 2004).  Components of the checkpoint signaling networks assemble into more complicated networks.  Sensors, transducers and mediators are shared when generating different responses including chromatin remodeling, altered gene expression and DNA replication.  The data demonstrate that many of the pathways are interrelated, and that pathway components exhibit previously unrecognized links between repair mechanisms and checkpoints.

**A CHEMICALLY-DEFINED MEDIA**

*C. elegans* also has many characteristics that make it an excellent model system for use in space.  *C. elegans* reproduces as a self-fertilizing hermaphrodite, is small (adults are approximately 1 mm long), and thus easily grown in a small space.  The life-cycle is short, approximately one-week under conditions at the International Space Station (ISS), and the progeny numbers high, a few hundred per hermaphrodite.  The normal food source for *C. elegans* is bacteria; however for space experimentation it can be fed a chemically defined, axenic media (CeMM) adapted for space travel by C. Conley’s laboratory at NASA, Ames (Lu and Goetsch 1993; Szewczyk N J et al. 2003).  The worms will survive for several months in CeMM media, and can be transferred to fresh media and maintained apparently indefinitely.  In addition, under conditions of overcrowding and limited food larvae can enter a dormant “dauer larva” stage.  These dauers can survive for several

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**ANALYSIS OF GENE EXPRESSION**

There are a number of resources for studying global gene expression in *C. elegans*.  Microarray analysis was first used by Reinke et al. (2000) to produce a profile of germline expression.  It has been used subsequently for a number of descriptions of differential gene expression, including a profile of the genes differentially expressed in the chemically defined CeMM media compared to the commonly used nematode growth media (NGM) (C. Conley & N. Szewczyk, NASA, p. comm.).  In addition to microarray analysis, SAGE profiles of each of the developmental stages of *C. elegans*, including the dauer larvae stage have been done (Jones et al. 2001; www.wormbase.org).  Expression of individual genes which are turned on in specific developmental stages has been characterized by GFP promoter analysis (McKay, et al. 2004).  In this case, a fluorescent reporter lights up when the gene is transcriptionally active (expressed).  For example, a muscle-specific promoter expresses only in muscle (Fig. 1).  The potential exists to adapt this approach to a reporter detection system in space, which would record which genes are turned on under particular conditions, for example, responses to lift-off and solar flares.

**REPAIR SYSTEMS**

In the case of assaying the response to radiation, reporter analysis of repair genes would be of interest.  *C. elegans* has a large number of repair genes which function in highly conserved pathways, that is the protein sequences are conserved with both yeast and higher organisms, including man.  A review of the repair pathways (Rose, unpublished), and many other aspects of *C. elegans* biology, will be accessible in the upcoming worm book, *C. elegans* III which will be available on-line in 2005 at www.wormbase.org.  Genes involved in responding to radiation damage were first identified by Hartman and Herman (1982).  The review presents a summary of the genes for which mutant phenotypes have been described, including components of the pathways for nucleotide excision repair, mismatch repair, DNA damage checkpoint, non-homologous end joining, homologous recombination repair, and chromosomal structure surveillance. A broad perspective on genes involved in DNA repair has been gained using high throughput, genome-wide analysis of RNAi phenotypes (Piano et al., 2002; Kamath et al. 2003; Pothof et al., 2003; Lettre et al., 2004; vanHaaften et al. , and protein interactions (B) and protein interactions (Boulton et al. 2002; Li et al.).  As part of the interactome analysis, known proteins implicated in replication, nucleotide excision repair, mismatch repair, base excision repair, nonhomologous end joining, homologous recombination and checkpoint pathways were used in yeast 2-hybrid experiments to identify physical interactors in the predicted proteome (Boulton et al. 2002; Li et al. 2004).  Components of the checkpoint signaling networks assemble into more complicated networks.  Sensors, transducers and mediators are shared when generating different responses including chromatin remodeling, altered gene expression and DNA replication.  The data demonstrate that many of the pathways are interrelated, and that pathway components exhibit previously unrecognized links between repair mechanisms and checkpoints.

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months at ambient temperature and will resume their normal life-cycle when introduced to fresh axenic media.

For experimental purposes, samples can be prepared either on the ISS or on the ground for subsequent data analysis. At the re-entry site the samples can be preserved alive in a frozen state in liquid nitrogen and be recovered later for biological analysis.

**C. ELEGANS IN SPACE**

Johnson and Nelson (1991) first proposed using *C. elegans* as a model system for space biology studies. Since then *C. elegans* has flown on several missions to Earth orbit, and was shown to develop and reproduce normally, making it an excellent model system for biological research in space. Nelson et al. (1989; 1994a; 1994b) investigated mutations induced by cosmic rays in *C. elegans* on Spacelab in low Earth orbit. Their analysis was for short-term (8 days) radiation exposure. Currently nothing is known about longer term exposure to the different types of radiation in space, nor about the effects of exposure to the range of radiation in the space environment. In his review, Nelson (2003) states that “The unique feature of the space radiation environment is the dominance of high-energy charged particles (HZE or high LET radiation) emitted by the Sun and galactic sources, or trapped in the Van Allen radiation belts. These charged particles present a significant hazard to space flight crews, and accelerator-based experiments are underway to quantify the health risks due to unavoidable radiation exposure”. Recently, Nelson et al. (2002) examined the effect of different types of radiation, gamma rays, accelerated protons, and iron ions at the same physical dose. Using RT-PCR differential display and whole genome microarray hybridization experiments, they described unique transcriptional profiles for the different radiation treatments. The genes affected by each radiation species were associated with unique regulatory clusters, highlighting our limited knowledge of the biological responses to radiation exposure.

**THE ICE-FIRST PROJECT**

In this context, we took part in a recent International collaboration to use *C. elegans* as a model system for biological studies in space, ICE-First (Fig. 2). The project was coordinated by Michel Viso of the Centre Nationale d’Etudes Spatiales (CNES) with the help of the European Space Agency (ESA) and the Space Research Organization of the Netherlands (SRON) and was flown on the Dutch Science Mission (DSM) to the International Space Station during April 19-30 2004. Researchers from France, Japan, USA and Canada participated. The scientific projects included validation of liquid culturing in the space flight environment; studies on muscle protein growth and maintenance; whole genome microarray responses to spaceflight; and morphology of larval development during space flight (Table 1). The effects on nematodes being grown for three to four generations in CeMM media and of being in space for the 10 days of the mission is being analyzed by A. Rose’s laboratory for eT1-balanced mutations (Zhao et al., ‘Spaced-out Worms’ abstract available at http://elegans.swmed.edu/) and by D. Baillie’s laboratory for changes in RNA expression (unpublished).

**ACCUMULATED MUTATION RATE MEASUREMENT**

The most common type of easily identified mutation is that affecting expression of an essential gene (lethals). Lethals will not accumulate if normal animals are used. We have developed a system to measure the accumulated mutation rate, the eT1-system (Rosenbluth and Baillie, 1981; Rosenbluth et al., 1983) was used. eT1(III;V) (eT1) is a reciprocal translocation that recombinationally balances the left half of *Linkage Group V* [LG(V)] and the right half of *Linkage Group III* [LG(III)] (Rosenbluth and Baillie 1981) which is nearly 20% of the...
nematode’s genome. eT1(III) breaks in unc-36 thus giving eT1 a visible phenotype. LGV(left) contains approximately 7% (23 m.u.) of the recombinational distance in the genome and approximately 10% of its DNA. It is relatively straightforward to calculate forward mutation rates using this system, and that has been done for mutagens routinely used in the laboratory, such as EMS and gamma radiation (Rosenbluth et al., 1983; 1985); formaldehyde (Johnsen and Baillie 1988) and UV radiation (Stewart et al., 1991). In the analysis of exposure to gamma radiation Rosenbluth et al., (1983) observed that at low doses, the curve was non-linear (Fig. 3). The data show that low doses of radiation are non-damaging, apparently due to repair mechanisms, which may be very good news for those spending long periods of time in space, if the radiation exposure is low level.

The eT1 methodology can also be used to determine what types of mutations were generated. That is, are they putative point mutations or are they predominately small or large rearrangements? The mapping can determine if the new mutations occurred randomly or whether there were some mutational “hotspots”. Any putative point mutations can also be identified as new alleles of known genes or as newly identified genes. The mutational rate and the types of mutations generated and preserved in a proposed accumulating dosimeter system could be quickly analyzed. There is a caveat to this that must be taken into account when analyzing mutations in the accumulating dosimeter. That caveat is the loss of worms that die as a result of mutations in essential genes due to purifying genetic selection. Therefore it is important to carefully screen for semi-viable and morphological mutations because these should not be eliminated as quickly through purifying selection. The majority of point mutations and rearrangements will be identified by analyzing semi-viable and morphological mutants. Many rearrangements will not include essential genes and thus not be subject to rapid purifying selection. Mutations induced by ionizing radiation are mainly rearrangements [Nelson et al. (1989; 1994); Rogalski, Moerman and D.L. Baillie (1982); Rosenbluth, Cuddeford and Baillie (1983)]. Therefore we expect that the majority of mutations captured in the accumulating dosimeter will be rearrangements, which could be efficiently analyzed for the entire genome using the method of comparative genomic hybridization similar to that developed by Ishkanian et al. (2004) for identifying minute genomic rearrangements in the human genome. The method consists of the construction of a DNA microarray containing overlapping BACs, PACs, YACs or in our case cosmid clones that cover the entire regions of interest. These microarrays are sensitive enough to detect single copy change and so can detect heterozygous deficiencies and duplications.

**SUMMARY AND CONCLUSIONS**

The wide variety of research resources, available for biological analysis in C. elegans, provide a promising backdrop for the development of specific systems to study the effects of traveling and living in space. A high priority could be the development of an accumulating dosimeter. NASA, for example, has called for protocols whose objective is to “determine the effects that long-term exposure to the space environment has over multiple generations in space”.

In summary, the resources and knowledge of the *C. elegans* system make it an excellent biological model for both studies of gravitational effects on muscle gene expression and mutational consequences of radiation exposure.

**ACKNOWLEDGEMENTS**

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INTRODUCTION

All biological processes are based on the use of a selected set of proteins to perform the many steps that underlie the final coherent set of events. It is an implicit assumption of modern biology that every activity of every organism can be viewed in this way. Given this recognition, two fundamentally different approaches to identifying these proteins can be applied. The biochemical approach takes the route of actually isolating the proteins themselves and characterizing them, first in vitro and more recently, with the advent of recombinant DNA technology, by reintroducing versions of the gene encoding a given protein back into the cell to test the roles hypothesized for the protein. In contrast, the genetic approach begins at the level of the organism with the proposition that the proteins involved in a particular process can be identified by first identifying their genes. The genes are identified by their ability to disrupt the process in question when mutated. In other words, if a mutation to a particular gene disrupts a particular process, then the protein encoded by that gene must play a role in that process. With this approach, much activity is spent in determining what type of protein is encoded by each mutated gene.

Although the route from mutation to sequenced gene may be long and hard, genetics is an extremely powerful approach because it can be applied to much more complex biological processes than biochemistry. Clearly for fundamental, universal processes that can be studied in cell-free extracts, the biochemical approach is highly effective. But for complex processes, such as developmental events or behavioral responses, in which many components of the whole organism are in play, genetics offers perhaps the only viable route to dissecting out the protein components.

The genetic approach has been used to probe gene/protein function in many organisms. However, the extent to which genetic methods and tools have been developed for Drosophila melanogaster far exceeds that for any other complex multi-cellular organism. As a result, Drosophila stands alone in terms of its ability to provide insight into complex biological processes. Many of the dramatic advances in understanding the genetic basis of development and behavior have come, and will continue to come, from Drosophila.

In this review we will first delineate the historical and practical reasons that have led to Drosophila becoming such a pre-eminent model organism and then discuss some of the unique advantages and molecular methodologies that it now offers for studying gene/protein function. We will then provide examples of biological processes where genetic work in Drosophila has opened up molecular understanding with relevance across all of evolution and has permitted insights into our own molecular makeup. In particular, recent work that uses Drosophila to address gravity-related phenomena will be discussed.

THE DEVELOPMENT OF DROSOPHILA AS A GENETIC SYSTEM

Mendel's genetic studies of the garden pea were the serendipitous consequence of his duties in the monastery garden. In contrast, Drosophila came to be a central organism in genetics as a result of careful consideration by T. H. Morgan, who, in the early 1900's, was looking for a suitable species in which to perform studies of heredity. Drosophila met all his criteria; a small species with a short life cycle that could be easily reared to produce large numbers of progeny. The intense studies of Drosophila that Morgan and his highly talented students (C. B. Bridges, H. J. Muller and A. H. Sturtevant) went on to perform gave rise to many of the concepts that are
fundamental to genetics today. The recognition of genes as linear arrays on the chromosomes grew directly from their research, as did the demonstration of recombination between homologous chromosomes and the chromosomal basis of sex determination (Sturtevant, 1965).

But beyond these theoretical contributions, the genetic tools evolved from these studies have placed Drosophila in its unparalleled position in terms of understanding gene/protein function. In particular, two types of special chromosomes were developed as result of their work. Balancer chromosomes were first created by Muller (18). These chromosomes with multiple re-arrangements cannot recombine with a homologous chromosome partner, and therefore their use allows particular arrangements of mutations on a given chromosome to be maintained through many generations. Balancer chromosomes are available for all three of the major Drosophila chromosomes and their use is an essential component of most genetic manipulations in the organism. Deficiency chromosomes, each lacking a defined chromosome region, were another offshoot of their work. These chromosomes are invaluable in genetic mapping since a recessive mutation will show a phenotype when paired with a deficiency chromosome that is lacking the region containing the mutated gene. At this point in time, sets of deficiency chromosomes are available from the Drosophila stock centers that cover the entire genome and allow straight forward mapping of a mutation to a unique small chromosome region. These sophisticated tools are not available for any other organism.

In addition to these "man-made" genetic tools, the organism itself has conferred further advantages for genetic studies. The rich array of external appendages (multiple bristle types, wings, eyes, antennae and so on) that can be mutated without producing lethality has allowed for the isolation of many marker mutations that are the work-horse tools of most genetic schemes. In addition, several unusual aspects of Drosophila biology have proved extremely valuable. These biological eccentricities were not known, or anticipated, at the time of Morgan's decision to work on Drosophila. Along with insects from several other evolutionary groups, members of the Genus Drosophila do not show meiotic recombination in the male. This provides another useful mechanism in genetic schemes for avoiding rearrangements in arrays of mutations assembled on particular chromosomes. In addition, Drosophila belongs to a group of organisms that makes extensive use of an unusual growth mechanism in certain tissues: DNA replication proceeds without nuclear division or cytokinesis such that polyplody nuclei, often with thousands of copies of the chromosomes, are produced in huge cells. In some tissues the chromosome copies are retained together in perfect register such that giant, so-called polytene, chromosomes with intricate, reproducible patterns of bands are produced. The polytene chromosomes of the Drosophila larval salivary glands are particularly fine and have been intensely studied (Figure 1). The maps created for these chromosomes by Bridges (1935) are still in use and the positions of many of the deficiencies (and duplications) in the chromosomes used today in genetic mapping were identified by the changed banding patterns seen in the polytene chromosomes. A method for hybridizing DNA fragments encoding particular genes to the polytene chromosomes was developed by M.-L. Pardue (Pardue and Gall, 1969; Pardue et al., 1970) and with the advent of recombinant DNA this provided an even more rapid route to positioning a gene within the genome - by hybridizing its DNA to the polytene chromosome set.

![Figure 1. The polytene chromosome set of the Drosophila larval salivary glands.](image)

**THE TRANPOSON BASED TOOL KIT FOR IN-DEPTH MOLECULAR GENETIC ANALYSIS IN DROSOPHILA**

The revolution initiated by the invention of DNA cloning technology has enhanced gene function analysis not just in Drosophila but in all organisms under study. One special consequence in Drosophila, however, was that this methodology permitted the molecular basis of an unusual genetic phenomenon termed hybrid dysgenesis, identified and studied by M. Kidwell, to be explained. Kidwell (Kidwell et al., 1977) discovered that high levels of mutagenesis occurred when certain strains (termed P and M strains) of Drosophila were crossed to one another, but only if the P strain was used to provide the male parent, not the female. O'Hare and Rubin (1983) established that the behavior of a class of transposable DNA elements,
present only in P strains and hence termed P elements, is the underlying basis of hybrid dysgenesis. Movement of P elements is repressed in the P strains but in crosses to M strains, which lack the elements, repression is released. Transposition of the elements to new locations in the genome is thus allowed, and as a result, new mutations are generated.

Since their discovery, the biology of P element transposons has been exploited to generate an impressive variety of molecular tools for in vivo analysis of gene function in Drosophila. In initial ground-breaking work, Spradling and Rubin (1982; Rubin and Spradling, 1982) made modified versions of P elements and demonstrated that these could be used to reintroduce cloned genes back into the organism. The benefits of this so-called transformation technique are multiple. First, it permits genetic rescue of mutations - that is, reversion of a mutant phenotype to the normal wild type state by re-introduction of a "good" version of the gene. But more importantly, when combined with the extensive mutant collections available in Drosophila, it permits a detailed analysis of gene function not possible in other organisms. Thus, for many genes, a completely non-functional mutation (null mutation) of the gene has been isolated previously. By introducing different modified versions of a gene into its null genetic background through P element transformation, the ways in which the modified gene can support the complete set of functions of the normal gene can be addressed. For example, by this approach an investigator could address the function of particular regions of the protein coding sequence in vivo, by determining the ability of appropriately modified gene constructs to restore the mutant phenotype to normalcy.

But P elements have been put to even more sophisticated genetic uses. Three powerful P-based methodologies deserve special mention. One technique addresses the central quest in genetics - that of determining, once a mutant phenotype of interest has been identified, which gene is affected by the mutation. Most of Drosophila genetics to date has relied upon chemically induced mutations. Such mutagens typically produce single base changes in DNA. Given that the genome of Drosophila is ~ 1.65 x 10^8 bases in total, identifying the location of that single base change has some of the quality of finding a needle in a hay stack. Without the sophisticated genetic/molecular methods developed in Drosophila, this task would be impossible, but even with these tools, it can take years of effort to identify the genes affected by such

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**The Gal4 system**

The upper diagram shows a Gal4 P element transposon (pGawB - see Brand and Perrimon, 1993) inserted into the Drosophila genome in a typical position close to an enhancer element that would normally regulate an adjacent gene (shown here to the right of the transposon). The Gal4 coding sequence within the transposon will be expressed in the pattern dictated by the enhancer element. In addition to the Gal4 gene, pGawB contains the white + gene (a marker used to identify successful integration of the transposon into the genome) and bacterial plasmid sequences (amp ori) to allow cloning of the DNA adjacent to the insertion site. In order to identify the Gal4 expression pattern produced by the enhancer, flies carrying the pGawB insertion are crossed to flies carrying a second P-based insertion that has the bacterial β-galactosidase (lacZ) gene linked to a promoter region carrying five Gal4 binding sites (UAS sequences) (see A in figure). The progeny of this cross will carry both constructs in their genomes and thus will express lacZ in the pattern of the Gal4. Given that lacZ is very easily detected histochemically, this allows simple determination of the Gal4 expression pattern. Once the pattern is known, it can be used to express any gene of interest (see B in figure) by a similar procedure.
mutations. P elements, by inserting themselves into the genomic DNA, naturally act as mutagens. In order to be able to determine very rapidly which gene is affected by a particular P element insertion, versions of P elements that permit the rapid and simple cloning of the DNA adjacent to their insertion point have been generated (Cooley et al., 1988). Given that the Drosophila genome is completely sequenced and at an advanced level of annotation, in most cases, sequencing of the P-element adjacent DNA permits immediate and unambiguous positioning of the P insertion within the genome. This ability to make "tagged" mutations readily lead to the affected gene is a powerful advantage that is now being used throughout the Drosophila community.

A second P element based technique allows the identification of the regulatory elements associated with particular genes. As a result of the molecular analysis of the insertion points of thousands of P elements, it has become clear that they preferentially insert into regions of genes that contain sequences regulating the expression of the genes. This is an intriguing finding and presumably reveals some aspect of chromatin function that has yet to be understood. Many of these regulatory sequences, termed enhancers, lie in the 5' flank of the transcribed region. Modified versions of P elements have been generated that will report the expression pattern dictated by an enhancer flanking a P insertion site (O'Kane and Gehring, 1987). These modified versions contain the coding sequence for a bacterial protein β galactosidase (lacZ) but with no associated transcription regulatory elements. As a result there is no expression of the lacZ in Drosophila unless its host P element is inserted so as to allow an adjacent enhancer to confer an expression pattern on lacZ. The pattern of lacZ expression has been examined in hundreds of these so-called "enhancer trap" lines and thus regulatory sequences that give gene expression in many different tissues have been identified.

Building on this approach, a further powerful P element based system (the GAL4-UAS system -see Figure 2) has been developed that allows patterns of enhancer-driven expression to be converted into the expression of any gene of interest (Brand and Perrimon, 1993; Duffy, 2002). This is achieved by using the gene for a yeast transcription factor, Gal4, in place of lacZ, in the "enhancer trap" P construct and then introducing into the genome, in a second P element, the gene of interest under the control of a promoter that will be activated by Gal4 binding. Thus the gene of interest will be expressed wherever Gal4 is expressed. The resulting ability to express particular genes in pre-selected tissues has many valuable applications. For example, although a gene of interest may be expressed in many tissues, the phenotype of a particular mutation to the gene may result from defects in only one of these tissues. This hypothesis can be tested by expressing the wild type, non-mutant, version of the gene in particular tissues of the mutant animal and determining in which tissues the mutant phenotype is rescued.

An example from the Beckingham lab will illustrate this point (Wang et al., 2002). We identified a mutation to the universally expressed calcium signaling protein calmodulin that affected muscle behavior in the larval/pupal stages. We wished to determine whether these defects reflected problems in the musculature itself, in the nervous system innervating the muscles, or perhaps elsewhere in the animal. By expressing wild type calmodulin in either the muscles or the nervous system using this technique, we were able to show convincingly that the defects originated entirely within the muscles themselves.

Although the Gal4-UAS system allows directed gene expression in particular tissues it does not permit temporal control over when, in the development of that tissue, the gene will be expressed. However, systems are now evolving to address this need to control gene activation in terms of not just tissue but also time (reviewed in McGuire et al., 2004). Modification of the Gal4-UAS system so that Gal4 is activated only in response to feeding the hormone analog RU486 is one approach; use of a temperature sensitive version of the Gal4 repressor protein Gal80, which represses Gal4 at permissive temperatures but allows Gal4 expression upon shift to higher temperatures, is another.

Many proteins have vital roles in different tissues at different stages of development. A null mutation in such a gene will produce death at the first point in the life cycle of the organism at which the protein is essential. Thus, a classic null mutation cannot be used to address the roles of the protein beyond this point of death. For any recessive mutation, this problem can be overcome by so-called mosaic analysis. In this approach, the whole organism is heterozygous for the mutation and therefore phenotypically wild type. Then, some method is used to render the mutation homozygous in particular cells of the body so that the effects of the mutation can be addressed in those cells. Although classical Drosophila genetics provided some techniques for generating mosaic animals they were cumbersome and not generally applicable to any gene. Another advance that has grown out of the ability to readily transfer gene constructs into the genome with P element vectors is a generally applicable system for generating mosaic animals (Xu and Rubin, 1993). This system, termed the FLP-FRT system (Figure 3) uses a recombinase enzyme (FLP) from yeast that will induce sequence-specific recombination at a site termed FRT. FRT sequences are appropriately positioned on two homologous chromosomes one of which carries a mutation of interest. FLP activity is then used to induce an illegal recombination event that will result in a cell in which both homologous chromosomes carry the mutation. By using versions of the FLP enzyme that are under the control of either tissue-specific enhancers or a promoter induced by heat-shock, cell lineages homozygous for the mutation can be induced in either particular tissues, or throughout the animal at particular times.
It is important to recognize that, effective as these transposon-based methods are for gene analysis, any gene reintroduced into the organism in this way is not inserted so as to replace the version of the gene already present in the genome. Hence as alluded to above, to examine the function of the transgenic construct without interference from the endogenous chromosomal version of the gene requires the pre-existence of a null mutation of the gene in question. In contrast, in the mouse and in yeast, the methods for re-introducing genes into the genome target their integration by homologous recombination so that they are substituted for the endogenous gene at the correct chromosomal locus (Thomas and Capecchi, 1987; Petes et al., 1991). Recently methods for this type of targeting of genes have also been developed in Drosophila and will be valuable for those genes for which a null mutation is not available (Rong and Golic, 2000).

Figure 3. The FLP-FRT system for generation of mosaic tissues. The upper panel shows a pair of homologous chromosomes in a single cell of an animal that has been generated to allow mosaic analysis of a particular mutation (represented here by a star symbol on one of the chromosomes). The cell is heterozygous for the mutation and therefore phenotypically normal. The chromosomes are shown in G2 of the cell cycle, when sister chromatids for both chromosomes are present. FRT sequences are present at identical positions on the two chromosomes. Flp recombinase, expressed from another chromosome in the cell, causes an illegal recombination event between the FRT sequences on chromatids of the homologous pair. As a result, the two chromatids containing the mutation are now attached to different centromeres (see middle panel). After mitosis, when chromatids derived from each chromosome pair have segregated to the daughter cells (lower panel), one cell will be homozygous for the mutation and the other wild type. Reproduced with permission from Nature Reviews in Genetics 3, 176-188, 2002 (www.nature.com/reviews) copyright 2002, Macmillan Magazines Ltd.
THE INITIAL UNLEASHING OF THE POWER OF DROSOPHILA GENETIC SCREENS

The mutations initially studied by Morgan's group were naturally occurring ones. Indeed the discovery of the first white-eyed mutant fly by Morgan was a defining moment in Drosophila genetics. The critical advance of directly generating mutations was pioneered by Muller, when he initiated the use of ionizing radiation as a mutagen (Muller, 1927). Subsequently, more easily usable chemical mutagens - in particular DNA alkylating agents such as ethylmethane sulfonate - have been identified and used. The ability to generate mutants in a scattershot manner throughout the genome, led to the concept of the genetic screen; that is, a two-stage experimental protocol whereby mutants are generated and then those that affect a particular process of interest are isolated and studied. Although genetic screening of this type was established in microbial systems relatively early, it was not pursued in complex multi-cellular animals initially for two compelling reasons: first, in most species existing genetic analysis was too primitive to permit in-depth analysis of mutants, and second, the resources and time necessary for multi-generational analysis of hundreds or thousands of mutant lines made such screening completely impractical in most species.

In essence, Drosophila was the only complex animal species for which these two criteria could be met and in the 1970's directed screening efforts to identify mutants in particular processes were initiated. Three of these early screening efforts deserve particular mention for the far-reaching impact of their findings.

a) Mutants in visual processes

Pak (1979; 1995) used an electrophysiological assay to identify mutants which altered the electroretinogram of the fly and thus, by implication, to find genes with roles in Drosophila phototransduction and other vision-related events. Several of these mutations have proved to affect genes with general roles in intracellular signaling processes and thus they provide insights with broad significance into intracellular regulation. The trp mutations in particular have played an enormously important role in advancing understanding in neural signaling in mammalian systems. Originally named for the electrophysiological effect of the mutants (a transient receptor potential,) the trp gene proved to encode a novel kind of ion channel. Trp is now recognized as the founding member of a vast and varied family of related channels used throughout the nervous system across evolution and with roles in many sensory processes such as taste, touch, pain and temperature perception (Clapham, 2003; Huang, 2004).

b) Mutants in behavioral processes

Benzer, after his successful exploitation of bacteriophage genetics to address the nature of the genetic code, turned his attention to Drosophila genetics as a route to exploring the biological phenomenon that interested him most - that of behavior. Although Benzer received considerable criticism for attempting to unravel complex phenomena one gene at a time, the genetic screening initiated in his lab. identified mutants that have played ground-breaking roles in understanding such universal phenomena as circadian rhythms, courtship and mating activity, and learning and memory. Mutants of the gene period were isolated by Konopka in Benzer's lab (Konopka and Benzer, 1971) and subsequent cloning of the period gene by the labs of Hall (a Benzer post-doc) and Rosbash (Reddy et al., 1984) has led to recognition of its pivotal role in regulating diurnal activity across all evolutionary orders. The first learning mutant, dune, isolated in the Benzer lab by Quinn (Dudai et al., 1976) proved to encode an enzyme involved in cyclic nucleotide metabolism. Along with rutabaga, another Quinn mutation that affects adenylyl cyclase (Livingstone et al., 1984) these learning mutants have provided part of the now large body of evidence demonstrating the role of cyclic AMP in learning and memory in all biological systems.

c) Mutants in embryonic patterning

Nusslein-Volhard and Wieschaus as young new independent investigators at the EMBL labs in Heidelberg, undertook a large scale screen to identify genes that affect formation of the Drosophila embryonic body plan. A large number of mutations were produced, some with remarkable effects on the final form of the animal (Nusslein-Volhard and Wieschaus, 1980; Nusslein-Volhard et al., 1984; Jurgens et al., 1984). For example, the even-skipped and odd-skipped mutations generate larvae in which every other body segment is missing. The impact of this screen cannot be over emphasized. Determining what kinds of genes were behind such unusual phenotypes was of great interest. Further, this work appeared just at the moment when cloning the gene affected by a given mutation was becoming feasible. The cloning and further study of genes identified by this screen is one of the seminal contributions of Drosophila to modern biology. Many of these genes are conserved in higher species and over the past 20 years we have seen the mammalian homologs identified and their fundamental roles in vertebrate cell function established. Indeed whole conserved pathways used across evolution to execute similar functions have been identified. The Hedgehog signaling pathway (Lum and Beachy, 2004) is a striking example of the impact of this screen: besides roles in embryonic development in all species, this pathway plays a critical part in regulation of tissue growth in adult vertebrates and is implicated in the transition to tumor growth in several cancers (Scott, 2003). Three key members of this pathway (Hedgehog, Patched and Smoothened) were identified by the Nusslein-Volhard/Wieschaus screen.
FURTHER DEVELOPMENT OF GENETIC SCREENING IN DROSOPHILA

Numerous Drosophila screens have been carried out since this period and genes with roles in processes as diverse as olfaction (Woodward et al., 1989), hearing (Eberl et al., 1997), sensitivity to ethanol (Guarnieri and Heberlein, 2003) and development of various organ systems have been identified. As proved true for the embryonic patterning screens, the extensive screening in the Rubin lab. focused on eye development (e.g. Therrien et al., 2000; Greenwald and Rubin, 1992) has uncovered genes and pathways that are universally conserved. The so-called Pax-Six-Eya-Dach signaling network is one such conserved pathway to emerge from this work (Ozaki et al., 2004). In addition, a "second order" class of screens has been developed that allows further proteins in a given pathway to be identified once one pathway member has been isolated through mutation. These so-called genetic interaction (or enhancer/suppressor) screens involve searching for second site mutations that suppress or enhance the phenotype of a given mutation in a starting gene of interest. The underlying assumption here is that only mutations that affect protein products that interact with one another, or at least have roles in the same process, could modify one another's phenotype in this way.

As discussed above, a major advance for genetic screening has involved the development of modified P transposons that can be used as insertional mutagens that then allowed rapid cloning of the adjacent DNA (Cooley et al., 1988). This approach is now widely in use, and our own studies of gravitaxis took advantage of this approach (see below). But in addition, genetic screening methods have been developed that take advantage of some of the more sophisticated P element based tools discussed above. One such technique allows the identification of genes with roles in particular processes by examining the effects of their over-expression or mis-expression in particular tissues (Rorth, 1996). The technique uses Gal4 expressed in a specific pattern to activate randomly inserted UAS-type promoter elements and thus to initiate expression of any endogenous gene adjacent to the insertion in the pattern determined by the Gal4 expression characteristics. For a fraction of the genes in Drosophila, even null mutations do not produce a phenotype, perhaps as a result of functional redundancy. This new method is valuable in that it permits functional analysis of such genes.

By combining aspects of the Gal4-UAS system, the FLP-FRT system and mutagenesis extremely sophisticated screens are now being developed and executed in Drosophila. One example will suffice. In order to identify genes that might contribute to the transition from a non-invasive to a metastatic tumor, Pagliarini and Xu (2003) devised a scheme in which FLP-FRT was used to generate mosaic cell lines in developing eye tissue. As a result of a single FLP-induced recombination event they could generate a clone of cells that simultaneously expressed a fluorescent marker protein (GFP -for cell identification) and a gene that causes the cells to transition to non-invasive tumorous growth. In addition, this recombination event rendered the cells homozygous for one of a collection of random mutations being tested for their effects in a non-invasive tumor cell. Those mutations that caused a transition to metastatic growth could then be easily identified since fluorescent cells were no longer confined to the eye, but rather were spread throughout the organism. No other organism allows this kind of sophisticated, directed approach to identifying genes with roles in metastasis. The implications of this particular screen in terms of understanding human cancer are obvious.

DROSOPHILA IN THE GENOMIC ERA

With the sequencing of the genomes of several model organisms, a new so-called genomics era has arrived. Interestingly, the genomes of the vertebrate model organisms have proved to be "quasi polyploid" in that three or four highly related copies of many genes are present. Pleasingly this is not true of Drosophila; most Drosophila genes are unique and the complication of genetic redundancy is thus greatly diminished.

The new central approach of the genomics era is whole genome gene expression analysis using probes for all the genes in the organism displayed by micro-array technology. This approach compares expression patterns for two different experimental situations, such as a particular tissue before and after some experimental treatment, or at two different stages of development. Again, Drosophila brings particular advantages to this type of approach. Most notably, the ability to pursue array-based expression data by subsequent study of mutants and altered gene constructs adds a depth of analysis not possible in most organisms.

A wealth of Drosophila community resources has developed over the years including bioinformatic tools to handle the large data sets now accumulating from genomic studies. Several stock centers around the world maintain both classical and transgenic mutants. A variety of international consortia make datasets and molecular reagents available to the community at large. The FlyBase database (FlyBase@flybase.net) maintains a list of Drosophila researchers, references, genome and mutant information, and many user-contributed articles and tutorials. Of particular note is the Berkeley Drosophila Genome Project (BDGP: http://www.fruitfly.org/) which maintains developing genomic resources such as banks of cDNAs and stocks of newly generated gene disruption mutants. There is now also an initiative to maintain extensive raw datasets in a central, searchable database; FlyMine (http://www.flymine.org). FlyBase is a central repository for links to developing bioinformatics tools.

With the genome completely sequenced, the Drosophila community has begun development of tools for the
ultimate phase of genomic research - a full understanding of the function of each individual gene. Two useful tools are already available for this work. First, a large fraction of the proteins of the Drosophila proteome have been tagged with GFP by genomic insertion of an appropriate reporter construct (Kolm et al., 2004; http://flytrap.med.yale.edu). The GFP tag permits the expression pattern and subcellular location of the protein from a given gene to be easily investigated. Second, as a complement to standard mutations, libraries of DNA constructs that can be used to create mutant phenotypes by RNAi-mediated mRNA destruction have been generated (http://www.hgmp.mrc.ac.uk/geneservice). Currently, constructs for ~90% of the Drosophila genome are available. In addition, a large collection of new gene/small genomic deletions has been made by the company Exelixis, using more recently generated transposons such as piggybac, razorbac and warthog. These will aid studies of uncharacterized genes (http://drosophila.med.harvard.edu/index.php?option=content&task=view&id=5&Itemid=27).

DROSOPHILA AND HUMAN DISEASES

The promise of using the capacity for deep genetic analysis in Drosophila to advance molecular understanding of specific human diseases has recently begun to be realized. The major approach to date in modeling a human disease in Drosophila has been to express the protein product associated with a particular disease state in the fly and then to establish whether the changes associated with the disease are mimicked in this organism. For three major classes of neurodegenerative diseases, Alzheimer's, Parkinson's disease and the polyglutamine class of diseases (exemplified by Huntington's disease) this approach has been successful and the model disease state generated in the fly is being used for further analysis (Bonini and Fortini, 2003; Iijima et al., 2004). Candidate genes that might modify the severity of the disease-related phenotype (see suppressor and enhancer screens above) are being investigated. For both Parkinson's and the polyglutamine diseases, this approach has already established that chaperone proteins such as hsp70, which regulate protein folding, can ameliorate the disease phenotype (Bonini and Fortini, 2003). Current indications also suggest that general insights into the biological function of the disease protein (or its interaction partners) will come from this strategy of expressing in Drosophila. For example, over-expression in Drosophila of APP, the protein associated with Alzheimer's disease, produces a blistered wing phenotype (Fossgreen et al., 1998). This defect is associated with failed cell-cell adhesion in the wing epithelia and suggests that the processing enzyme that modifies APP (which is already present in Drosophila) might also play a role in processing critical cell adhesion molecules.

However, the great power of this Drosophila disease modeling approach lies in large scale genetic screening for mutations that enhance or suppress the disease phenotype. By this route, completely novel genes involved in disease progression will be identified. In time, this will provide new potential targets for development of therapeutic drugs.

DROSOPHILA'S CONTRIBUTION TO RESEARCH INTO GRAVITY-BASED RESPONSES

a) Earth-based studies

Interestingly, geotaxis (now more correctly termed gravitaxis) was one of the first behaviors investigated in Drosophila at the beginning of the last century (Carpenter, 1905). However, further work was not undertaken again until the late 1950's/early 1960's when Hirsch began extensive studies of the phenomenon. At that time, the issue of inheritance of behavioral traits was of intense interest because of its far-reaching social implications in terms of human behavior. Hirsch's primary goal was thus to identify defined behavioral responses and then to address their heritability. He designed vertical multi-choice mazes to examine gravitaxis behavior in walking, as opposed to flying, flies (Hirsch, 1959). By selecting flies from a wild type population that emerged either at the high or the low maze exits he was able to demonstrate stable heritance of the maze behavioral traits over many generations with maximal behavioral separation between high and low lines occurring after 48 generations (Hirsch and Erlenmeyer-Kimling, 1961). Today, after more than 700 generations, these lines still breed true in terms of their maze behavior.

Erlenmeyer-Kimling and Hirsch (1961; Hirsch and Erlenmeyer-Kimling, 1962) established that the determinants for these traits were dispersed throughout the chromosome set, but prevailing methodology precluded actually identifying any of the loci involved. More recently however, Toma et al. (2002) have used the genomics approach described above to identify these loci. They compared patterns of gene expression in the high and low strains by DNA microarray technology and identified several genes for which expression levels differed significantly between the two strains. For three genes, they were able to use previously identified mutants of the loci to demonstrate that the genes had roles in gravitaxis. Thus mutants at these genes showed altered behavior in the maze.

We have taken an alternative approach to identify genes with roles in gravitaxis (Armstrong, Texada, Munjaal, Baker and Beckingham, manuscript submitted). We have used the gravitaxis maze as the central assay of a genetic screen to identify mutants with altered behavior in this gravitaxic response. By examining mutations caused by a Gal4 expressing "enhancer trap" type P transposon (see above), we have been able to quickly move from the mutants we have identified to the affected genes. The actual sense organs that detect gravitational force are not known in Drosophila. However, recognizing that Gal4 will be expressed in those tissues that are the likely...
origins of the mutant phenotype, we have been able to identify candidate peripheral sense organs that might mediate graviperception. In particular, a set of sensory structures on the Drosophila head show strong expression of Gal4 in many of our mutant lines (Figure 4).

Bhattacharya and collaborators are addressing another behavioral response to gravity in Drosophila (Sanchez, Stowers, Shenasa, Fahlen and Bhattacharya, manuscript in preparation). They have established that upon subjecting flies to hypergravitational force (1.8g-8g) a highly stereotypic locomotor response is initiated, the features of which suggest that increased g force stimulates an adaptive program of gene expression. Whole genome micro-array studies and candidate gene approaches are being used to identify genes important for this locomotor response. The mushroom body in the Drosophila brain, which is important for olfactory learning and memory and for locomotion, has been shown to be involved in regulating this behavioral response to hypergravity. Our de novo forward genetic screen studies have identified several novel genes with roles in gravitaxis. But a striking aspect of all three of these approaches is that many of the gravity-related genes identified have clear homologs in human beings. As shown in Table 1, several of these genes have predicted roles in neural signaling or morphogenesis. Interestingly, gravity-related genes with roles in regulating circadian activity have been identified by both Toma et al. and Bhattacharya and colleagues (Table 1). The Bhattacharya lab has also identified a role for conserved genes of the cAMP pathway (dunce, rutabaga) known to be involved in learning and memory in addition to genes affecting longevity and stress (methuselah and I’m not dead yet). The roles of these various genes are thus much broader than the original studies of them might have indicated. Further, it is hard to imagine any route by which the roles of these genes in gravity-based responses could have been identified in any mammalian system, given the limited methodology currently available in these organisms. But with the characterization of these genes in Drosophila, the possibility for mammalian studies is now open. Hopefully future studies will address the roles of these proteins in gravitaxic responses in mammals.

b) Micro-gravity experiments

The features of Drosophila that make it ideal for genetic studies of a multi-cellular organism also make it ideal for experiments in the micro-gravity environment. On the International Space Station (ISS), where mass and volume are critical issues, the small size and fecundity of Drosophila make it possible to use large numbers of individuals for experiments - at least two orders of magnitude more than could be used with mammals. Further, the ease with which Drosophila can be reared in
### TABLE 1

**Genes with Roles in Gravitaxic Behavior in *Drosophila* that Have Conserved Homologs in Humans**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Function</th>
</tr>
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<tbody>
<tr>
<td>aPendulin (Pen)</td>
<td>Nuclear importin</td>
</tr>
<tr>
<td>aCryptochrome (cry)</td>
<td>Photopigment protein with role in circadian rhythms</td>
</tr>
<tr>
<td>aPigment-dispersing Factor (Pdf)</td>
<td>Neuropeptide that mediates circadian locomotor activity</td>
</tr>
<tr>
<td>bBroad (br)</td>
<td>Zinc-finger class transcription factor</td>
</tr>
<tr>
<td>bOff-track (otk)</td>
<td>Receptor tyrosine kinase with role in neural pathfinding (Human homologs are Trk neurotrophin receptors)</td>
</tr>
<tr>
<td>bDiscs large (dlg1)</td>
<td>MAGUK class protein with guanylate kinase, PDZ, SH3 and P-loop domains with a role in synapse structure (Human homolog is chapsyn-110)</td>
</tr>
<tr>
<td>bEscargot (esg)</td>
<td>Zinc-finger class transcription factor with role in peripheral nervous system development (Human homolog is SLUG)</td>
</tr>
<tr>
<td>bConnector of kinase to AP-1 (Cka)</td>
<td>WD-40 domain protein, part of JNK signaling cascade (Human homologs are striatin, zinedin and cell cycle autoantigen SG2NA)</td>
</tr>
<tr>
<td>cPeriod (per)</td>
<td>Transcription factor that regulates circadian rhythm (Human homolog is PER3)</td>
</tr>
<tr>
<td>cTimeless (tim)</td>
<td>Transcription factor that regulates circadian rhythm (Human homolog is hTimeless)</td>
</tr>
<tr>
<td>cDunce (dnc)</td>
<td>Phosphodiesterase that regulates cAMP levels (Human homolog is cAMP-specific 3', 5'-cyclic phosphodiesterase 4D)</td>
</tr>
<tr>
<td>cRutabaga (rut)</td>
<td>Adenylate cyclase responsible for cAMP synthesis (Human homolog is brain adenylate cyclase 1)</td>
</tr>
<tr>
<td>cI’m not dead yet (indy)</td>
<td>Sodium dicarboxylate cotransporter implicated in longevity (Human homolog is NADC3)</td>
</tr>
<tr>
<td>cG-salpha60A</td>
<td>Component of transmembrane receptor signal transduction cascade involved in associative learning in mushroom body (Human homolog Guanine nucleotide-binding protein G-s-alpha 3)</td>
</tr>
</tbody>
</table>

\[ a = \text{Toma et al. (2002); } b = \text{Armstrong et al., submitted (see text); } c = \text{Sanchez et al., in preparation (see text).} \]
small, closed, containers allows design of experiments that need very little attention from the astronaut crew. Drosophila experiments in microgravity that take advantage of the powerful genetic methods available in this organism are planned in the near future. A collaborative experiment by Beckingham, Bhattacharya and Armstrong will investigate the behavior and gene expression changes evoked by microgravity in both wild type and mutant flies. An experiment by Thompson and Woodruff will use the genetic manipulations possible in Drosophila to gain information about the mutagenic effects of radiation levels on the ISS. Details of these experiments may be obtained from the NASA Task Book site (http://peer1.nasaprs.com/peer_review/index.cfm).

**CONCLUSION**

Based on appearance we might not readily conclude that *Drosophila melanogaster* and *Homo sapiens* have a great deal in common. But with the sequencing of both genomes it is now clear that more than half the genes in Drosophila have homologs in human beings. What's more, not just genes, but whole molecular pathways, originally delineated in Drosophila as a result of its sophisticated genetics, are now known to be conserved intact in mammalian systems. The value of using Drosophila to investigate fundamental biological processes with the distinct goal of uncovering insights directly relevant to human beings is thus evident. The major contributions of Drosophila to date have been in terms of molecular understanding of development and embryogenesis. But current research activities indicate that use of Drosophila to study human disease and behavioral phenomena is expanding and we may expect further substantial contributions in these areas. Despite its small size, Drosophila has a complex repertoire of behavioral responses including activities that relate readily to the human condition such as courtship and male-male aggression. The continually expanding array of resources in Drosophila will ensure that it will be a major source of insights into these more complex aspects of organismal biology. In terms of gravitational biology, Drosophila is already providing results that have implications for gravity-based responses in people. The use of Drosophila as a model organism to probe gene expression and behavior in micro-gravity fulfills two valuable goals in parallel. On one hand, data that can be interpreted in depth thanks to the wealth of genetic data available for the organism will be generated. Simultaneously, information pertinent to human responses in the microgravity environment will be produced.

**ACKNOWLEDGMENTS**

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ABSTRACT

Animal models have been used to study the effects of space flight on physiological systems. The animal models have been used because of the limited availability of human subjects for studies to be carried out in space as well as because of the need to carry out experiments requiring samples and experimental conditions that cannot be performed using humans. Experiments have been carried out in space using a variety of species, and included developmental biology studies. These species included rats, mice, non-human primates, fish, invertebrates, amphibians and insects. The species were chosen because they best fit the experimental conditions required for the experiments. Experiments with animals have also been carried out utilizing ground-based models that simulate some of the effects of exposure to space flight conditions. Most of the animal studies have generated results that parallel the effects of space flight on human physiological systems. Systems studied have included the neurovestibular system, the musculoskeletal system, the immune system, the neurological system, the hematological system, and the cardiovascular system. Hindlimb unloading, a ground-based model of some of the effects of space flight on the immune system, has been used to study the effects of space flight conditions on physiological parameters. For the immune system, exposure to hindlimb unloading has been shown to result in alterations of the immune system similar to those observed after space flight. This has permitted the development of experiments that demonstrated compromised resistance to infection in rodents maintained in the hindlimb unloading model as well as the beginning of studies to develop countermeasures to ameliorate or prevent such occurrences. Although there are limitations to the use of animal models for the effects of space flight on physiological systems, the animal models should prove very valuable in designing countermeasures for exploration class missions of the future.

INTRODUCTION AND OVERVIEW

Animal models have been used extensively to study the effects of spaceflight on physiological conditions. The rat has been the animal used most extensively, but some studies have also been carried out utilizing mice and rhesus monkeys (Sonnenfeld, 2005). Invertebrates have also been extensively used, as have fish and amphibians. Studies have been carried out on just about every physiological parameter that could be studied, including the musculoskeletal system, the cardiovascular system, neurovestibular system, the immune system, and overall developmental biology. Animal models have been most useful when carrying out experiments that could not be carried out on human subjects (Sonnenfeld, 2005). These experiments involve exposure to conditions that would be dangerous for humans. Also, developmental biology studies have been carried out in animals flown in space that could not have been accomplished using humans. Additionally, since the number of crewmembers is small and their time is extremely limited, animal models have been utilized to provided preliminary data on which to base future human studies. Therefore, animal models have proven to be extremely useful in providing information on detrimental effects of space flight conditions on physiological function as well as for the development of countermeasures to ameliorate or prevent such detrimental effects. No doubt, animal models will play a crucial role in the realization of the initiative that has been announced to develop exploration class missions for the exploration of space.

The nature of the changes induced by space flight in animal physiological system has been included in a definitive review volume, and they will not be replicated here (Sonnenfeld, 2005). Instead, recent work using animal to study the effects of space flight conditions on the immune system will be highlighted in this review. Additional background information on the overall use of animals to study the effects of space flight on physiology can be found in the review volume (Sonnenfeld, 2005).

Additionally, since the opportunities to carry out spaceflight animal studies are rare and expensive, ground-based systems have been developed that model some of the effects of space flight on animal physiology (Giron et al., 1967; Il'in and Novikov, 1980; Morey, 1979; Musacchia et al., 1980; Space Studies Board, 1998). The need for these models has been heightened as we have embarked upon the era of planning for exploration class space missions (Sonnenfeld, 2005). The limited ability to carry out space flight animal experiments during the construction phase of the international Space Station compounded by the difficulties in carrying out flight experiments when the activities of the Space Shuttle fleet are limited or suspended, has enhanced our need for the ground-based models. Without these, it seems unlikely that sufficient information will be available to allow for planning of human exploration class space flight missions.

There are several ground based animal models available, including low pressure chambers and centrifugation hypergravity models (Giron et al., 1967; Oyama and Platt, 1965) for studying the continuum of the force of gravity, but the most commonly utilized ground based model is hindlimb unloading of rodents (Il'in and Novikov, 1980;
Morey, 1979; Musacchia et al., 1980; Space Studies Board, 1998). This model is also known as hypokinetic, hypodynamic antiorthostatic suspension or tail suspension. In the model, rats or mice are suspended so that their hindlimbs are not weight bearing and with a head down tilt so that there is a fluid shift to the head. The forelimbs remain load-bearing. This results in conditions similar to some of those observed during space flight, and effects on physiological systems are most often similar to those observed during space flight (Il’in and Novikov, 1980; Morey, 1979; Musacchia et al., 1980; Space Studies Board, 1998). Chapes et al., (1993) reviewed the effects of hindlimb unloading on the immune system and showed that hindlimb unloading was an acceptable, but not perfect, model for the effects of space flight on the immune system. Most of the effects of space flight on the immune system were replicated using the hindlimb unloading model, with the exception of leukocyte subset distribution in rats.

**SPACE FLIGHT CONDITIONS, GROUND-BASED MODELS AND THE IMMUNE SYSTEM**

Exposure to space flight has been shown to modify many immunological parameters (Sonnenfeld and Shearer, 2002; Sonnenfeld, Butel and Shearer, 2003). These parameters have also been recently extensively reviewed, and have been demonstrated using rats, mice and rhesus monkeys. In brief, the immunological factors shown to be affected by space flight in animals include: leukocyte subset distribution, leukocyte blastogenesis, the response of bone marrow cells to colony stimulating factors, cytokine production, natural killer cell activity, and neutrophil activity. The development of these immunological responses in offspring of pregnant rats flown in the Space Shuttle were not affected (Sonnenfeld and Shearer, 2002; Sonnenfeld, Butel and Shearer, 2003). Exposure of rodents to the hindlimb unloading model has also resulted in changes in the immune system similar to those observed during space flight (Sonnenfeld and Shearer, 2002; Sonnenfeld, Butel and Shearer, 2003). Again, these have been recently reviewed (Sonnenfeld and Shearer, 2002; Sonnenfeld, Butel and Shearer, 2003), and details will not be given here. Functional immune responses have been shown to be altered after rodents have been placed in the hindlimb unloading model, including the same functional immune response that have been shown to be affected by space flight such as cytokine production, leukocyte blastogenesis, response of bone marrow cells to colony stimulating factors, neutrophil activity, and resistance to infection. Leukocyte subset distribution was not affected in the same way by hindlimb unloading as it was by space flight, suggesting that the model is not useful for lymphocyte cell distribution studies (Sonnenfeld and Shearer, 2002; Sonnenfeld, Butel and Shearer, 2003).

**SPACE FLIGHT CONDITIONS, GROUND-BASED MODELS AND RESISTANCE TO INFECTION**

Although it is clear that exposure of animals to space flight conditions results in alterations of immunological parameters, the question can still be asked, does this affect the actual health and well-being of the host? This question could only be answered by carrying out studies using infectious disease or tumor models. For obvious reasons, such studies could not be carried out using humans. Animal models have proved invaluable for carrying out infectious disease studies and have increased our understanding of the biological significance of the effects of space flight conditions on the immune system.

Since space flight experimental opportunities for animal studies have been so limited, infection studies have not been carried out in space. Therefore, the hindlimb unloading model has proven to be invaluable for carrying out experiments on the effects of space flight conditions on resistance to infection (Table 1).

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Effect of Hindlimb Unloading</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Encephalomyocarditis virus D</td>
<td>Compromised resistance</td>
<td>Gould et al., 1987</td>
</tr>
<tr>
<td>Propionibacterium acnes</td>
<td>Decreased innate immunity</td>
<td>Fleming et al., 1990</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Compromised resistance; Could be protected if pretreated with active hexose correlated compound (AHCC)</td>
<td>Belay et al., 2002; Aviles et al., 2003b, 2004</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Compromised resistance</td>
<td>Aviles et al., 2004</td>
</tr>
</tbody>
</table>

Table 1. Effect of hindlimb unloading of mice on resistance to infection.
In an early study, female Swiss/Webster mice were inoculated with the D variant of encephalomyocarditis virus (EMC-D virus). Females of the Swiss/Webster strain normally are totally resistant to infection with EMC-D virus (Gould et al., 1987) with the resistance mediated, at least in part, by interferon (Gould et al., 1987). Hindlimb-unloaded mice became susceptible to infection, whereas mice that were restrained without head down tilt and carrying full load on their hind limbs were still resistant to infection (Gould et al., 1987). The ability of mice to produce interferon-α/β correlated with the alteration in resistance to EMC-D virus.

An additional experiment using mice subjected to hindlimb unloading was carried out by Fleming et al., (1990). The results of the study showed that hindlimb-unloaded mice had impaired ability to produce superoxide, decreased ability to kill phagocytosed bacteria (Propionibacterium acnes), and altered corticosterone levels. This suggested a compromise of innate immune defenses, particularly neutrophils, by hindlimb unloading.

Mice that were placed in the hindlimb unloading model showed enhanced resistance to primary infection with Listeria monocytogenes, an intracellular pathogen (Miller and Sonnenfeld, 1993, 1994). This was probably due to enhanced macrophage function and production of cytokines produced by macrophages that resulted in enhanced clearance of the pathogen (Miller and Sonnenfeld, 1993, 1994). At the same time that the resistance to the primary L. monocytogenes infection was strengthened by hindlimb unloading, the ability to generate long-term T cell mediated immunologic memory to the L. monocytogenes was inhibited (Miller and Sonnenfeld, 1993, 1994). Therefore, although initial resistance to an obligate intracellular organism was enhanced by hindlimb unloading, the ability to develop long-term T cell-mediated resistance to challenge with the organism was inhibited (Miller and Sonnenfeld, 1993, 1994). Therefore, it is possible that in space flight conditions, although initial resistance to some organisms might be enhanced, over the long-term, that resistance would be likely to be compromised.

Although the previous studies generated evidence that resistance to infection could be compromised by hindlimb unloading, the organisms studied were not likely to be pathogens during a space flight mission. EMC-D virus and L. monocytogenes are excellent organisms for studying resistance to infections in models, but they are not pathogens for humans that would be found in space flight conditions. Therefore, additional studies were carried out using potential pathogens.

Klebsiella pneumoniae is a gram negative enteric bacteria that is a major problem in immunocompromised surgical patients (Polk et al., 1992). It is part of the normal microbiota in the intestine, and when the host is immunocompromised, can spread from the gut to cause sepsis and death (Polk et al., 1992). Therefore, it is a possible pathogen form space travelers who might develop a compromised immune system. Pseudomonas aeruginosa is an opportunistic gram negative pathogen that has already caused infectious difficulties during a space flight mission (Hawkins and Ziegelschmid, 1975; Taylor, 1974). One of the astronauts in the Apollo 13 mission developed a urinary tract infection with P. aeruginosa during the mission (Hawkins and Ziegelschmid, 1975; Taylor, 1974). For these reasons, these two organisms were chosen for additional studies on the effects of hindlimb unloading on resistance to infection.

A study was undertaken to determine the effects of hindlimb unloading on resistance of mice to infection with K. pneumoniae (Belay et al., 2002). Mice were infected with K. pneumoniae. Mortality, as expected, was 50% for control mice that were normally caged and housed and received 1 LD₅₀ (lethal dose for 50% of the animals) of K. pneumoniae. Mortality was 43% for control mice that were restrained and received 1 LD₅₀ of K. pneumoniae., which was not significantly different from control mice. Mortality was 86% for experimental mice that were hindlimb-unloaded and received 1 LD₅₀ of K. pneumoniae, which was a statistically significant different from the other groups of mice (Belay et al., 2002). Mean time to death was also significantly reduced in the hindlimb-unloaded mice. Additionally, the hindlimb-unloaded mice could not readily clear the bacteria from the blood and other organs, suggesting that the innate immune system was compromised in this infection (Belay et al., 2002). Therefore, it appears that exposure of mice to hindlimb unloading decreased resistance K. pneumoniae.

Additional studies were also completed to determine the effects of hindlimb unloading on resistance of mice to P. aeruginosa (Aviles et al., 2003a). Hindlimb-unloaded mice had significantly enhanced mortality when infected with P. aeruginosa in a fashion similar to that obtained with K. pneumoniae (Aviles et al., 2003a). The hindlimb-unloaded mice that were infected had a prolonged elevation of corticosterone, indicating that a stress response might play some role in decreasing resistance to infection (Aviles et al., 2003a).

If resistance to infection is compromised in mice by hindlimb unloading, could a countermeasure be developed that could minimize the deleterious effects of hindlimb unloading on resistance to infection? This is a question of some importance, because it is possible that such a countermeasure could be further developed to protect humans from infections during space flight missions.

Studies were begun with active hexose correlated compound (AHCC). AHCC is an extract prepared from co-cultured mycelia of several species of Basidiomycete mushrooms (Kidd, 2000). It contains polysaccharides, amino acids, and minerals and is orally bioavailable. It has been shown to have a beneficial effect on the immune

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system of normal humans (Matsui et al., 2002) and rodents, including enhancement of natural killer cell activity (Burikhanov et al., 2000; Matsushita et al., 1998; Wakame et al., 1999; Yagita et al., 1998, 2002). AHCC is available “over-the-counter” as a nutritional supplement.

In the study carried out with AHCC, mice pre-treated for one week by gavage with AHCC and then hindlimb-unloaded with continued AHCC treatment were significantly protected from infection with K. pneumoniae (Aviles et al., 2003b). In fact, protection against infection was greater in hindlimb-unloaded mice than in normally caged mice, suggesting that more beneficial effects would be observed in immunocompromised hosts (Aviles et al., 2003b). This surprising result stimulated additional mechanistic studies with AHCC. Hindlimb-unloaded mice infected with K. pneumoniae could not clear the infection from the blood, but hindlimb-unloaded mice pre-treated with AHCC and infected with K. pneumoniae were able to clear the bacteria from the blood (Aviles et al., 2003b). These results suggested that the immune system was stimulated by pre-treatment with AHCC, resulting in clearing of the infection.

Additional mechanistic studies have been carried out to determine the role of AHCC in enhancing resistance to infection. Hindlimb–unloaded mice infected with K. pneumoniae and pre-treated with AHCC showed enhanced cytokine production, spleen cell blastogenesis and peritoneal cell nitric oxide production (Aviles et al., 2004). These results again supported that AHCC treatment enhanced innate immunity in immunocompromised hosts, but also showed a possibility that AHCC treatment could enhance acquired immunity (Aviles et al., 2004).

CONCLUSIONS

The results of the infectious disease studies show that exposure of mice to hindlimb unloading conditions resulted in compromised immune responses and compromised resistance to infection. AHCC may be a useful countermeasure to protect mice from the compromising effects of hindlimb unloading on resistance to infection. This certainly raises an issue that will require further study in actual space flight experiments.

The above results could not have been obtained without the use of the hindlimb unloading animal model. The space flight studies that should be carried out will not be able to be completed without the use of animals. The countermeasures that must be developed must have their efficacy and safety tested in animal models.

The need for these models is not limited to the immune system. Similar problems as well as the need for development of countermeasures exist in all disciplines (Sonnenfeld, 2005). Therefore, it is extremely important, as we go forward with planning for exploration class human space flight missions, that we include the use of both ground-based and flight animal models to enhance our ability and enable us to protect future space travelers from any detrimental effects of the space flight environment.

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Symposium II: Pharmacological Countermeasures to Physiological Changes Induced by Space Flight

Joan Vernikos, Editor
EXERCISE AND PHARMACOLOGICAL COUNTERMEASURES FOR BONE LOSS DURING LONG-DURATION SPACE FLIGHT
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ABSTRACT

Bone loss in the lower extremities and lumbar spine is an established consequence of long-duration human space flight. Astronauts typically lose as much bone mass in the proximal femur in 1 month as postmenopausal women on Earth lose in 1 year. Pharmacological interventions have not been routinely used in space, and countermeasure programs have depended solely upon exercise. However, it is clear that the osteogenic stimulus from exercise has been inadequate to maintain bone mass, due to insufficient load or duration. Attention has therefore been focused on several pharmacological interventions that have been successful in preventing or attenuating osteoporosis on Earth. Anti-resorptives are the class of drugs most commonly used to treat osteoporosis in postmenopausal women, notably alendronate sodium, risedronate sodium, zoledronic acid, and selective estrogen receptor modulators, such as raloxifene. There has also been considerable recent interest in anabolic agents such as parathyroid hormone (PTH) and teriparatide (rhPTH [1-34]). Vitamin D and calcium supplementation have also been used. Recent studies of kindreds with abnormally high bone mineral density have provided insight into the genetic regulation of bone mass. This has led to potential therapeutic interventions based on the LR P5, Wnt and BMP2 pathways. Another target is the RANK-L/osteoprotegerin signaling pathway, which influences bone turnover by regulating osteoclast formation and maturation. Trials using such therapies in space are being planned. Among the factors to be considered are dose-response relationships, bone quality, post-use recovery, and combination therapies—all of which may have unique characteristics when the drugs are used in space.

INTRODUCTION

The human skeleton has evolved in an environment where the force of Earth’s gravity has been a continual presence. It is, therefore, not surprising that removal of gravity during long-duration space flight results in a loss of homeostasis in the skeleton, which adapts to the new environment by shedding calcium (Lang et al., 2004) at a rate that is almost 10 times greater than that in a postmenopausal woman (Iki et al., 1996; Sirola et al., 2003). This adaptation to microgravity renders the skeleton “at risk” for fracture, increases the risk of renal stones (Whitson et al., 1999), and poses potential long-term health risks for astronauts on their return to Earth with reduced bone mass.

In this article, we will examine the evidence for loss of bone mass during long-duration space flight, discuss the mechanisms for such loss, review countermeasures that have been attempted to date, and examine the potential of pharmaceutical countermeasures in the future. The implications of recent findings regarding the genetic determinants of bone mass will also be discussed.

BONE LOSS IN SPACE: THE EVIDENCE

Bone loss during space flight has been a concern since the Gemini flights (1-14 day missions, 1962-1966). Mack and colleagues (Mack et al., 1967; Mack and LaChance, 1967), reported what they called “small but significant” bone loss. If one extrapolated their results to long-duration flights, these changes would have been alarming—ranging from 5.3% per month in the calcaneus during Gemini 7 to 89% per month in the finger phalanges during Gemini 4. The observations were based on the use of densitometry of plain X-rays, which is now regarded as an inaccurate methodology (Rambaut et al., 1975). It is, however, interesting to note that these authors also measured urinary and fecal loss of calcium in a group of bed rest subjects and reported a correlation of -0.7 between loss of bone mass and mean calcium intake.

Soviet researchers (Biriukov and Krasnykh, 1970; Krasnykh, 1969—reported by Rambaut et al., 1975) found average losses of 4.7% per month in the os calcis during 8-10 weeks of bed rest reported to the loss of bone mass. In the Apollo flights (6-14 day missions, 1968-1972), neutron activation analysis of fecal specimens (Brodzinski et al., 1971) (Apollo VII-XI; 6-11 days), densitometry from plain radiographs (Mack and Vogt, 1971) (Apollo VII and VIII; 11 and 6 days, respectively), and single-photon absorptiometry of calcaneus and forearm (Rambaut et al., 1975) (Apollo XIV-XVI; 9, 12, and 11 days, respectively) were used to assess changes in bone status.

Brodzinski et al. (1971) called the Gemini findings of Mack and LaChance (1967) “dubious,” and their own
measurements of calcium loss in Apollo VII-XI crew members suggested less substantial changes. They estimated that loss of total body calcium could be as little as 7.5% per year of space flight, but they suggested that a calcium balance experiment should be conducted on Skylab, and this was in fact accomplished (see below).

Using similar methodology to their earlier studies, Mack and Vogt (1971) reported average losses of 11.6% per month in the lower extremity and 22.6% losses in the upper extremity of six Apollo VII and VIII crew members. As discussed above, in retrospect, these changes in “bone density” measured from plain radiographs were clearly erroneous; control subjects on Earth did not show such large changes.

The single gamma photon absorptiometry of Rambaut et al. (1975) found a loss of 5.1% per month in the lower extremity, a gain of 0.6% per month in the radius, and a loss of 4.6% per month in the ulnae of nine Apollo XIV-XVI crew members.

One of the most complete series of calcium balance studies in space was conducted during the Skylab missions (Skylab 2, 28 days, 1973; Skylab 3, 59 days, 1973; Skylab 4, 84 days, 1973-1974—Rambaut and Johnston, 1979; Smith et al., 1977; Smith et al., 1998; Smith et al., 1999; Tilton et al., 1980; Whedon et al., 1977). Commencing 21 days prior to flight, during flight, and for 18 days post flight, the intake of 30 nutrients were monitored, 24-hour pooled urine collections were made, and fecal samples were vacuum dried for analysis. Weekly plasma samples were also taken. A 56-day ground-based control experiment was also conducted. During the Skylab 4 mission, average negative calcium balances of -100 (+25), -180 (+36), -229 (+60), -223 (+42), -88 (+52) were reported for the pre-flight, flight values, indicating that space flight is associated with increased bone resorption.

Rambaut et al. (1979) pointed out that “the chain of events leading ultimately to bone loss inflight remains elusive.” In an article almost 20 years later, in which the urine of Skylab crew members was re-analyzed, Smith et al. (1998) shed light on this mechanism by demonstrating that urinary excretion of collagen breakdown products during the Skylab 4 mission was 40-45% higher than pre-flight values, indicating that space flight is associated with increased bone resorption.

Using computer tomography (CT), Oganov et al. (1990) measured mineral density of lumbar vertebrae in four Salyut-7 crew members before and after extended flights (5-7 months’ duration). These authors reported that bone mineral density (BMD) diminished only in some of the test subjects and emphasized that the magnitude of changes was not correlated with flight time, presumably due to individual differences in rates of bone loss.

McCarthy et al. (2000) used three techniques (dual energy X-ray absorptiometry [DXA], ultrasonic measurements of velocity [SOS], and broadband attenuation [BUA] of the calcaneus) to evaluate changes in bone during two missions, of 180 and 20 days, to the Mir space station, involving three subjects. DXA measurements resulted in significant variation between different sites in the body for changes in BMD, with the greatest losses occurring in the lumbar spine and proximal femur.

The application of modern imaging techniques to bone changes during space flight was first accomplished by LeBlanc and colleagues (LeBlanc et al., 1996; LeBlanc et al., 1998; LeBlanc et al., 2000). In 1989, they installed a Hologic 1000W dual X-ray absorptiometry (DXA) scanner at the cosmonaut training center in Star City, Moscow, in the former USSR. Between 1990 and 1995, they studied 18 cosmonauts who had flown for between 126 and 438 days (LeBlanc et al., 1996; LeBlanc et al., 2000). These measurements showed regional losses during flight of between 1.06% and 1.56% per month in the spine, pelvis and proximal femur, but no significant changes in the upper extremities (Figure 1a). Losses were parallel, but smaller, during bed rest, except in the arms, where losses were greater than during flight. These data showed, for the first time, a pattern of lower-extremity loss and upper-extremity preservation during flight. The authors concluded that the in-flight exercise programs were not sufficient to completely ameliorate bone loss during flight (no countermeasures were used during bed rest).

Lang et al. (2004) provided data from DXA, volumetric quantitative computer tomography (vQCT), and quantitative ultrasound (QUS) on crew members from the Expeditions 2-6 to the International Space Station (ISS; 2001-2003, 130-197 days). The authors’ data confirmed...
that little progress had been made in preventing loss of bone mineral in the 30 years since Skylab. Notably, vQCT allowed an examination of the loss in both trabecular and cortical fractions of bone and also estimates of the volumetric BMD (vBMD) as well as the conventional areal BMD (aBMD). These data confirmed the large losses in the spine and proximal femur (Figure 1b), and indicated that the rate of loss of bone mineral content (BMC) in trabecular bone in the proximal femur was approximately twice that of the cortical loss. Since trabecular bone cannot be replaced after loss of trabecular continuity (Langton et al., 2000), this later finding is of particular concern. The authors also found that calcaneal estimates are not good surrogates for central or upper extremity skeletal measures and concluded that there was a continuing need to improve countermeasures to bone loss, as it has become clear that current efforts are inadequate.

**Figure 1b:** Data showing change in regional bone (in percent change per month; negative values represent loss) from 13 crew members on the International Space Station. Data adapted from Lang et al. (2004).

It is interesting to note that long-duration space flight continues to be a male bastion, and thus we do not have adequate data on gender differences in bone loss in space. For the 32 subjects for whom DXA data are available, there are only two women: one in the LeBlanc et al. (2000) series, who was reported to have similar responses to the mean of the group, and one in the Lang et al. (2004) series, whose data were not uniquely identifiable. Presumably, privacy issues prevented this disclosure, but one would hope that all crew members would make such data available in the future in the interest of science.

**PRIOR COUNTERMEASURES**

The only countermeasure that has so far been used in space for bone loss, albeit unsuccessfully, is exercise. Astronaut-physician William E. Thornton was a tireless proponent of exercise countermeasures, and his accounts of exercise countermeasures and devices (Thornton, 1989a; Thornton, 1989b; Thornton, 1989c) are required reading in order to understand the history of use of this modality. There is also a good description of exercise and other countermeasures in Nicogossian et al. (1994). The countermeasure tradition began in the confined space of the Gemini capsule, where a bungee cord held by a loop to the feet was pulled to exercise the arms and legs (Dietlein, 1965). There is no record of its efficacy, although measurements of heart rate, blood pressure, and respiration rate were taken during exercise to record cardiac response to exercise in space (Dietlein and Rapp, 1966).

The Soyuz 9 flight (18 days, 1970), on which bungee and expanders were also used for exercise (Nicogossian et al., 1994), highlighted the need for more effective countermeasures to combat the general loss of conditioning (Yegorov et al., 1972). Subsequently, some of the Salyut Space orbital stations (Salut 1, 1971 to Salyut 6, 1985) were equipped with a passive treadmill, a bicycle ergometer, and a gravity simulation suit for long wear (Gazenko et al., 1976). The efficacy of this “Penguin Suit” (Nicogossian et al., 1994) has not been confirmed.

The Skylab astronauts used several on-board exercise devices, including a bicycle ergometer and a Teflon® plate, not available until Skylab 4, on which they performed an unusual form of tethered locomotion (Figure 2a; Thornton and Rummel, 1977; Thornton, 1989a). They also had a Mini Gym exercise device, which allowed concentric muscular exercise to be performed, primarily benefiting the arms and trunk. Although this device probably transmitted higher forces to the legs than those from the bicycle ergometer, the force levels were still considered inadequate (Thornton and Rummel, 1977). No systematic record of the use of these devices by Skylab crew members is available in the literature, although it is likely that such records were kept.
Thornton is thought to be the first man to run around the world in low Earth orbit. This feat was performed during one complete orbit of STS-8 (1983) on a treadmill that Thornton helped to design. Because the mid-deck of the Space Shuttle was not particularly spacious, the passive treadmill had to be stowable in a locker, a fact that severely limited its belt length (Figure 2b). The subject was tethered by bungee cords, which applied an unknown tension to return the crew member to the treadmill surface. Kinematic analysis of on-orbit film taken during running on the treadmill (Thornton et al., 1998) indicated that there was restricted range of motion at the lower-extremity joints and a plantar-flexed “tip-toe” gait. No measurements of the foot forces were made.

Cosmonauts on Mir have been said to perform exercise “up to 3 hours per day” (Nicogossian et al., 1994), while others believe that the exercise was 2-3 hours on 3 of 4 days (LeBlanc et al., 2000). The passive treadmill was considered the “stadium” from which exercise was performed. While the subject was tethered in place using bungees, he not only walked and ran, but also performed calisthenics and upper-body exercises using additional bungee cords for resistance (Figure 3). The data from LeBlanc et al. (2000) showed clearly that this protocol, even if faithfully performed, is not an effective countermeasure for bone loss.

The exercise facilities available on the ISS through Expedition 12 consist of a Treadmill Vibration Isolation and Stabilization System (TVIS; Figure 2c; McCrory et al., 1999), a cycle ergometer with vibration isolation (CEVIS; Figure 2d), and the Interim Resistive Exercise Device (iRED; Figure 2e; Schneider et al., 2003). There is also a bicycle ergometer available in the Russian segment. None of these devices has a force measurement capability, and there is very little published information about their performance characteristics. When running on the treadmill, a subject must be tethered using a subject load device (SLD) to restrain him on the treadmill surface, and, optionally, a subject position device (SPD) is used to keep the subject in an area of the treadmill where a pitch oscillation of the treadmill will not be initiated. Each crew member is assigned a period of 2.25-2.5 hours every day for exercise—including set-up and break-down time, which can consume more than 50% of the assigned period. The work of Lang et al. (2004) showed that these devices as they are presently used are not effective as a countermeasure for bone loss during long-duration flights.

As we shall discuss below, prolonged bed rest is considered to be a viable analog of space flight. Shackelford et al. (2004) conducted a program of vigorous resistance training (averaging 74% of one repetition maximum) in nine individuals during a 17-week confinement. The exercise was found to have a beneficial effect on BMD during bed rest compared to controls, specifically in the lumbar spine (+3% vs. -1%), total hip (+1% vs. -3%), heel (+1% vs. -3%), total body (0% vs. -1%), and pelvis (-0.5% vs. -3%). However, the high levels of load imposed on the muscle groups studied have never been achieved in space, and it is unlikely that in-flight exercise devices currently in use will permit such loads to be achieved.
Figure 2c: The International Space Station Treadmill with a Vibration Isolation and Stabilization System (TVIS). (NASA photography)

Figure 2d: The Cycle Ergometer with Vibration Isolation and Stabilization System (CEVIS) in use on the International Space Station. (NASA photography)

Figure 2e: The Interim Resistive Exercise Device (iRED) in use on the International Space Station (ISS). (NASA photography)
Figure 3: A page from the Mir cosmonaut exercise instruction manual showing a 24-stage exercise session performed on the treadmill.
WHY HAVE EXERCISE COUNTERMEASURES IN SPACE NOT BEEN EFFECTIVE?

Since exercise has been the only countermeasure to bone loss so far attempted in space, and since considerable bone loss has occurred on all flights to date, it would be tempting to conclude that exercise is not an appropriate countermeasure. There are, however, several reasons why such a conclusion may be premature: (1) There has never been a controlled study of exercise, either in space or during bed rest. The lack of such a study in the more than 40 years that this problem has been recognized is highly perplexing to the current authors and perhaps reflects the fact that NASA has traditionally been an engineering rather than a science agency; (2) The loads applied to the body by any piece of exercise equipment were not measured prior to 2003, so it is not known whether or not equipment exerted 1-g-like loads; (3) Exercise adherence may have been less than optimal and, contrary to common belief, the ISS program was the first time a mandatory exercise program was instituted as part of a flight plan; (4) It is not known if a single daily concentrated “dose” of exercise in 0-g can effectively replace a “dose” that in 1-g is distributed throughout the day; (5) The duration of exercise programmed to date may not have been adequate to achieve the desired result; (6) There is considerable debate in the scientific community about the optimal loading strategy that will provide an osteogenic stimulus to bone (Turner, 1998; Turner and Pavalko, 1998). Evidence in the literature ranges from a few intermittent large loads per day (Lanyon, 1996) to 18,000 small-amplitude vibrations in a 10-minute period (Rubin et al., 2002a; Rubin et al., 2002b). There is also debate regarding the relative role of force and rate of change of force (Cullen et al., 2001; Linde et al., 1991; Mosley and Lanyon, 1998).

Our own experiments using force-measuring insoles during exercise on the ISS (Rice et al., 2004) have suggested that neither the load nor the duration of treadmill exercise in the current ISS exercise program is adequate to replace 1-g exercise.

Only when all six issues noted above have been carefully examined can the role of exercise as a countermeasure to in-flight bone loss be determined. Until such time, it is reasonable that the flight medicine community is looking to explore the use in space of pharmacological options that are being used on Earth to prevent postmenopausal osteoporosis.

The remainder of this review will examine the cellular and molecular targets for such therapy, present the currently available options, and discuss the limitations of knowledge required for the implementation of these therapies in space.

BONE REMODELING

Bone is an active tissue that is constantly being remodeled, principally by the action of two cell types: osteoclasts, which resorb bone, and osteoblasts, which build new bone (Figure 4). It is estimated that all of the bone in the adult skeleton is replaced every 10 years (Marx, 2004). Homeostasis of bone is only maintained if the opposing—or perhaps complementary—actions of osteoblasts and osteoclasts are balanced. A defect in either process can result in accumulation of bone (as in osteopetrosis) or in a net loss of bone (as in osteoporosis) (Helfrich, 2003; Phan et al., 2004). The mineral phase of bone is of primary importance to density, and therefore BMD has been used in the past as a main indicator of bone status (Kanis, 2002). However, there is now increasing interest in measures of bone “quality” that include structural as well as compositional information (Ammann and Rizzoli, 2003; Turner, 2002), and it is likely that a composite measure will eventually replace BMD as the parameter of choice.

The majority of current therapeutic interventions could be classified as resorption-prevention drugs. A number of advances in understanding how osteoclasts differentiate, mature, and are activated have recently been made (Boyle et al., 2003; Marx, 2004). Prevention of osteoclast formation (osteoclastogenesis) and development has been a prime target through a number of different pathways (see below). On the formation side of the equation, preventing osteoclast cell death (apoptosis) is also of interest, and a number of other “anabolic” or bone-building drugs with uncertain mechanisms are also being explored (Bisello et al., 2004; Deal and Gideon, 2003).

SUPPLEMENTATION

Traditionally, supplementation of daily intake of vitamin D and calcium (current recommended daily allowances [RDAs] 400 IUs and 1500 mg, respectively) have been considered mainstays of osteoporosis prevention. Adequate calcium is needed for mineralization, and vitamin D plays a role in the regulation of calcium deposition for bone mineralization. Both of these agents have weak antiresorptive properties (compared, for example, to bisphosphonates [Reginster, 2004]—see below), but combined therapy for 18 months (1200 mg calcium plus 800 IU vitamin D₃ [cholecalciferol]) has been shown to be effective in reducing hip fracture in elderly women who were Vitamin D deficient (Chapuy et al., 1992). The bioavailability of the various forms of calcium used in supplementation (calcium carbonate, citrate, phosphate, lactate, and formate) have been shown to be different (Hanzlik et al., 2005).

Since it is not always clear whether or not dietary intake of these agents is adequate, most drug trials routinely include calcium and vitamin D supplementation in control, placebo, and treatment arms. Astronaut diets can...
be closely controlled, so inclusion of RDA and supplementation in diet can be easily accomplished.

HORMONE REPLACEMENT THERAPY

Estrogen, in the form of 17β-estradiol, has a complex agonistic action on estrogen receptors (ERs) in the nucleus of osteoblastic cells (Riggs and Hartmann, 2003), which in turn affect estrogen receptor elements (EREs) in target genes. In estrogen deficiency, resorption outpaces formation, resulting in net bone loss. Estrogen also stimulates breast epithelial cell production and has been implicated in breast cancer risk (Riggs and Hartmann, 2003).

Hormone replacement therapy (HT) using estrogen (unopposed HT) or estrogen-progestin (opposed HT) was widely recommended for postmenopausal women until the landmark Women’s Health Initiative (WHI) study (Rossouw et al., 2002) demonstrated a number of adverse responses (increased risk of coronary artery disease, stroke, thromboembolism, and breast cancer) in subjects using opposed HT. Riggs and Hartmann (2003) stated that estrogen was the most widely prescribed drug in the world and that it was taken by 38% of postmenopausal women in the United States. In addition to reducing the risk for nonvertebral fractures (Torgerson and Bell-Syer, 2001), HT also had the added advantage of relieving a number of perimenopausal symptoms. Because of the increased risk of adverse side effects, HT is no longer recommended for prevention or treatment of osteoporosis (Kessel, 2004).

Estrogen also has significant effects on skeletal metabolism in men. Traditionally, estrogen was considered the regulator of the female skeleton and testosterone the male regulator. The discovery of mutations in the aromatase gene in men and concurrent abnormalities in skeletal metabolism (osteopenia and unfused epiphyses) have focused attention on the importance of estrogen physiology. Aberrations in osteoclast activity due to deficiency of inhibitors may attend the loss of estrogen with aging in both men and women and cause increased bone turnover (Carani et al., 1997; Khosla et al., 2002; Khosla et al., 2004).

SELECTIVE ESTROGEN RECEPTOR MODULATORS (SERMS)

Because of the side effects of HT, there has been increased interest in this class of nonhormonal drugs that target the ER. SERMs can have both agonist and antagonist effects in different tissues (e.g., tamoxifen [Tamofen], used in the treatment of ER-positive breast cancer, is an antagonist that slows the proliferation of tumor cells, whereas raloxifene [Evista] is a bone agonist that has an antiresorptive effect) (Riggs and Hartmann, 2003). Different SERMs that have similar effects on bone (e.g., raloxifene and idoxifene [investigational]) appear to have their modes of action through different molecular pathways (Nuttall et al., 2000). Because raloxifene, which is administered orally once per day, has a preferential effect on vertebral fracture risk reduction (Ettinger et al., 1999), it is possible that there are differences between the action of SERMs on trabecular vs. cortical bone.

There are some indications that raloxifene therapy decreases cardiovascular events in women with risk factors at baseline (Barrett-Connor et al., 2002) but carries...
with it a small increase in the risk for thromboembolism (Daly et al., 1996). SERMs do not appear to alleviate postmenopausal symptoms (National Osteoporosis Foundation, 2002; Cranney et al., 2002).

The common risk for both therapies is that of deep vein thrombosis, especially in conditions of clotting abnormalities. This risk is small but nonetheless present statistically. In other situations the drugs have divergent risks. Breast hyperplasia and breast cancer are not found with the SERM agents as they are with estrogen. Moreover, the SERM drugs do not cause cervical endometrial hyperplasia, menstrual bleeding, or cervical cancer.

The SERM drugs may offer an option for treatment of prostate cancer. In the presence of decreasing androgens with aging, estrogen induces prostatic hyperplasia and neoplasia. Antiestrogens and SERMs suppress prostate carcinogenesis. Some preliminary studies suggest that SERMs may not be useful as a general treatment for male osteoporosis, but there are some male patients, small in number, with the requisite balance of estrogen and testosterone for whom SERMs may be beneficial (Steiner and Raghow, 2003; Doran et al., 2001).

### ANTiresorPTIVE Drugs

The largest class of antiresorptive drugs is the bisphosphonates (such as alendronate [Fosamax], etidronate [Didronel], ibandronate [Boniva], pamidronate [Aredia], risendronate [Actonel], zoledronate [Zometa], and tiludronate [Skelid]). The drugs are distinguished by their potency, which is usually positively affected by the presence of a nitrogen atom (e.g., etidronate [low] to zoledronate [high]), by their mode of delivery (e.g., intravenously for pamidronate and zoledronate, orally for alendronate, orally, intravenously, or by injection for ibandronate), and by the frequency and size of dosing (e.g., 5 or 10 mg daily or weekly for alendronate, 2.5 mg daily for ibandronate, 10-90 mg annually for zoledronate). An excellent review of these drugs is provided by Reginster (2004).

Bisphosphonates are powerful and specific inhibitors of osteoclasts (Figure 5). They were originally thought to exert their action via incorporation in the skeleton by mimicking pyrophosphate and binding to the hydroxyapatite crystals in the bone matrix (Licata, 2005), especially at sites of remodeling, the bone multicellular units (BMUs) (Russell et al., 1999).

![Figure 5: Schematic of bisphosphonate action (Rodan and Fleisch, 1996). Where bisphosphonate (BP in the diagram) has been incorporated into the bone matrix, osteoclastic resorption of bone cannot occur. Reprinted with permission.](image)

Their actions have since been shown to be complex, however. The amino bisphosphonates inhibit osteoclastic cholesterol synthesis and membrane function and increase cellular apoptosis. The non-amino bisphosphonates produce ineffective ATP analogs and inhibit osteoclast function by “energy starving” the cell. Once incorporated, bisphosphonates remain bound at the bone surface and exhibit extremely low serum concentrations, thus limiting side effects. In general, the third generation (N₂-containing) bisphosphonates have shown approximately...
40-50% reduction in the risk of vertebral and nonvertebral fractures compared with placebo in postmenopausal women (Black et al., 1996; Chesnut et al., 2004; Harris et al., 1999) and have also resulted in increased BMD in the lumbar spine, total hip, and trochanter in women with and without osteoporosis (Cooper et al., 2003; Mortensen et al., 1998; Ravn et al., 1999).

There are two bed rest studies involving bisphosphonates that are relevant to the space program (LeBlanc et al., 2002; Watanabe et al., 2004). LeBlanc et al. (2002) administered 10 mg of alendronate daily to eight male subjects undergoing 17 weeks of horizontal bed rest. Compared with concurrent and historical controls, BMD loss was significantly attenuated (or eliminated) in the alendronate treatment group in the lumbar spine, femoral loss was significantly attenuated (or eliminated) in the compared with concurrent and historical controls, BMD that are relevant to the space program (LeBlanc et al., 2002; Watanabe et al., 2004). LeBlanc et al. (2002) administered 10 mg of alendronate daily to eight male subjects undergoing 17 weeks of horizontal bed rest. Compared with concurrent and historical controls, BMD loss was significantly attenuated (or eliminated) in the alendronate treatment group in the lumbar spine, femoral, neck, trochanter, and pelvis (but not calcaneus). Most markers of bone collagen breakdown and resorption (cross-linked N-telopeptide of type I collagen [NTx], pyridinium [Pyd], and deoxypyridinium [D-Pyd]) increased in both groups, but significantly less so in the treated group than in controls. Markers of bone formation (alkaline phosphatase, bone-specific alkaline phosphatase, and osteocalcin) were unchanged in controls, but were decreased in the treated group because of the reduced bone turnover. These results demonstrate that the drug does not ablate the bone loss totally, thus the observed clinical effects may require simultaneous mechanical stress.

Watanabe et al. (2004) administered 60 mg of pamidronate to seven male subjects 14 days before 90 days of 6-degree head-down bed rest. These authors also showed that alendronates, in addition to their osteoprotective properties, decrease the risk of renal stones. Compared with sedentary and resistance training controls, the pamidronate-treated subjects not only maintained significantly more bone in the proximal femur and lumbar spine, but also showed no evidence of urolithiasis (stones in the urinary tract). In the other groups, six subjects were found to have radiographic evidence of stone formation during bed rest. All but one of these stone-forming subjects had baseline hypercalciuria (>250 mg per day). Such patterns of stone formation may be a feature of all bed rest studies, and perhaps of long-duration space flight, that has been previously overlooked. However, it is extremely unusual for healthy patients with no prior stone risk to become “at-risk” in such a short time, and these results, although cautionary, need to be replicated.

Shapiro et al. (personal communication), in an as yet unpublished study, showed a reduction of bone loss in the lower extremities of patients with spinal cord injuries who had been administered intravenous zoledronate. The paradigm of spinal cord injury has been suggested to be another analog of space flight, although the absence of muscular action may tend to make it even more severe from a disuse point of view. The side effects of bisphosphonate therapy from the major study series have generally been mild (adverse effects in the upper gastrointestinal (GI) tract, constipation, flatulence, hypocalcemia, and diarrhea), but severe esophageal reactions have been reported with alendronate (Schnitzer et al., 2000). Consequently, its use is not recommended for patients with a history of upper GI complaints.

There is some concern that bone formed during the administration of bisphosphonates may not have the same “quality” as normal bone, thus negatively affecting on the mechanical integrity of the skeleton. Animal studies with bisphosphonates have shown a delay in fracture healing in rats and rabbits and an increase in the presence and persistence of microcracks and reduced remodeling, suggesting a potential change in biomechanical factors (Li et al., 1999; Li et al., 2001; Mashiba et al., 2001; Lehman et al., 2004). In addition, it is notable that Ruggiero et al. (2004) have identified a cluster of patients on chronic bisphosphonate therapy that had an associated risk of osteonecrosis of the jaw. This condition is also seen in the myeloma patients treated with i.v. zoledronic acid monthly (Lugassy et al., 2004), which is not the way it is used for treating osteoporosis. It is possible that such patients may have immune compromise supporting local dental infection and subsequent bone destruction.

If sequential combination therapy of different drugs is planned, Gasser et al. (2000) showed in studies of rat bone that the response to an anabolic drug (see below) was delayed in animals pretreated with bisphosphonates. However, in clinical studies, long-term use of alendronate and risedronate for 7-10 years shows no similar findings. Both drugs still suppress fractures, which argues against the adverse effects seen in animal models. Furthermore, histomorphometry shows no abnormal characteristics in patients after 3 or more years of use.

**ANABOLIC DRUGS**

Drugs in this class exert their mode of action by increasing bone formation rather than by inhibiting resorption. The important role of parathyroid hormone (PTH) in regulating bone and mineral metabolism has been known for more than 70 years (Bisello et al., 2004), but classical teaching identifies PTH as a powerful mobilizer of skeletal calcium into the serum in the presence of hypocalcemia (i.e., a state of secondary hyperparathyroidism). Evidence from animal experiments has shown that daily injection of PTH had anabolic effects on bone, and recent work has resolved these apparently paradoxical effects by showing a dependency on the pattern of exposure. Chronic elevation of PTH (as in primary hyperparathyroidism) leads to increased bone resorption, whereas intermittent elevation (as in once-daily injections with a short half-life) leads to increased formation. The mechanism of action of PTH appears to be the stimulation of existing osteoblasts via surface PTH receptors and interaction with RANK-L (NF-kB; see below) (Deal and Gideon, 2003). It is known that the amino-terminal region of PTH (the first 34 amino acids) is necessary and sufficient for full activity,
and the only anabolic agent that is currently Food and Drug Administration (FDA) approved for use in the treatment of osteoporosis is recombinant teriparatide (rhPTH [1-34] [Forteo]).

Administration of teriparatide (daily subcutaneous injection 20 µg or 40 µg for 19 months) to women with low bone mass and a history of prior fracture resulted in an almost 10% increase in vertebral BMD; treatment reduced the risk of a second vertebral fracture by approximately 65% and that of a nonvertebral fracture by approximately 50% compared with placebo. There is some concern that high doses of teriparatide (up to 60 times greater than approved human doses) have caused osteosarcoma in rats, but not monkeys. No similar complications have been observed in human studies, but an initially promising trial conducted in men (Orwoll et al., 2003) was terminated because of concern regarding the animal results.

It is also known that PTH-related peptide (PTHrP), a protein with some homology to PTH that is produced by tumors and leads to hypercalcemia, shares many of the actions of PTH but has receptors that are much more widely distributed (Bisello et al., 2004). The authors have initial evidence from human studies that PTHrP has the potential to be a powerful anabolic agent, and clinical trials to explore this possibility are ongoing.

It is possible that the mechanism for the differential effects of intermittent vs. continuous levels of PTH is in the modulation of the osteoprotegerin (OPG)/receptor activator of nuclear factor-κB ligand (RANK-L) ratio (see below) (Locklin et al., 2001).

**RANK-L/OPG**

In 1997, a new pathway regulating bone resorption was identified (serendipitously) by a group looking for novel genes in the rat intestine (Simonet et al., 1997). The transgenic mouse overexpressing one particular gene was found to have osteopetrosis and a deficiency of osteoclasts (Khosla, 2001) and the responsible protein was called osteoprotegerin for its protective role in maintaining bone mass. Simultaneously, Yasuda et al. (1998) found the same protein in a targeted search for the signaling link that had been previously hypothesized to exist between osteoclasts and ostoblasts (Rodan and Martin, 1982). The pathway that has been identified as a result of these and subsequent studies is shown in Figure 6. OPG is secreted as a soluble protein from bone marrow stromal cells and appears to be a decoy receptor, which binds to RANK-L. Since RANK-L is a major factor in osteoclast differentiation, activation, and apoptosis inhibition, it follows that the binding of RANK-L to OPG, rather than to its target RANK on the osteoclast precursor cell, will prevent bone resorption. Because RANK-knockout mice also exhibited osteopetrosis and absence of osteclasts (Li et al., 2000), the existence of a new OPG/RANK/RANK-L pathway in the control of bone resorption was confirmed. Several genetic mutations of this pathway are associated with bone diseases such as the family of hyperphosphatasias, Paget’s disease, and possible bone loss in inflammatory arthritis (Boyle et al., 2003; Khosla, 2001).

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**Figure 6:** Schematic of the OPG/RANK-L pathway (Khosla, 2001). Note that OPG acts as a decoy receptor preventing RANK from attaching to its ligand RANK-L and therefore inhibiting osteoclast differentiation. Copyright 2001, The Endocrine Society. Reprinted with permission.
OPG was an obvious choice as a clinical therapeutic agent to prevent osteoporosis, and indeed two forms of the protein were examined by Amgen in clinical trials of osteoporosis (Bekker et al., 2001) and multiple myeloma and breast carcinoma (Body et al., 2003). A presumed combination of concerns regarding efficacy, safety, treatment duration and manufacturing factors has resulted in OPG’s no longer being examined for clinical use. OPG does, however, continue to be explored for the treatment of bone tumors (Wittrant et al., 2004). A fully human monoclonal antibody for RANK-L, AMG 162, is being developed as an osteoporosis treatment instead (Bekker et al., 2004; McClung et al., 2004). Phase III clinical trials were initiated in late 2004 for AMG 162.

**CALCITONIN**

The peptide calcitonin exerts a complex inhibitory action on osteoclast function (Kajiya et al., 2003). It has been used in trials of both men and women with low bone mass and has been shown to stabilize (or prevent) bone loss (Toth et al., 2005) and, in women, to decrease vertebral fracture rate (Munoz-Torres et al., 2004). One attractive feature of calcitonin is that it can be administered by many routes, including nasally in the form of a daily (or intermittently administered [Tekeoglu et al., 2005]) spray.

**BONE GENETICS**

In the last 5 years, early insights into some of the genetic determinants of bone mass have been obtained. Ralston (2003) and Recker (2004) have recently reviewed the status of present knowledge in this area. Johnson et al. (2004) have commented regarding “how little we really know about the genes that control bone mass.” The genetic basis for diseases caused by a defect in osteoclasts is discussed by Helfrich (2003).

Gong et al. (2001) found that that the LRP5 gene, which encodes the low-density lipoprotein receptor-related protein 5, is important in bone mass accrual. They reported that loss-of-function mutations in LRP5 caused the autosomal recessive disorder osteoporosis-pseudoglioma syndrome and that Wnt-mediated signaling via LRP5 affects bone accrual during growth and peak bone mass (Figure 7).

Subsequently, mutations in the same gene were also found to be associated with diseases in which there was high bone mass (Boyden et al., 2002; Little et al., 2002). The lack of inhibitory action of the protein Dkk-1 on the Wnt signaling pathway suggested this protein as a potential therapeutic target for modulating bone mass. A review of LRP5 and Wnt signaling is presented by Johnson et al. (2004). Genes regulating lipoxygenase are also believed to influence bone mass (Klein et al., 2004).

There are indications that the Wnt signaling pathway is activated in response to mechanical loading (Johnson, 2004), and this may be a key element in the elusive mechanotransduction that has long been hypothesized to exist.
An alternative approach to the human linkage and association studies described above is the use of mouse models in quantitative trait locus (QTL) analysis (Liu et al., 2003; Rosen et al., 2001). QTL is basically a statistical analysis, sometimes of the entire genome, to identify which regions of the genome contain loci that influence the phenotype of interest.

The genes encoding type I collagen (COLIA1 and COLIA2) are mutated in osteogenesis imperfecta and may be useful markers of other osteoporotic phenotypes (Mottes et al., 1998).

The estrogen receptor gene may regulate some aspects of bone density since the discovery of a male patient with a gene mutation and osteoporosis (Gennari et al., 2005).

Finally, the early discovery of the vitamin D receptor gene helped introduce the notion that bone mass had a genetic basis (Eisman, 1995).

The complexity of BMD as a trait and the importance of gene-environment interactions have been emphasized in a study of risk factors for low spine and hip BMD involving 12 candidate gene loci and lifestyle factors by Lau et al. (2005).

While these various studies of genetic influence on bone mass are in their early stages, there is a high likelihood that they will eventually identify new therapeutic targets.

Table 1. The major classes of osteoprotective therapeutic drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Manufacturer</th>
<th>Class</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alendronate sodium</td>
<td>Merck</td>
<td>Bisphosphonate</td>
<td>Inhibit osteoclasts</td>
</tr>
<tr>
<td>(Fosamax)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zoledronic acid</td>
<td>Novartis</td>
<td>Bisphosphonate</td>
<td>Inhibit osteoclasts</td>
</tr>
<tr>
<td>(Zometa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risidronate</td>
<td>Procter &amp; Gamble / Aventis</td>
<td>Bisphosphonate</td>
<td>Inhibit osteoclasts</td>
</tr>
<tr>
<td>(Actonel)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raloxifene</td>
<td>Eli Lilly</td>
<td>SERM</td>
<td>Inhibit osteoclast</td>
</tr>
<tr>
<td>(Evista)</td>
<td></td>
<td>development</td>
<td></td>
</tr>
<tr>
<td>Estrogen</td>
<td>(Several mfrs.)</td>
<td>Sex steroid</td>
<td>Inhibit osteoclast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>development</td>
<td></td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Novartis</td>
<td>Peptide hormone</td>
<td>Inhibit osteoclasts</td>
</tr>
<tr>
<td>Teriparatide</td>
<td>Eli Lilly</td>
<td>PTH fragment</td>
<td>Anabolic</td>
</tr>
<tr>
<td>(Forteo)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTHrP</td>
<td>Osteotrophin</td>
<td>PTH relative</td>
<td>Anabolic</td>
</tr>
<tr>
<td>AMG 162</td>
<td>AMGEN</td>
<td>RANK-L antibody</td>
<td>Inhibit osteoclast</td>
</tr>
<tr>
<td></td>
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<td>development</td>
<td></td>
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SPECIAL CONSIDERATIONS FOR THERAPEUTIC DRUG USE IN SPACE

The use of therapeutic drugs in space requires both the provider and the patient to accept a different set of standards, assumptions, and approvals compared with the use of the same drugs on Earth. For example, the primary criterion that the FDA uses for approval of drugs designed to treat osteoporosis is a demonstrated reduction of fracture risk, usually hip or vertebral fracture. Such evidence usually comes from a clinical trial of postmenopausal women with evidence of osteoporosis that is blinded, placebo controlled, and randomized. This approach may not be appropriate for decisions regarding drugs for use in long-duration space flight, since the astronaut corps comprises primarily younger men (see remarks above regarding the number of women astronauts/cosmonauts who have undergone long-duration space flight), and such individuals are likely to be in good bone health at the time of treatment. Such clinical trials typically take many years to accomplish (for example, the WHI study, mentioned above, was scheduled for 8.5 years), and the time frame could slow the identification and application of effective therapies.

Given current NASA priorities, it is almost certain that there will not be a sufficient number of astronauts to allow a placebo-controlled dose-ranging on-orbit trial with sufficient statistical power to be mounted in the next decade. It is, therefore, likely that the decision to use a therapeutic drug for astronauts will be based on evidence...
from a bed rest study supported by experience in a few individual volunteers who will take the drugs prior to and/or during space flight.

Among the questions that will need to be answered in these human trials are: (1) What is the bioavailability of the various drug therapies in 0-g? (2) Are the dose-response curves similar in 0-g to those established in 1-g? (3) What are the post-flight consequences for bone health of taking osteoprotective drugs? (4) If drugs need to be taken on-orbit, how should they be stored for maximum effectiveness? (5) How will a drug’s effectiveness be determined on-orbit so that doses can be modulated? (6) What is the best combination of drug and exercise countermeasures?

SUMMARY AND CONCLUSIONS

This review has defined the current status of exercise and therapeutic drug countermeasures for bone loss during long-duration space flight. The available data indicate that exercise countermeasures to date have not been effective and crew members continue to lose significant bone mass in the lower extremities and lumbar spine. Better-designed studies are needed to determine if the entire distributed daily dose of exercise that occurs in 1-g can be successfully replaced by short periods of high-intensity exercise on-orbit. Exercise dose on-orbit must also be quantified.

Drug therapeutics for bone have not yet been used in space, and, given the considerable experience using several classes of osteoprotective drugs on Earth (mostly in postmenopausal women with low bone mass), it seems wise to explore such interventions for use during space flight. However, the many differences between the 1-g clinical studies and the 0-g individual prescription must be carefully considered. Many new therapies can be expected in the future as investigators achieve a better understanding of the genetic regulation of bone mass, and genetic screening may offer a means of selecting crew members with a low susceptibility to bone loss.

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Prescription for Long-Duration Space Flight
Proceedings of the 1986 Workshop on Exercise


Watanabe, Y., Ohshima, H., Mizuno, K., Sekiguchi, C., Gravitational and Space Biology 18(2) June 2005 57


CONSEQUENCES OF CARDIOVASCULAR ADAPTATION TO SPACEFLIGHT: IMPLICATIONS FOR THE USE OF PHARMACOLOGICAL COUNTERMEASURES

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ABSTRACT

There is little evidence obtained from space flight to support the notion that occurrence of cardiac dysrhythmias, impaired cardiac and vascular function, and manifestation of asymptomatic cardiovascular disease represent serious risks during space flight. Therefore, the development of orthostatic hypotension and instability immediately after return from spaceflight probably reflect the most significant operational risks associated with the cardiovascular system of astronauts. Significant reductions in stroke volume and lower reserve for increasing peripheral vascular resistance contribute to ineffective maintenance of systemic arterial blood pressure during standing after spaceflight despite compensatory elevations in heart rate. The primary mechanism underlying reduced stroke volume appears to be a reduction in preload associated with less circulating blood volume while inadequate peripheral vasoconstriction may be caused partly by hyporeactivity of receptors that control arterial smooth muscle function. A focus for development of future countermeasures for hemodynamic responses to central hypovolemia includes the potential application of pharmacological agents that specifically target and restore blood volume (e.g., fludrocortisone, electrolyte-containing beverages) and reserve for vasoconstriction (e.g., midodrine, vasopressin). Based on systematic evaluations, acute physical exercise designed to elicit maximal effort or inspiratory resistance have shown promise as successful countermeasures that provide protection against development of orthostatic hypotension and intolerance without potential risks and side effects associated with specific pharmacological interventions.

Key words: blood volume; blood pressure; heart rate; stroke volume; cardiac output; peripheral vascular resistance

INTRODUCTION

The effects of extended exposure to microgravity environments on the cardiovascular system are well documented (Convertino, 2002). Although cardiovascular adaptations appear benign during a space mission, they have been manifested in reduced physiological or physical function upon return to Earth. As a result, a major focus of space-related research has been directed to the systematic development and evaluation of potential countermeasures. Among numerous treatments, specific pharmacological agents designed to enhance hemodynamic and autonomic functions have been considered or tested.

In 2000, the National Aeronautics and Space Administration (NASA) published their first draft of the Bioastronautics Critical Path Roadmap (BCPR) with the purpose of defining areas of biomedical research required for future long duration space flight. Specifically, the objective of the BCPR for human health and countermeasures was to focus on “understanding, characterizing, and counteracting the whole body’s adaptation to microgravity, enabling healthy astronauts to accomplish mission objectives and return to normal life following a mission”. The BCPR outlined specific critical risks of serious adverse health or performance consequences that would result from space flight. The priority for cardiovascular risks identified by the BCPR included 1) occurrence of serious cardiac dysrhythmias; 2) diminished cardiac function; 3) manifestation of previously asymptomatic cardiovascular disease; 4) impaired cardiovascular response to orthostatic stress; and 5) impaired cardiovascular response to exercise stress. In 2004, a revised version of the BCPR reduced the identified priority for cardiovascular risks to include only the occurrence of serious cardiac dysrhythmias and diminished cardiac and vascular function.

The purpose of this paper is to provide an assessment of proposed risk(s) to the cardiovascular system during space flight based on a critical review of data documented in the literature. An emphasis will be placed on the efficacy of specific pharmacological treatment of mechanisms associated with cardiovascular adaptations that lead to compromised operational performance of astronauts. An attempt will be made to provide perspectives on limitations and interpretations of these data in an effort to present future directions for development and/or implementation of effective pharmacological and non-pharmacological countermeasures for cardiovascular adaptation to space flight.

ASSESSMENT OF RISK TO THE CARDIOVASCULAR SYSTEM DURING SPACE FLIGHT

Occurrence of serious cardiac dysrhythmias. Despite numerous anecdotal reports, there is little evidence of a potential for occurrence of heart rhythm disturbances during space flight that may result in a serious cardiac event. For instance, no arrhythmias were reported in a group of healthy astronauts during long-duration space missions despite a prolongation in QT interval (D’Aunno
No increase in cardiac dysrhythmias were reported from electrocardiogram (ECG) tracings collected on astronauts while performing their routine tasks and extravehicular activities (EVA) during short- (<14 d) or long-duration space missions (>14 d) (Fritsch-Yelle et al., 1996a; Rossum et al., 1997; Goldberger et al., 1994). Although a single isolated episode of a non-sustained, asymptomatic 14-beat ventricular tachycardia (VT) episode was reported in an astronaut during the second month of a mission on the Russian MIR space station (Fritsch-Yelle et al., 1998), further analysis raised the possibility that this VT episode might represent a normal variant if the ST elevation existing in the ECG tracing was also seen in the astronaut’s previous resting tracing (Ellestad, 1998). Finally, “no pathology in the myocardial bioelectrical activity” was reported in 59 cosmonauts during MIR space missions of greater than 6-month duration (Golubchikova et al., 2003). Taken together, there is little evidence to suggest that the occurrence of serious cardiac dysrhythmias is a high risk to the health and well-being of astronauts during short- or long-duration space missions.

**Manifestation of previously asymptomatic cardiovascular disease.** The basis of the hypothesis that long-duration space flight may exacerbate previously undetected cardiovascular disease (e.g., coronary artery disease) is dependent upon the existence of evidence that supports one or both of two premises: 1) there have been cases within the astronaut community of undetected cardiovascular disease that existed before space flight; and/or 2) extended exposure to microgravity in some way aggravates pre-existing cardiovascular disease. Unfortunately, there are no published data to support the occurrence of either condition and, therefore, no evidence to suggest that conditions of space flight might cause a pre-existing cardiovascular disease to become symptomatic or accelerate the progression of the disease. Likewise, there is no published documentation to suggest that any astronauts have displayed the presence of asymptomatic cardiovascular disease prior to long duration missions. Therefore, in the currently selected astronaut population who undergo extensive medical screening prior to selection and mission in an effort to exclude the existence of clinical conditions, the risk of exacerbating a pre-existing asymptomatic cardiovascular disease appears to be very low.

**Diminished cardiac function.** It is clear from space flight experiments that stroke volume is significantly reduced upon return to earth (Buckey et al., 1996; Bungo et al., 1987; Convertino, 1990, 1995; Henry et al., 1977; Levine et al., 1996, 2002; Mulvaugh et al., 1991). Echocardiographic data demonstrated that the lower stroke volume was associated with reduced cardiac size (Bungo et al., 1987; Mulvaugh et al., 1991). Although a reduction in circulating plasma and blood volume that occurs with space flight is associated with less cardiac filling, data obtained from magnetic resonance imaging measurements obtained from 4 astronauts who participated in the 10-d D-2 NASA space mission revealed an average 14% reduction in left ventricular mass (Perhonen et al., 2001). These data were the first obtained from humans to offer evidence that there is a possibility for cardiac remodeling during space missions that might compromise myocardial function and contribute to lower stroke volume. In addition, there is evidence from ground simulation experiments that diminished cardiac compliance might reduce diastolic function and compromise cardiac filling (Levine et al., 1997). However, recent evidence generated from ground-based and flight experiments on animals suggests that smaller cardiac size simply may reflect the impact of negative caloric balance and reduction of body mass routinely observed in astronauts during space flight and results in a constant cardiac mass to body mass ratio (Ray et al., 2001). Regardless of any evidence for cardiac remodeling, measures of myocardial function curves before and after the 84-day U.S. Skylab mission (Henry et al., 1977), ejection fractions measured before and during the 237-day Russian Salyut-7 mission (Atkov et al., 1987), and arterial pulse wave velocities measured before and during the Russian 23-day Salyut-1 and 63-day Salyut-4 missions (Convertino, 1990) all suggest that there is little impact of long-duration exposure to microgravity on cardiac function. The space flight data probably reflect the effectiveness and importance of performing current intense exercise countermeasures in the maintenance of normal cardiac function. Therefore, the current evidence suggests that the risk of diminished cardiac function during or following space flight appears negligible in the presence of the current effective exercise space flight countermeasures.

**Diminished vascular function.** Hemodynamic responses during stand tests conducted on 14 astronauts following 9-14 days of space flight revealed that the distinguishing feature between astronauts who could (finishers) or could not (nonfinishers) complete 10 minutes of standing after these space missions was a significantly lower vasoconstrictor response in nonfinishers (Buckey et al., 1996). The relationship between low vasoconstrictive response and failure to complete stand tests has been corroborated in an additional 87 astronauts after space flight (Fritsch-Yelle et al., 1996b; Meck et al., 2004; Waters et al., 2002). These results obtained from astronauts following space missions have advanced the hypothesis that diminished vascular function may represent a significant cardiovascular risk of space flight. This hypothesis may be further supported by evidence of reduced vascular smooth muscle contraction with associated atrophic and morphological alterations generated from ground base animal models (Delp et al., 1993, 1999, 2000; Zhang et al., 1997; Zhang, 2001).

In contrast to attenuated vasoconstrictive responses reported in pre-syncopal astronauts, astronauts who display orthostatic stability after spaceflight exhibit elevated vascular resistance compared to preflight (Buckey et al., 1996; Fritsch-Yelle et al., 1996; Meck et al., 2004; Waters et al., 2002; Levine et al., 2002). These results have been corroborated in subjects exposed to
ground simulations of microgravity (Convertino et al., 1994; Kimaya et al., 2004). Therefore, there appears to be a discrepancy in vasoconstrictive response between non-presyncopal and presyncopal astronauts. A reduction in the vasoconstrictive reserve has been identified as a mechanism that contributes to orthostatic intolerance (Fu et al., 2004) and thus may provide an alternative explanation for a limitation in vascular function following adaptation to spaceflight. The vasoconstrictive reserve is defined as the difference between baseline and maximum vasoconstriction (Engelke et al., 1996). Both spaceflight and ground experiments have provided evidence that vasoconstriction during supine rest after exposure to microgravity is increased and associated with hypovolemia (Convertino, 1996, 1999; Convertino et al., 1994; Engelke et al., 1996; Gabrielsen et al., 1995). Increased peripheral vasoconstriction after return from space flight reflects a sympathetically-mediated reflex compensatory response to a reduction in stroke volume and cardiac output (Convertino et al., 2004a). A linear relationship between increased muscle sympathetic nerve activity and stroke volume was maintained between pre-and post-space flight tilt tests, suggesting a tight coupling (signaling) between stroke volume and sympathetic nerve activity (Levine et al., 2002). Since maximal vasoconstriction is finite, the elevated resting vasoconstriction associated with low circulating vascular volume and stroke volume represents a reduction in vasoconstrictive reserve and lowers the capacity to buffer orthostatic hypotension (Convertino, 1999; Engelke et al., 1996). It is therefore unclear based on the current evidence that any cardiovascular risk associated with space flight reflects a diminished vascular function or simply lower vasoconstrictive reserve secondary to hypovolemia.

Impaired cardiovascular autonomic functions. Adaptations of autonomically-mediated baroreflex mechanisms that control cardiac chronotropic responses and peripheral vascular resistance may contribute to inadequate blood pressure regulation after exposure to microgravity. Hypoadrenergic responsiveness has been hypothesized as a contributing mechanism to post-flight orthostatic intolerance as evidenced by a relationship between low blood norepinephrine and less vascular resistance in presyncopal astronauts (Fritsch-Yelle et al., 1996; Waters et al., 2002). Since sympathetic nerve activity, circulating norepinephrine and peripheral vascular resistance are all elevated in orthostatically-stable astronauts after space flight (Levine et al., 2002; Fritsch-Yelle et al., 1996; Waters et al., 2002), sympathetic withdrawal that occurs at the point of presyncope (Cooke & Convertino, 2003; Iwase et al., 2000) in addition to blood sampling in the supine posture of only the presyncopal astronauts (Fritsch-Yelle et al., 1996; Meck et al., 2004) may offer an explanation other than hypoadrenergic responsiveness for lower circulating norepinephrine reported in presyncopal astronauts.

Attenuation of cardiac vagal nerve traffic withdrawal induced by carotid baroreceptor stimulation was associated with presyncope during stand tests following exposure to simulated and actual microgravity (Convertino et al., 1990; Fritsch et al., 1992). Although heart rate increased with standing in syncopal subjects, their tachycardia was less than half that observed in the nonsyncopal subjects. These data provided the first evidence that attenuated carotid-cardiac baroreflex function may impair the capacity of tachycardic mechanisms to maximize elevations in heart rate, and subsequently cardiac output, during standing. Therefore, attenuation of baroreflex-mediated cardiac chronotropic responses induced by exposure to microgravity may represent a cardiovascular risk of limiting reflex compensatory tachycardic responses necessary to maintain adequate cardiac output.

Impaired cardiovascular response to orthostatic stress. Orthostatic hypotension and compromise following return from space flight has been well documented since the U.S. Gemini program (Hoffler, 1977) and presyncopal symptoms have been reported in 28% to 65% of mission specialists or scientists studied during stand or tilt test after returning from specific life science space missions (Buckey et al., 1996; Fritsch-Yelle et al., 1996; Meck et al., 2004; Waters et al., 2002). Impaired orthostatic performance in astronauts following their return from space flight has been associated with lower circulating blood volume, decreased stroke volume and cardiac output, and limited capacity to elevate peripheral vascular resistance (Buckey et al., 1996; Convertino, 1996; Fritsch-Yelle et al., 1996; Meck et al., 2004; Waters et al., 2002). It is clear that the inability of an astronaut to stand and perform an emergency egress from a spacecraft after landing could result in a life-threatening event. Thus, impaired cardiovascular response to standing after return from space may represent one of the highest risks to the safety, well-being, and performance of astronauts.

Impaired cardiovascular response to exercise stress. Numerous experiments using human subjects exposed to ground simulations of microgravity have demonstrated significant reduction in aerobic capacity (Convertino, 1995). More recently, a 22% reduction in aerobic capacity was demonstrated in 6 astronauts following only 9 or 14 days of space flight and was associated with reduced stroke volume (Levine et al., 1996). It is also clear that the reduced stroke volume during physical work in space is affected directly by lower cardiac filling, i.e., end-diastolic volume (Atkov et al., 1987). The relative reduction in maximal oxygen uptake following cardiovascular adaptation to ground simulations of microgravity is correlated highly with the relative magnitude of reduced circulating blood volume (Convertino, 1995), suggesting a close coupling between blood volume and cardiac filling. However, there is no evidence in the literature to suggest that a loss of 20% to 25% of aerobic capacity has impaired operational performance during or after space flight.

Summary of cardiovascular risks associated with space flight. There is little evidence obtained from space flight...
to indicate that occurrence of cardiac dysrhythmias, impaired cardiac function, and manifestation of asymptomatic cardiovascular disease represent serious risks during space flight. Data from the literature provide the most convincing argument that impaired cardiovascular responses to orthostatic and exercise stresses represent the primary operational risks to astronaut health, safety and performance following space flight. Figure 1 illustrates the changes and interactions of mechanisms underlying the effect of cardiovascular adaptation to microgravity on orthostatic and exercise performance. It is clear from Figure 1 that the development of countermeasures should focus on restoring central blood volume, stroke volume and reserve for increasing peripheral vascular resistance.

**Figure 1.** Diagram outlining adaptations in underlying mechanisms of cardiovascular functions and their impact on operational functions in astronauts that result from exposure to microgravity (spaceflight).

### PHARMACOLOGICAL COUNTERMEASURES FOR CARDIOVASCULAR ADAPTATION TO SPACE FLIGHT

Extensive experiments conducted in both space flight and ground simulations provide a compelling argument that the most effective pharmacological countermeasures for protection of orthostatic and physical work performance should target plasma and/or blood expansion, autonomic dysfunction, and/or impaired vascular reactivity. Current clinical practices include the use of agents such as fludrocortisone or electrolyte containing beverages that expand circulating blood volume (Benditt et al., 1999; Raviele et al., 1996; Robertson & Davis, 1995); beta-adrenergic blockers such as propranolol, metoprolol, atenolol, nadolol, and esmolol in an effort to diminish the degree of cardiac mechanoreceptor activation or oppose peripheral vasodilatory effects of epinephrine (Benditt et al., 1999; Raviele et al., 1996); disopyramide in an effort to avoid vasovagal responses by counteracting parasympathetic activity (Benditt et al., 1999; Raviele et al., 1996); serotonin reuptake blockers such as fluoxetine hydrochloride and verlafaxine hydrochloride in an effort to reduce the effects of serotonin-mediated vasodepressor effects (Benditt et al., 1999); alpha-adrenergic agonists such as ephedrine, etilephrine or midodrine in an effort to increase venous tone and venous return as well as elevating peripheral vascular resistance by inducing arteriolar constriction (Benditt et al., 1999; Raviele et al., 1996; Robertson & Davis, 1995). Based on operational efficacy, the discussion of pharmacological agents used as potential countermeasures against deleterious effects of cardiovascular adaptation(s) to space flight will focus on specific experimentation and testing of blood volume expanders and vasoconstrictors.

**Pharmacological expansion of circulating blood volume.** Microgravity-induced hypovolemia contributes to orthostatic compromise after space flight. To counter this effect, U.S. astronauts currently adhere to a regimen of consuming a maximum of eight 1-g salt tablets with approximately 912 ml of fluid designed to make an isotonic saline drink approximately 2 h prior to reentry in an effort to restore blood volume (Bungo et al., 1985). Although a reduced orthostatic tachycardia following short duration space missions was encouraging during the initial use of saline loading (Bungo et al., 1985), exposure to microgravity for longer than 7 days failed to ameliorate orthostatic compromise in astronauts (Vernikos & Convertino, 1994; White et al., 1991). Despite the continued use of saline loading by astronauts in the U.S. space program, there is little evidence to suggest that taken alone it is effective against the development of post-flight orthostatic intolerance (Buckey et al., 1996).

The use of the mineralcorticoid fludrocortisone has been used clinically for nearly 40 years with some success to treat orthostatic hypotension, particularly in patients with an etiology linked to hypovolemia (Benditt et al., 1999; Robertson & Davis, 1995). Fludrocortisone acts to enhance sodium and fluid retention and has been reported to sensitize alpha-adrenergic receptors (Benditt et al., 1999). However, fludrocortisone appears to be most effective when consumed over days to weeks rather than on the day it is first administered (Robertson & Davis, 1995). Consequently, in a preliminary investigation, Vernikos and co-workers (1991) were the first to report that the administration of fludrocortisone with three doses over the final 24 hours of exposure to 7 days of simulated microgravity restored plasma volume in all subjects and protected orthostatic tolerance in 4 of 7 subjects who had previously become syncopal after head-down bed rest. In a subsequent investigation (Vernikos & Convertino, 1994), a more rigorously controlled experiment was conducted to compare the effectiveness of the current astronaut saline loading regimen to fludrocortisone as countermeasures for reduced plasma volume and orthostatic intolerance after spaceflight. Eleven healthy male subjects underwent a 3-day ambulatory baseline period followed by exposure to 7 days of 6° head-down bed rest. Treatments consisted of two volume expansion groups. One group (5 subjects) consumed 8 salt tablets (1 g NaCl per tablet) and 960 ml of water 2 hours prior to ambulation. The second group (6 subjects) consumed 0.2 mg oral dose of fludrocortisone at 0800 and 2000 h the day before and 0800 h the day the subjects got out of bed (2 hours before standing). After treatments, all subjects
attempted a 15-min unsupported stand test. Plasma volume decreased by 12% on day 7 of bed rest, and was restored by fludrocortisone but not by saline load (Fig. 2). Despite similar elevation in heart rate between the two groups, the group treated with saline loading experienced significant orthostatic hypotension compared to the fludrocortisone group (Fig. 3). Protection of arterial blood pressure during standing with fludrocortisone treatment was associated with restored vasoconstriction reserve and cardiac baroreflex function. Only 1 of 6 subjects showed syncopal symptoms in the fludrocortisone-treated group, whereas 4 of 5 subjects did so in the saline-load group. Acute fludrocortisone treatment appeared to have distinct advantages as a protective measure for orthostatic intolerance after exposure to ground simulation of microgravity.

![Figure 2. Plasma volume before and at the end of head-down bed rest, and after treatment with saline (open circles and broken lines) and fludrocortisone (closed circle and solid line). Symbols represent mean (± SE). Asterisk indicates P < 0.05 compared to pre-bed rest baseline level. Data are modified from Vernikos and Convertino [1994].](image)

![Figure 3. Responses of heart rate (top panels) and mean arterial pressure (bottom panels) in subjects during supine baseline and at the end of 15 min of standing before (left panels) and after (right panels) bed rest after treatments with saline (closed circles and solid lines) and fludrocortisone (closed circles and broken lines). Symbols represent mean (± SE). Asterisk indicates P < 0.05 compared to saline treatment. Data are modified from Vernikos and Convertino [1994].](image)

Subsequently, the fludrocortisone countermeasure was tested on 7 male astronauts whose orthostatic responses were compared to 18 astronauts who received a placebo (Shi et al., 2004). Astronauts took either 0.3 mg fludrocortisone or placebo orally 7 hours prior to landing. Treatment with this single dose of fludrocortisone resulted in some protection of plasma volume but no protection of orthostatic tolerance. In the transition to spaceflight operational implementation, an effective dose of 0.2 mg taken 3 times during 24 hours prior to standing in the ground experiment was altered to a single dose of 0.3 mg taken 7 hours prior to landing in conjunction with the operational saline fluid loading countermeasure that was taken approximately 5 to 6 hours before landing. The difference in results of fludrocortisone application between ground and space may simply reflect significant alterations in the operational transition from the ground experiment to spaceflight testing. In the end, the use of
intolerance following spaceflight awaits the results of a potential countermeasure for prevention of orthostatic postflight orthostatic intolerance. The use of midodrine as compared to the astronaut’s post-spaceflight response similar to the preflight response and dramatically lower posture after space flight with midodrine treatment was restoration of vascular resistance in the presence of higher stroke volume and cardiac output (Fig. 5). Despite the effect of midodrine on arterial vasoconstriction, the elevation of peripheral vascular resistance during upright posture after space flight with midodrine treatment was similar to the preflight response and dramatically lower compared to the astronaut’s post-spaceflight response following her initial mission when she experienced hypotension and presyncope (Fig. 5, lower right panel). Rather than arterial vasoconstriction, the lower peripheral vascular resistance in the presence of higher stroke volume suggests that the primary effect of midodrine after space flight in this astronaut was restoration of vasoconstrictive reserve by the improvement of central blood volume and cardiac filling (i.e., enhanced venoconstriction and venous return). These results may underscore the importance of central blood volume rather than autonomic dysfunction(s) as a primary mechanism of postflight orthostatic intolerance. The use of midodrine as a potential countermeasure for prevention of orthostatic intolerance following spaceflight awaits the results of continued successful implementation to future spaceflight missions.

Use of adrenergic-receptor agents. The association of impaired peripheral vascular constriction with development of post-spaceflight orthostatic hypotension and syncope motivated consideration for the use of pharmacological agents that target the response of vascular adrenoreceptors. The administration of the non-specific β-adrenoreceptor antagonist propranolol has been proposed as a countermeasure targeted at increasing peripheral vascular resistance by inhibition of vasodilatory effects of circulating epinephrine on vascular smooth muscle (Sandler et al., 1985). However, this approach was abandoned when benefits of peripheral vasoconstriction were overridden by inhibitory chronotropic and inotropic effects that led to reduced orthostatic tolerance in ground experiments.

Most recently, the α1-agonist drug midodrine was administered to 6 subjects one hour before a tilt stand test after they had completed exposure to 16 days of 5° head-down tilt (Ramsdell et al., 2001). Midodrine stimulates both arterial and venous constriction. Compared to the responses of 4 control subjects who received a placebo, midodrine significantly ameliorated development of hypotension and presyncope during the tilt test. Subsequently, this countermeasure was tested on a female astronaut who had become hypotensive and presyncopal during a stand test following her first 9-day space mission (Platts et al., 2004). After a second 11-day space flight, this astronaut received a single 10-mg dose of midodrine administered orally 1 hour prior to a tilt test. Her hemodynamic responses to the post-spaceflight orthostatic tests were compared. Compared to the supine posture, midodrine treatment was associated with stable systolic, diastolic and pulse pressures in contrast to dramatic reductions in these pressure in the absence of midodrine (Fig. 4). Stabilization of upright blood pressure with midodrine was associated with attenuated reductions in stroke volume and cardiac output (Fig. 5). Despite the effect of midodrine on arterial vasoconstriction, the elevation of peripheral vascular resistance during upright posture after space flight with midodrine treatment was similar to the preflight response and dramatically lower compared to the astronaut’s post-spaceflight response following her initial mission when she experienced hypotension and presyncope (Fig. 5, lower right panel). Rather than arterial vasoconstriction, the lower peripheral vascular resistance in the presence of higher stroke volume suggests that the primary effect of midodrine after space flight in this astronaut was restoration of vasoconstrictive reserve by the improvement of central blood volume and cardiac filling (i.e., enhanced venoconstriction and venous return). These results may underscore the importance of central blood volume rather than autonomic dysfunction(s) as a primary mechanism of postflight orthostatic intolerance. The use of midodrine as a potential countermeasure for prevention of orthostatic intolerance following spaceflight awaits the results of

Potential limitations and side effects of pharmacological intervention. The primary concern for using pharmacological intervention for space flight countermeasures is timing of drug administration and side effects. In the operational space flight environment, the time that the drug is administered is critical to its effectiveness. For instance, most of the blood pressure raising effect of fludrocortisone results from sodium retention that develops over several days, with the full pressor action being observed in 1 to 2 weeks (Robertson & Davis, 1995). When attempts were made to administer fludrocortisone to astronauts daily over the final 3 to 5 days of the mission, crewmembers complained of painful pressure behind the eyes and discontinued use of the drug [Shi et al., 2004]. This should not be unexpected since headaches in addition to hypokalemia are a common side effect of fludrocortisone (Robertson & Davis, 1995). When applied to astronauts only on the final day of flight, there was no effect on orthostatic responses. Thus, the combination of administration schedule and side effects have rendered fludrocortisone an ineffective countermeasure.

Since plasma concentrations of the most potent metabolite of midodrine peak at 1 hour (Robertson & Davis, 1995), the drug would be most effective operationally if taken before re-entry. With an α1-adrenoreceptor agonist action, the primary side effect of midodrine is hypertension, particularly in the supine (non-orthostatic) posture. It is therefore likely that the administration of midodrine before re-entry would result in high blood pressure while in orbit.
Figure 5. Heart rate, stroke volume, cardiac output and peripheral vascular resistance responses from supine to upright postures preflight (open triangles and dotted line), postflight without midodrine (open circles and broken line) and postflight with midodrine (solid circles and solid lines). Data are modified from Platts et al. [2004].

NON-PHARMACOLOGICAL COUNTERMEASURES FOR CARDIOVASCULAR ADAPTATION TO SPACE FLIGHT

Use of a single bout of maximal exercise. The use of physical exercise as a potential countermeasure against post-space flight orthostatic intolerance has been long considered because of the recognized effect of physical activity on circulating blood volume and baroreflex functions. More than a decade ago, specific attention was given to a single exposure of graded exercise designed to elicit maximal effort performed within 24 hours before re-entry from a space mission. In addition to the potential benefits of protecting aerobic capacity (Convertino, 1987), orthostatic tolerance was restored following 16 days exposure to a ground simulation of microgravity when tested 24 hours after a maximal exercise countermeasure was applied (Engelke et al., 1996). Improved physiological functions affected within 24 hours by acute maximal exercise and associated with blood pressure regulation included restoration of blood volume (Convertino et al., 1996), vasoconstrictive reserve (Engelke et al., 1996), and cardiac baroreflex sensitivity (Engelke et al., 1996). Subsequent to ground experiments, a single bout of maximal cycle ergometer exercise was performed by astronauts within 18 to 24 hours prior to landing during space flight missions to test this countermeasure as a possible treatment for post-space flight orthostatic hypotension and intolerance (Moore et al., 2001). Echocardiographic measurements made on the astronauts involved in the testing of the maximal exercise countermeasure demonstrated that stroke volume and cardiac output were restored to pre-flight levels in the exercise group during post-space flight standing, but fell in the control group in a similar fashion as that reported in the ground investigation (Convertino, 2002). Therefore, this exercise regime was successful in targeting the primary mechanisms associated with post-space flight orthostatic intolerance. It is also operationally attractive because it could be performed within 24 hours before the end of a mission and required minimal time of the astronauts (less than 20 minutes only once).

Use of an impedance threshold device (ITD). Recent investigations have focused on the application of a simple concept that central blood volume may be increased acutely by transforming the thorax into a more active vacuum and drawing venous blood from extrathoracic cavities into the heart and lungs. Building on this concept, an inspiratory impedance threshold device (ITD) designed to elevate intrathoracic negative pressure, i.e., create a vacuum, within the chest each time the chest expands...
during the inspiratory phase of breathing has been described (Convertino et al., 2005). With the use of an ITD, we have demonstrated in several human experiments that inspiratory resistance can: (a) reset cardiac baroreflex function to a higher operating range for blood pressure (Convertino et al., 2004b); (b) increase stroke volume, cardiac output and arterial blood pressure in normovolemic (Convertino et al., 2004c) and orthostatic (Convertino et al., 2005) subjects; (c) reduce peripheral vascular resistance (i.e., increase vasoconstrictive reserve) (Convertino et al., 2004c); (d) increase cerebral blood flow (Convertino et al., 2005); and (e) reduce orthostatic symptoms (Convertino et al., 2005). Again, the ITD represents a successful approach to targeting the primary mechanisms associated with post-space flight orthostatic intolerance. It is also operationally attractive because it requires little training and easily could be used by an astronaut to avoid cardiovascular collapse associated with post-flight orthostatic instability. Currently the ITD is under consideration for placement in the flight medical bag and on the International Space Station.

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Table 1. Qualitative summary of the effects of microgravity and five pharmacological and non-pharmaceutical countermeasures on cardiovascular functions associated with risk to astronaut health, safety and performance after a space mission.

SUMMARY

Impaired cardiovascular responses during standing and performing physical work represent operational risks to astronaut health, safety and performance following space flight. Cardiovascular functions associated with these operational risks include attenuated autonomic baroreflex functions, lower central blood volume, stroke volume and cardiac output, and vasoconstrictive reserve (Table 1). Plasma volume expanders and vasoconstrictors represent the primary pharmacological treatments that have been tested for management of post-flight orthostatic intolerance. Table 1 summarizes the effects of three pharmacological countermeasures in relation to cardiovascular alterations induced by adaptation to microgravity. Saline loading has had little success in reversing adverse cardiovascular adaptations. Fludrocortisone showed promise in ground experiments, but alterations in dose implementation for operational use in space missions showed little positive effects. Midodrine was successful in improving orthostatic tolerance in ground experiments and in one application to space flight. Although pharmacological intervention offers an alternative to treatment of post-flight orthostatic intolerance, there is compelling evidence that intense physical exercise or application of mechanical devices can provide similar physiological effects on acute expansion of baroreflex function(s), central blood volume and vasoconstrictive reserve (Table 1). Since the possibility of side effects or interaction with other drugs exists, the use of pharmacological agents as countermeasures to cardiovascular dysfunctions following space flight should be considered only after the application of more physiologically natural techniques are exhausted.

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REFERENCES


DIET AS A FACTOR IN BEHAVIORAL RADIATION PROTECTION FOLLOWING EXPOSURE TO HEAVY PARTICLES
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ABSTRACT

Major risks associated with radiation exposures on deep space missions include carcinogenesis due to heavy-particle exposure of cancer-prone tissues and performance decrements due to neurological damage produced by heavy particles. Because exposure to heavy particles can cause oxidative stress, it is possible that antioxidants can be used to mitigate these risks (and possibly some health risks of microgravity). To assess the capacity of antioxidant diets to mitigate the effects of exposure to heavy particles, rats were maintained on antioxidant diets containing 2% blueberry or strawberry extract or a control diet for 8 weeks prior to exposure to 1.5 or 2.0 Gy of accelerated iron particles at Brookhaven National Laboratory. Following irradiation rats were tested on a series of behavioral tasks: amphetamine-induced taste aversion learning, operant responding and spatial learning and memory. The results indicated that the performance of the irradiated rats maintained on the antioxidant diets was, in general, significantly better than that of the control animals, although the effectiveness of the diets ameliorating the radiation-induced deterioration in performance varied as a function of both the specific diet and the specific endpoint. In addition, animals fed antioxidant diets prior to exposure showed reduced heavy particle-induced tumorigenesis one year after exposure compared to the animals fed the control diet. These results suggest that antioxidant diets have the potential to serve as part of a system designed to provide protection to astronauts against the effects of heavy particles on exploratory missions outside the magnetic field of the earth.

INTRODUCTION TO SPACE RADIATION RISKS AND THEIR MANAGEMENT

The field of space radiation health has recently been singled out as one of two major initiatives within the research programs of the U. S. National Aeronautics and Space Administration (NASA, 2002). The goals of space radiation health research are to understand qualitatively and quantitatively the ionizing radiations present in the space environment, identify qualitatively and quantitatively the risks associated with these radiations, and discover countermeasures to mitigate these risks (Tobias and Todd, 1974). This article first introduces the basic science and definitions of quantities underlying radiation health research and protection then summarizes recent research related to a specific countermeasure (dietary antioxidants) to a specific risk (neurological performance decrement).

Many things come in threes. Omniae homiliae in tres partes divisae sunt.

In this section, briefly, we introduce
- Three kinds of space radiation
- Three space radiation risks
- Three forms of radiation risk management
- Three forms of biological countermeasures

THREE KINDS OF SPACE RADIATION

Space radiations consist of (1) energetic protons from the sun, (2) protons and electrons from the sun that are trapped in the earth’s magnetic field, and (3) cosmic rays that include energetic nuclei of H, He, C, N, O and Fe atoms.

Solar Particles. When the sun is very active, such as just before and just after sunspot maxima (every 11 years) magnetohydrodynamic effects allow the escape of intense clouds of energetic protons that can deliver doses of 0.3 to 3.0 Gy over a period of about 3 days (Townsend et al., 1991; Parsons et al., 1999). Otherwise the sun is constantly releasing lower energy protons that pass from the solar magnetosphere to Earth’s magnetosphere.

Trapped Radiations. Energetic electrons and protons arriving at Earth from the sun are trapped in Earth’s magnetic flux lines, where they spiral back and forth between the north and south magnetic poles, in which case they are said to comprise “trapped radiation belts”.

Galactic Cosmic Rays. The presumed celestial origin of the high-energy, high-charge particles causes them to be called “galactic cosmic rays”, or GCR. They are also called “HZE” particles owing to their high charge and energy. A few such particles pass every cm² every few seconds above the Earth’s atmosphere, but these are attenuated by the Earth’s magnetic field and atmosphere and never reach sea level. All of these three categories of radiations produce secondary radiations such as neutrons and gamma rays when they interact with matter. This fact is relevant to problems of shielding spacecraft and their contents; not only is shielding heavy, it could generate more dangerous radiation.
THREE SPACE RADIATION RISKS

The risks presented to space travelers by these radiations include (1) cancer due to chronic proton and cosmic-ray exposure, (2) immune and/or hematopoietic failure due to high-dose solar proton storms, and (3) possible neurological effects caused by single tracks of cosmic-ray heavy nuclei. Other well-known effects of heavy ions, such as cataracts and retinal flashes, are not considered mission- or life-threatening. Until more is known about the biological effects of chronic heavy-ion exposure it remains to be determined which of these is the most serious biological risk or whether these three risks need to be considered in parallel, independently.

Cancer and cellular effects due to heavy-ion exposure. It has been noted that (Curtis and Letaw, 1989; Setlow, 1999) on a round trip to Mars, the nuclei of about half of all of a crew member's cells will have been traversed by at least one energetic multiply charged cosmic-ray particle unless extraordinary shielding measures are implemented. The irradiation of cells in vitro with heavy ions has for several decades served as a model for studying the potential effects of cosmic-ray particles on cells. Most of our earliest understanding of high LET particle radiobiology was derived from studies of cell killing, carcinogenesis and mutagenesis in vitro. It has been amply demonstrated that surviving cells traversed by heavy particles are transformed to malignancy (Yang, 1985; Kronenberg, 1994) or mutated (Evans et al., 2001). Modern molecular methods of studying mutagenesis and in vitro and in vivo carcinogenesis are being applied to this problem with the discovery of interesting, previously unappreciated phenomena such as genome instability and "bystander effects" on non-hit cells (Deshpande et al., 1996). Delayed effects on the progeny of irradiated cells, such as mutation, carcinogenesis and impaired growth rate are not expected to be reflected in human responses during a deep space mission; these processes carry post-flight risks to individual health and constitute the most significant risk only if the other two risks are less important or less probable.

Immune/Hematopoietic System Failure due to High-Dose Proton Storms. The most efficient action of ionizing radiation is the killing of cells. A hit cell is typically 10,000 times as likely to undergo reproductive death as it is to be transformed to malignancy or to express an assayable mutation. The probability of cell death per unit dose increases with LET up to a maximum (Barendsen et al., 1960; Todd, 1967). Granulopoietic and lymphopoietic cells in the bone marrow are exquisitely sensitive to ionizing radiation, and the reduction of their progeny in the circulation to about 2% of normal cell counts is considered life threatening (Bond et al., 1965). In this context, a proton dose as low as 2.0 Gy could be life threatening, and, considering the compromising effects of space flight, including low gravity, on the immune system (Konstantinova, 1991), even lower doses should be considered dangerous (Todd et al., 1999).

Neurological Effects of Heavy Ions. The earliest biological studies with cyclotron beams targeted neural tissue damage and the accompanying physiological effects (Malis et al., 1957). The general thought was that heavy ions, due to the geometry of their energy loss, were capable of directly killing non-dividing neurons and vital glial cells whereas only very large doses of x or gamma rays had this capability (Zeman et al., 1961). A wide variety of morphological and physiological changes in neural systems have been reported following heavy-ion irradiation, usually at rather high doses, typically exceeding 1 Gy (Joseph et al., 1998). Recalling that about half of all cells are hit by at least one cosmic ray in a 2-3-year deep-space mission although the dose is only a few cGy, it is still necessary to ask if the neurological functioning of crew members will be impaired to such an extent as to jeopardize a mission and/or the lives of the crew members. Critical switchyards in the central nervous system, sparsely populated with neurons (hippocampus, corpus callosum, etc.) are particularly vulnerable points for the induction of behavior decrements due to the destruction of a small number of cells. Laboratory animal studies are now underway to determine, via quantitative neurochemistry and behavior analysis, the nature and level of effects of nervous function (Rabin et al., 2000), since functional effects constitute end-points of relevance to mission performance while morphological effects may or may not relate to critical functions. This risk is the subject of this article.

THREE FORMS OF RADIATION RISK MANAGEMENT

With three categories of radiation and three categories of biological response radiation health in space is more complicated than that on Earth; therefore, one of the goals of space radiation health research is the reduction of uncertainties. However, there are predictable (trapped protons, galactic cosmic rays) and stochastic (solar event protons) radiation sources. Likewise the biological responses include stochastic (carcinogenesis, neurological effects) and those with predictable dose-response curves. These combine to create risk management dilemmas (Todd, 2003).

Potential countermeasures have been classified into three categories (Cucinotta et al., 2001). These are (1) operations (which establish flight schedules and orbital strategies), (2) shielding (which increases spacecraft up-mass), and (3) biological, such as medication consisting of radical scavengers (that must be taken immediately before exposure), anti-oxidant consumption (which must be maintained continuously), cytokines (which may ameliorate immune and hematological effects specifically after exposure), and cell transplants (which should be isologous).

Operations. It is unwise to be in the wrong place at the wrong time if this can be avoided. Scheduling deep-space missions to miss periods of solar proton storm activity
and choosing trajectories and spacecraft orientations are examples of risk management by operations.

**Shielding.** In the case of shielding, several approaches have been considered, some very creative. In addition to the skin of a spacecraft the normal contents of space vehicles (water tanks, waste containers, avionics instruments) also constitute shielding. Active shielding, such as with a plasma or strong magnetic fields, has been entertained for several years.

**Biological Countermeasures.** In the case of biological countermeasures, all of the alternatives mentioned below are under exploration.

**THREE FORMS OF BIOLOGICAL COUNTERMEASURES**

Biological countermeasures against near-term and late effects of ionizing radiations consist of (1) radical scavengers, (2) cytokine treatment and (3) pharmacological and nutritional countermeasures against reactive oxygen species (ROS). At least 2 of these categories might also mitigate microgravity effects.

**Radical scavengers.** These consist of compounds, typically alcohols and sulfhydryls that function at the moment of irradiation to chemically react with free radical species produced in the radiation’s path. Most radical scavengers have been tested in vitro, and most of them have been found toxic in vivo.

**Cytokine treatment.** The radiosensitive leukopoietic and hematopoietic systems are known to be responsive to interleukins and hematopoietic factors. These are relevant in managing both space-flight stress (low gravity) and radiation damage (cell killing and carcinogenesis)

**Pharmacological and Nutritional countermeasures against persistent ROS.** Research over the past several years has been conducted to discover biological countermeasures to the above three risks: carcinogenesis by HZE particles, immune system effects due to high doses plus life in low gravity, and subtle neurological and behavioral effects that might jeopardize a deep-space mission. The general findings to date indicate that dietary antioxidants can constitute a line of defense against all of these radiation risks.

**NEUROBEHAVIORAL EFFECTS OF EXPOSURE TO HZE PARTICLES**

Exposing rats to HZE particles can affect performance on a variety of neurobiological and behavioral endpoints. Following exposure to $^{56}\text{Fe}$ particles rats show a reduction in potassium-stimulated dopamine release and in the behaviors that are dependent upon the integrity of the dopaminergic system (Joseph et al., 1992).

Deficits have been observed in both motor and cognitive behaviors. The effect of exposure on motor behavior is shown as a decrease in upper body strength, measured by the length of time rats can maintain their grip on a wire suspended above the ground (Joseph et al., 1992). Similarly, exposure to low doses of HZE particles will prevent the acquisition of a conditioned taste aversion (CTA) produced by the dopamine agonist amphetamine (Rabin et al., 1998). A CTA is produced by pairing a novel taste solution (10% sucrose) with an unconditioned stimulus (amphetamine). As a result of this pairing the rat will avoid ingestion of the solution at a subsequent presentation. Because amphetamine is a dopamine agonist, the development of an amphetamine-induced CTA requires an intact dopamine system. In this regard, the effects of exposure to $^{56}\text{Fe}$ particles are similar to those produced by the dopamine antagonist haloperidol (Rabin et al., 1998) in that both treatments disrupt dopaminergic function and interfere with the acquisition of an amphetamine-induced CTA.

Exposure to HZE particles can also affect cognitive performance (Shukitt-Hale et al., 2000). The Morris water maze is a standard test of cognitive ability in which rats are required to use spatial cues to locate a platform placed just below the surface of the water. There are no differences in performance between the non-irradiated controls and the irradiated rats in the initial acquisition of the task. However, when the platform is moved to a different location in the maze the irradiated rats show significantly poorer performance than the control rats. Similarly, when the platform is absent during probe trials, the irradiated rats spend significantly less time in the quadrant in which the platform had been located than do the non-irradiated control rats. These results indicate that the irradiated rats are deficient in their ability to perform a task requiring the use of spatial cues.

A second cognitive behavior which is affected by exposure to HZE particles is operant conditioning (Rabin et al., 2002), in which the organism learns to make a response in order to obtain reward or avoid punishment. Operant conditioning is broadly construed to include all forms of complex learning. The specific task that was utilized was responding on a fixed-ratio (FR) reinforcement schedule. On an FR schedule, a rat is required to make a fixed number of responses (level presses) in order to secure a reinforcement (45 mg food pellet). On an FR-1 schedule, the rat is rewarded for every lever press, while on an FR-35 reinforcement schedule the rat is required to make 35 responses in order to be rewarded with a single food pellet. When tested 3 months following exposure to $^{56}\text{Fe}$ particles, only the rats exposed to 2.0 Gy (but not 1.0 or 1.5 Gy) showed a disruption of responding at schedules of reinforcement of FR-20 or greater. There were no effects of irradiation at schedules less that FR-15. When tested 8 months later (11 months post-irradiation) all irradiated groups showed significantly decreased performance compared to the non-irradiated controls.
RELATIONSHIP TO AGING AND OXIDATIVE STRESS

The neurochemical and behavioral deficits detailed above are also observed in old rats. As a result, it has been proposed that exposing rats to HZE particles produces “accelerated aging” (Joseph et al., 1992). Specifically, old rats show deficits in potassium-stimulated dopamine release and related deficits in motor behavior (Joseph et al., 1978). Similarly there are significant decreases in the performance of old rats on the Morris water maze, indicating decreased ability to utilize spatial cues in a learning task (Shukitt-Hale et al., 1999). In addition, the partial loss of dopaminergic neurons produced by treatment with the neurotoxin 6-hydroxydopamine which does not affect the performance of young rats on an ascending fixed-ratio schedule does cause a significant impairment in the performance of older rats (Lindner et al., 1999).

Previous research has shown that maintaining rats on antioxidant diets containing blueberry or strawberry extract can ameliorate the neurochemical and behavioral changes that are characteristic of the aging process. This observation is consistent with current theories which suggest that oxidative stress and the production of reactive oxygen species (ROS) are key factors in the aging process (Finkel and Holbrook, 2000).

Oxidative stress occurs when endogenous and exogenous sources of ROS exceed the capacity of the antioxidant systems to remove them. In addition to the production of ROS by endogenous sources, such as the aerobic metabolism of mitochondria and the destruction of dopamine by monoamine oxidase, oxidative stress can also be produced by exogenous sources such as exposure to ionizing radiation, including exposure to HZE particles (Denisova et al., 2002). Acting to mitigate the effects of oxidative stress are a variety of endogenous antioxidant defense systems (superoxide dismutase and glutathione peroxidase) and exogenous sources of antioxidants (vitamins and flavonoid antioxidants). Where the production of ROS exceeds the antioxidant capacity of these systems, the consequences of oxidative stress include aging (Finkel and Holbrook, 2000), carcinogenesis (Oberly, 2002) and a variety of neurodegenerative disorders such as Parkinson’s and Alzheimer’s diseases (Halliwell, 2001).

One treatment that has been effective in ameliorating the neurobehavioral effects of aging has been the use of dietary antioxidants, such as are found in fruits and berries. Measured as Oxygen Radical Absorbance Capacity, the free radical scavenging capacity of blueberries and strawberries is much higher than that of vitamin E (Prior et al., 1998; Wang et al., 1996). Research using old animals has shown that maintaining rats on diets containing 2% blueberry or strawberry extract prevents the age-related changes in potassium-stimulated dopamine release and in the behaviors that depend upon the integrity of the dopaminergic system (Bickford et al., 2000; Joseph et al., 1998, 1999).

DIETARY COUNTERMEASURES AGAINST SPACE RADIATION RISKS

To the extent that oxidative stress mediates the neurobehavioral effects of exposure to HZE particles, then antioxidant treatments may function to ameliorate the effects of irradiation. Given the similarity in the neurobehavioral effects of aging and irradiation, it is possible that maintaining rats on diets containing flavonoid antioxidants may also counteract the effects of exposure to HZE particles.

Blueberries and strawberries contain a variety of compounds which may function as antioxidants. The polyphenolics contained in fruits include the hydroxycinnamates and the flavonoids such as the anthocyanins and flavonols. As indicated by HPLC analysis (Joseph, unpublished), the relative amounts of these compounds in different fruits vary, which may account for differences in the antioxidant capacity. Additional work will be needed in order to determine the active compounds and their effects on specific neurobehavioral endpoints. Nonetheless, as summarized below, maintaining rats on diets containing either blueberry or strawberry extract can ameliorate the effects of exposure to HZE particles on specific neurochemical and behavioral endpoints.

When rats are maintained on diets containing 2% blueberry or strawberry extract for two months prior to exposure to $^{56}$Fe particles (1.5 Gy, 1 GeV/n), the radiation-induced decrease in potassium-stimulated dopamine release in the striatum is prevented (Joseph et al., unpublished). These results are similar to those obtained with aged rats maintained on identical diets (Joseph et al., 1998, 1999).

Concordant with the neurochemical effects, antioxidant diets also ameliorate the cognitive/behavioral deficits produced by exposure to HZE particles, although the effectiveness of the blueberry and strawberry diet varies as a function of the specific endpoint. For CTA learning, the rats maintained on either the blueberry or strawberry diet failed to show the $^{56}$Fe particles-induced disruption of an amphetamine-induced CTA (Rabin et al., 2002). Following exposure to either 1.5 Gy or 2.0 Gy the irradiated rats maintained on either diet for two months prior to exposure showed the acquisition of a CTA following injection of the dopamine agonist amphetamine. As shown previously (Rabin et al., 1998, 2000), the irradiated rats fed a control diet failed to acquire an amphetamine-induced taste aversion.

Similar results were obtained in the initial test of spatial learning and memory using the Morris water maze (Shukitt-Hale, unpublished). The irradiated rats maintained on either the blueberry or strawberry diets showed a significant reduction in the latency to find the
location of the submerged platform on the probe trial on Day 2 compared to the irradiated rats fed a control diet. By Day 3, however, the performance of the irradiated rats fed the strawberry diet was not significantly different from that of irradiated rats fed the control diet, whereas the irradiated rats fed the blueberry diet continued to show a reduced latency compared to the irradiated rats fed the control diet.

The effects of antioxidant diets on operant responding also varied as a function of the specific diet. Seven months following exposure to 1.5 Gy of $^{56}$Fe particles, there was no effect of irradiation on operant responding. When the rats were tested eleven months following irradiation, the animals fed either the control or blueberry diets showed significantly poorer performance on an ascending fixed-ratio reinforcement schedule than the non-irradiated rats (Rabin et al., in press). The performance of the rats fed the strawberry diet was not significantly different from that of the non-irradiated controls and significantly better than that of the irradiated rats fed the blueberry diet. Similar results were obtained following exposure to 2.0 Gy of $^{56}$Fe particles, except that the effects of irradiation and diet were observed when the rats were first tested five months following irradiation (Rabin et al., submitted).

As indicated above, exposure to HZE particles can produce cancer. Current theories ascribe a role for oxidative stress in carcinogenesis (Oberly, 2002). To the extent that oxidative stress does play a role in tumor development, the use of antioxidant diets should reduce the development of tumors following irradiation. Because the rats that were tested on the operant conditioning task were observed for up to 12 months following exposure to 1.5 Gy or 2.0 Gy of $^{56}$Fe particles, data were also collected on the effects of diet on tumor development. Preliminary analyses indicate that both blueberry and strawberry diets significantly reduced the occurrence of radiation-induced tumors (Joseph, in preparation).

CONCLUSIONS

Overall, the observations presented in this review indicate that exposing the heads of laboratory animals to doses of HZE particles can have deleterious effects on cellular and systemic functioning. On a cellular level, irradiation can produce mutagenesis or cell death. On a systemic level, there are changes in signal transduction processes in the central nervous system and related decrements of motor and cognitive performance which have the potential to affect the capacity of astronauts to successfully meet mission requirements. There are a number of strategies that may be used to counteract the effects of exposure to HZE particles. These include scheduling missions during times of reduced GCR flux or increasing the level of shielding. An alternative approach which may hold promise involves using dietary manipulations to reduce the levels of oxidative stress produced by exposure to HZE particles. By reducing oxidative stress and the generation of ROS, diets containing blueberry or strawberry extract may provide necessary protection against the deleterious effects of exposure to GCR, while also potentially mitigating other space flight stresses, and enabling astronauts to successfully fulfill mission requirements.

ACKNOWLEDGMENTS

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NOMENCLATURE AND GLOSSARY

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>GCR</td>
<td>Galactic Cosmic Rays, high energy nuclei from outer space</td>
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<tr>
<td>Gy</td>
<td>Gray, the SI unit of absorbed ionizing radiation dose (1 J/kg)</td>
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<td>HZE</td>
<td>High-charge, high energy particles (GCR)</td>
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<tr>
<td>LET</td>
<td>Linear Energy Transfer, energy deposited per unit distance in particle track</td>
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<tr>
<td>QF</td>
<td>Quality factor, for adjusting dose to equivalent dose based on radiation quality (LET)</td>
</tr>
<tr>
<td>rad</td>
<td>Radiation absorbed dose, cgs units (100 erg/g; 0.01 Gy)</td>
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<tr>
<td>rem</td>
<td>Biological equivalent cgs dose unit, rad x RBE (0.01 Sv)</td>
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<tr>
<td>RBE</td>
<td>Relative Biological Effectiveness</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>Sv</td>
<td>Sievert, the SI unit of biologically equivalent dose (Gy x RBE)</td>
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REFERENCES


B. Rabin — Diet as a Factor in Behavioral Radiation Protection


Short Papers

Advanced Life Support and Biotechnology (page 81)
Animal Development, Physiology and Gravity Response (page 91)
Cell Biology (page 99)
Plant Development and Gravity Response (page 113)
EVALUATION OF A SILANE QUATERNARY AMMONIUM SALT AS AN ANTIMICROBIAL SURFACE TREATMENT
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NASA guidelines for planetary protection aim to preserve the unaltered environments of both our own planet and all other planetary bodies in our solar system. Preventing the spread of Earth-based life forms to other planets and the contamination of our own planet by extraterrestrial life forms is an important consideration when planning space missions. Even with extensive sterilization techniques during the assembly of spacecraft, bacteria, mainly extremophiles, still survive.¹,²

Another pressing concern when discussing the control of microbes in space is the maintenance of astronaut health. Astronauts traveling in spacecraft are subject to close-quarter living for an extended period of time. Ideal conditions for microbe growth often arise as a result of irregularity in temperature and humidity control onboard the spacecraft. An impaired human immune system during spacflight and the lack of available medical care necessitate bacterial growth prevention measures.³

To prevent the survival and growth of microbes and to decrease the pathogenic stress on crewmembers, we tested whether the ÆGIS Microbe Shield™ could be used as an antimicrobial surface treatment for materials in and on spacecraft using a dynamic contact bacterial solution method.⁴

**Surface Preparation:** Four aluminum coupons (1.7 cm x 5.5 cm x 0.2 cm) were treated with a 1:100 water dilution of silane QAS (42% in methanol; AEM 5700) to 0.42% (Figure 2). The pieces were flipped after 7.5 minutes and soaked for a total of 15 minutes. The surfacing was performed in a Nuaire Biological Safety Cabinet at room temperature. The coupons were hung on a wire in the safety cabinet to dry overnight.

**Culture Preparation:** Cultures of Bacillus subtilis, Escherichia coli, and Staphylococcus epidermidis were prepared in Tryptic Soy Broth (TSB) overnight at 35 °C. The broth was diluted to a transmittance of 72% on a Vitek Colorimeter (1.5-3.0 x 10⁸ CFU/mL). This bacterial solution was mixed with 0.003 M KH₂PO₄ buffer to a 1:1000 dilution (1.5-3.0 x 10⁵ CFU/mL).

A company protocol was followed for testing the effectiveness of the QAS.⁴

**Surface Testing:** The bacterial suspension was transferred into 9 flasks in 50 mL portions; 4 flasks contained treated surface, 4 flasks untreated surface, and 1 flask no surface to act as a growth control. A sample was taken from the growth control at time=0 and plates were poured. The nine flasks were placed in a horizontal shaking water bath at 35 °C and ~120 RPM (Rate 6 on Lindberg/Blue M Agitator). After one hour, samples were taken from every flask, plated, and incubated at 35 °C overnight. Bacterial colonies were counted on the plates, population densities were calculated across all trials, and t-tests were completed. This information was used to determine the effectiveness of the QAS by examining its toxicity towards bacteria.

Aluminum coupons, treated and untreated, were autoclaved, rinsed with deionized water, and reused for the next trial. A confidence level of 95% was used to test the significance of the logarithmic data.

For each of the species tested, the numbers of live bacteria were significantly reduced on the coupons treated with QAS according to t-tests of the logarithmic data (Figure 3). The exception was the second trial of B. subtilis in which a p-value of 0.428 was obtained. The effectiveness of the QAS on E. coli was less than that obtained for the other bacterial species.
According to the claim of the manufacturer (ÆGIS Environments; Midland, MI.) that the QAS is permanent, repeated use of the treated surfaces was not expected to have an effect on the outcome. The microbe shield was also intended to withstand temperatures well over the autoclaving temperature of 121 °C. The reduction in activity of the product after autoclaving and rinsing increased the difficulty of comparing the QAS effectiveness across species of bacteria (Figure 4).

The results, except from the fourth use of the metal (second trial of B. subtilis), correlated with the expected antimicrobial effects of the QAS. However, company claims of the permanency of the product were not supported by results of the experiments. E. coli, expected to be particularly vulnerable to the QAS from previous testing and because it is non-spore-forming, was less affected by the treatment compared to the other species. Since E. coli was used in the final two trials, a degrading microbe shield would explain this result possibly attributed to the effect of autoclaving and rinsing the coupons between uses. The surface darkened after autoclaving in the spent media, suggesting that some sort of change occurred to the metal. Autoclaving could have simulated the effect of burning, which the product is not designed to withstand.

Even with the evidence of the loss of effectiveness over time, the results suggested that this product may be of some use in controlling microbes in future space travel. If surfacing techniques could be refined and the scope of the product better understood, this antimicrobial shield could be a very important tool in the prevention of bacterial growth on and in spacecraft of the future.

Since this was a preliminary analysis of the product only demonstrating effectiveness of the QAS in a solution of bacteria under dynamic contact conditions, much work still needs to be completed. While possible applications such as using the shield within water-filled pipes could arise from this testing, the ability of the microbe shield to prevent bacterial colonization in other spacecraft environments needs to be explored. Testing of bacteria using newly treated metal should be performed and the durability of the surfacing agent should be explored. Other investigations should include determining the concentrations of bacteria against which the product is effective and in what concentration the substance must be applied to the surface to retain antimicrobial properties.

REFERENCES

The field of cryobiology originated in 1949, when Polge, et al., described the cryoprotective property of glycerol in the freezing of spermatozoa (1). Lovelock and Bishop reported the cryoprotective ability of dimethyl sulfoxide (DMSO) on red blood cells in 1959 (2). Forty-two years later, DMSO was used to preserve the first cryogenically-stored cells launched onboard STS-105 for use on the first human cellular biotechnology payload, Cellular Biotechnology Operations Support System (CBOSS-01), on the International Space Station (ISS).

DMSO is the most common cryoprotective agent used in the laboratory. While DMSO is easily eliminated in ground-based experiments, its removal in flight-based experiments is more difficult due to microgravity, hardware limitations, and on-orbit constraints. Because of the deleterious effects of DMSO on cells at non-cryogenic temperatures, there was concern regarding the difficulties of removing the DMSO after thawing the cells in microgravity.

Our primary goal was to find an alternate cryoprotectant to DMSO to be used for future CBOSS Exploration Cell Science investigations. While systematically screening for potential permeating and non-permeating cryoprotectants, we used a human colorectal carcinoma cell line, MIP-101 (3) that has flown on several biotechnology payloads (4), including CBOSS-01. We utilized data from immediate post-thaw viability, culture recovery and clonogenic assay to determine a candidate alternate cryoprotectant to DMSO. Once alternates were identified, we explored the use of a binary system consisting of permeating and non-permeating cryoprotectants (5) at concentrations suboptimal in single cryoprotectant systems in an effort to maximize cryoprotective effect at lower concentrations.

MIP-101 cells were resuspended in cryopreservatives using RPMI culture media as a carrier solution. Afterwards, they were frozen according to a two-step procedure involving initial cooling at -1°C/min overnight in a Nalgene Cryocooler at 4°C followed by storage in liquid nitrogen (LN2) vapor, or by storing cells directly in the LN2 vapor phase at -10°C/min. The frozen cells were thawed by immersion and agitation in a 37°C water bath. A sample was taken immediately and assayed by Guava Viacount to determine the post thaw viability and cell density. Ability to preserve cellular function after cryopreservation was assessed by the recovery of viable cells in short- and long-term cell culture experiments.

Culture Recovery: Cells were plated in 6-well plates and cultured. After 96 hours, cells were harvested and viability was determined by Guava Viacount. In the assay to determine cell growth kinetics, cells were harvested at 12, 24, 36, and 48 hours. Clonogenic Assay: Cells were seeded in 35-mm tissue culture plates and cultured for 10-12 days. Individual colonies were counted after staining with crystal violet.

Binary Cryoprotectant: Suboptimal levels of ethylene glycol and raffinose were combined to cryopreserve cells.

**Figure 1a. Viability from Permeating Cryoprotectants.** The results show that cells cryopreserved in dimethyl sulfoxide and ethylene glycol have a post-thaw viability of ~80%.

**Figure 1b. Viability from Non-Permeating Cryoprotectants.** Among the cells cryopreserved in non-permeating agents, those cryopreserved in trehalose, raffinose and sucrose have post-thaw viabilities of 30-40%.

**Figure 2a. Recovery from Permeating Cryoprotectants.** The increase in cell growth of cells cryopreserved in dimethyl sulfoxide, glycerol, and ethylene glycol were ~12-fold over the course of 96 hours, whereas the increase of cells cryopreserved in propylene glycol was ~18-fold.
The performance of a cryoprotectant in either preserving post-thaw viability or culture recovery must be weighed to determine a candidate alternate to DMSO. Post-thaw viability is most important because it describes not only the number of cells that survived cryopreservation, but the starting cell density for subsequent culture. Culture recovery describes the growth potential of the cells; however, the overall cryoprotective action of a cryoprotectant hinges upon the post-thaw viability. We found that some cryoprotectants preserve post-thaw viability and others preserve post-thaw culture recovery.

As an alternate cryoprotectant, 10% ethylene glycol performed best in terms of viability and the clonogenic assay. The second best candidate was 10% propylene glycol, which did not preserve viability at the level of DMSO, but cells cryopreserved in propylene glycol had greater growth potential than DMSO and ethylene glycol.

It is interesting to note that while raffinose-preserved cells had a relatively low post-thaw viability, they had the highest protection in terms of post-thaw culture recovery after 96 hours. In addition, in the kinetics assay, raffinose-preserved cells are the only cells where the initial population increased without first declining, except for the control, unfrozen cells.

Trehalose did not perform well in terms of post-thaw viability, in relation to DMSO, and not as well as raffinose in post-thaw recovery.

Non-permeating cryoprotectants do not show much potential as single system cryoprotectants, but as shown in our binary cryoprotectant experiment, may be used in conjunction with permeating cryoprotectants in order to reduce cryoprotectant concentration, though more development of the binary system is required.

REFERENCES


**Pseudomonas aeruginosa** Growth and Production of Exotoxin A in Static and Modeled Microgravity Environments.


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*Pseudomonas aeruginosa* is a ubiquitous, water-borne bacterium and opportunistic pathogen, which may thrive in water and other environments. It may form a biofilm of cells in an extracellular polymeric matrix, on the mucous membranes of the lungs in cystic fibrosis patients and on many other surfaces. An important virulence protein of *P. aeruginosa* is Exotoxin A (ETA) (Campa et al., 1993). The microgravity environment of spaceflight (10^-4 to 10^-6 x g) can provide important information as to how the physiology of terrestrial organisms is affected by gravity. Modeled Microgravity (MMG) systems are used to simulate the gravitational effects of spaceflight on cells. Previous studies have shown changes in protein expression in *P. aeruginosa* PA103 (ATCC 29260) bacteria (Pulcini et al., 2004) and up-regulation of virulence factors in *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (Nickerson et al., 2004) in response to MMG. Variations in the protein expression of *P. aeruginosa* in MMG reflect alterations in metabolic and physiological functions, and also in putative pathways responsible for the production of *P. aeruginosa* virulence factors (Pulcini et al., 2004). The virulence of *P. aeruginosa* is suspected to be enhanced in MMG. However, the growth and physiology of *P. aeruginosa* has not been well studied in MMG conditions. The goal of this study was to establish the growth of this microorganism in MMG systems, and to determine how Exotoxin A production is related to different stages of growth. It is anticipated that similar experiments will be performed in spaceflight. Fundamental questions concerning the effects of spaceflight on microorganisms in relation to crew health risks remain unanswered.

The MMG system used to study the influence of gravity on the growth cycle was the clinostat. Samples in a clinostat still experience unit gravity (g), however, the constant rotation of the samples results in the g-vector being time-averaged to near-zero (Klaus et al., 2001). MMG is the term used to describe the resultant state of clinorotation. Vertical rotation (VERT) was utilized as a rotational control to the clinostat. In the vertical system, the resultant force gravity vector is parallel to the gravity vector of the Earth. Static control (STAT) conditions were achieved inside clinostat tubes in a stationary position.

To assess the effects of MMG, the lag, log and stationary phases of growth of the ETA producing strain, PA103, were studied. The experiment inoculum was prepared in two different ways. One was suspended in glycerol (20 % final concentration) and frozen at -80°C (FZN) while the other was suspended in water and refrigerated at 4°C (REF). Plate count results were used to adjust the inoculum suspension to ca. 5x10^8 CFU/ml before freezing or refrigeration. Both inocula were added to MSDM2 (Modified Simple Defined Media 2) at a ratio of 1:10 and used to inoculate 5 ml syringes each with 2 ml of medium to simulate flight growth chamber specifications. FZN inoculum growth cycle sampling times were time zero, 30 min, 3 h, 6 h, 9 h, 12 h, 15 h, 18 h, 21 h, and 24 h. For the REF inoculum, growth cycle sampling times were time zero, 6 h, 12 h, 15 h, 18 h, 21 h, and 24 h. For both inocula and every time point, syringes were placed in a STAT control and in a MMG environment. VERT controls were included only for the REF inoculum. Time points differ between the FZN and REF inocula due to space constraints in the VERT rotator and relevance to ETA production. MMG and VERT samples were rotated at 15 RPM and all samples incubated at 37°C. Samples were drop plated on R2A agar and incubated for 24 h at 30°C. Optical density (OD) readings were taken at 540 nm. The remaining culture was fixed with formalin (2.0 % final concentration). ETA was quantitated by an ELISA assay developed for spaceflight experiment BACTER on STS-107. Anti-*Pseudomonas* Exotoxin A (Sigma) was used as the primary antibody, Anti-Rabbit-HRP (Sigma) as secondary antibody and orthophenylenediamine (OPD) as the HRP indicator substrate. A dilution series of standard ETA (CalBioChem) was included. Optical density was read at 490 nm and sample concentrations were determined from the standard curve in ng/ml.
The growth cycle of _Pseudomonas aeruginosa_ was apparently not affected by the MMG conditions of the clinostat (Figure 1 and 2). The initial cell numbers in samples from the FZN inoculum diluted in MSDM2 were consistently lower than those from the REF inoculum. The FZN inoculum cultures also reached lower numbers in the stationary phase, and there was a decrease in CFU/ml between 18 and 21 h that recovered by 24 h (Figure 1). This decline and recovery did not occur with the REF inoculum (Figure 2). The overall ETA production rate was fairly constant during the log phase with both inocula. For the FZN inoculum, the ETA concentration in the MMG was essentially equivalent to the STAT control (Figure 3). With the REF inoculum, the ETA concentration was somewhat higher in the vertical rotation system when compared to the MMG and STAT samples (Figure 4). The difference between the bacterial populations of the FZN vs. the REF inocula apparently affected the overall ETA production throughout the growth cycle (Figures 1, 2, 3 and 4). Mean ETA concentrations in samples from the REF inoculum were generally higher than from the FZN inoculum. Differences between the FZN and REF inocula may have been the result of selection of a particular population during freezing.

As demonstrated here, due to the differences that can be induced in the experimental sample by pre-experiment storage conditions, it is crucial importance that all experiments, especially for spaceflight, are done with the inoculum prepared in the same way to obtain consistent and comparable results. Future work will focus on studying the effects of different speeds in the clinostat and another MMG system such as the Synthecon HARV (High-Aspect Ratio Vessel), on the growth cycle of PA103, including shear stress consequences on growth and virulence. In addition, proteomic analyses will be performed at intervals over the growth cycle to determine variations in protein expression which may be related to virulence.

**REFERENCES**


NASA has defined a metric to determine the necessary launch capacity for an advanced life-support system (ALS), designed to support humans for an extended duration beyond low earth orbit. This metric allows the comparison of techniques and technologies over time and between mission scenarios, yielding the optimum physical-chemical and bioregenerative array for human life support. The metric in use is Equivalent System Mass (ESM), which equates power, volume, cooling and crew time into a mass-equivalency unit (Kg) as shown by Equation 1 (Levri, 2003).

\[
\text{ESM} = M + (V \cdot V_{eq}) + (P \cdot P_{eq}) + (C \cdot C_{eq}) + (\text{CT} \cdot \text{D} \cdot \text{CT}_{eq})
\]

Where M, V, P, C, CT and D are the mass, volume, power, cooling, crew time and mission duration, respectively, for a given system, and the subscript multipliers are the equivalency factors used to convert these into units of mass equivalents.

Lighting for plant growth is estimated to account for 43% - 60% of the total ESM for biomass production, with much of this value due to the power requirements (Drysdale and Bugbee, 2003). Significant energy from overhead electrical lighting is lost by inaccurately targeting the light to all leaves in a crop canopy. When plants are young, light is lost by illuminating empty space around seedlings. If crops are planophiles (e.g. cowpea, soybean), which close their canopies as they age, then younger leaves shade out older leaves. This leads to a small percentage of leaves (approximately 10-15%) doing the photosynthetic work to support the entire crop stand.

To avoid wasting light and to utilize more of a crop’s photosynthetic capabilities, we envision an intracanopy lighting system that allows switching of lights in response to plant growth. A reconfigurable lighting system is the result of a jointly sponsored collaborative research agreement between Orbital Technologies Corp. (Madison, WI) and the ALS NSCORT Crop Production group at Purdue University. We have worked together to develop a light-emitting diode (LED)-based system from earlier proof-of-concept studies with fluorescent lighting (Frantz et al., 2001).

Orbitec had previously designed a 1-in-square “Chip-on-Board” LED “light engine” for plant growth containing up to 132 LEDs with 5 possible colors, and 2 photodiodes that can detect a variety of wavelengths. For this system, we utilized these light engines. However, due to our requirements, chips were populated only with red and blue LEDs, with green LEDs and photodiodes also in place but not currently utilized. Future generations could include different LEDs depending on individual crop requirements. Each lightsicle strip has 20 light engines which have 5 rows of LEDs, four rows of sixteen 640-nm red LEDs and one row of sixteen 440-nm blue LEDs giving a total of 80 LEDs per chip (Fig. 1 inset). Heat from the LED chips is removed through a channel running behind the strip and a fan-driven airflow system mounted in the lightsicle enclosure (Fig. 1). This cooling method allows plants to grow near or touch the strips without scorching.

Other components of the lighting system include a control enclosure containing a power supply and circuitry that control LED intensity via dimmer potentiometers, as well as incremental switching of light engines from the bottom to the top of lightsicles. Additionally, a timer allows preprogramming of desired photoperiod. A separate power and communications distribution assembly allows integration of the 16 strips.

A mounting system was developed to suspend the lightsicles within a crop-growth compartment. The growth area consists of an EGC (Chagrin Falls, OH) walk-in growth chamber with temperature, relative humidity, and CO2 control. A recirculating hydroponics system was constructed. The root-zone compartment is mounted on a table frame, and a framework of angle iron was constructed around the hydroponics system. After lighting installation was complete, walls made from reflective white poly film were hung between the top of the framework and the table. Lightsicle mounts were suspended from struts placed horizontally over the framework at intervals. Brackets from the struts were
attached to metal strips, and corresponding strips were
fastened to the lightsicle mounting bracket (Fig. 1). Velcro 
was used to join the two sets of strips together. This 
mounting system allows ease of lightsicle removal 
for configuration changes, height adjustment, and 
maintenance. Lightsicles were mounted in a 
configuration to maximize light coverage uniformity 
within the growth compartment. The power and 
communications assembly was mounted to the framework 
adjacent to the growth compartment. Cables run from 
each individual lightsicle to this assembly, and separate 
cables extend from the distribution assembly to the 
control enclosure outside of the chamber.

Photosynthetically active radiation (PAR) was 
measured at different positions within the canopy space. Measurements were taken while red and blue intensities 
were changed and incremental switching of light engines 
occurred. Fig. 2 shows the increases in PAR with light intensity at two different heights within the array.

The differences between PAR values in Fig. 2A and B are due to radiation reflected off the poly film wall, 
radiation from surrounding engines, and variations in the 
light engines and associated drivers. The maximum light output measured within the system was approximately 900 µmol/s/m² (data not shown).

In addition to PAR measurements, current draw was 
measured as light intensity was increased (Fig. 3). The 
correlation coefficient \( R^2 = 0.994 \) indicates good 
agreement between energy input to and PAR output from 
the system. The y intercept indicates the baseline current 
value for an energized system with no lights on. We 
measured this value at 2.6 Amps running with 24 volts 
DC.

We have developed a reconfigurable lighting array 
for plant growth in an ALS. This system is initially in an 
intracanopy arrangement for growth of planophile crops. 
Initial system tests indicate good performance with 
possible electronic modification in future systems to 
counteract component variations. Current drawn by the 
system strongly correlates with light output. The first 
cowpea crop grown in the system appeared healthy 
though somewhat elongated with weak stems. This test 
demonstrated the need for intense blue light early on to 
allow for de-etiolation and reduction in hypocotyl 
elongation. Other protocol modifications may include 
switching an extra light engine on to guide plant growth. 
Preliminary ESM calculations for an intracanopy lighting 
array on a m² and m³ basis are underway. These 
calculations will allow us to define the critical sensitivity 
points within the lighting subsystem that will yield a high 
return on investment when improved, i.e. big reductions 
in ESM.

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INTRODUCTION

Some pathological conditions described in astronauts, such as cardiovascular deconditioning, represent the adaptive response to the absence of gravity and are partially due to the effects exerted by microgravity (µg) at the cellular level. Since endothelial cells are crucial in the maintenance of the functional integrity of the vascular wall, it is noteworthy that we described endothelial dysfunction in response to simulated µg. In particular, we have shown that cultured Human Umbilical Vein Endothelial Cells (HUVEC) in the Rotating Wall Vessel (RWV) grow faster than controls, rapidly remodel their cytoskeleton and, after a few days, markedly down-regulate actin (Carlsson et al., 2002, Carlsson et al., 2003). The RWV system requires cells suspended or seeded on microcarriers. This is an obstacle to evaluate other endothelial functions, among which cell migration, a crucial event in vasculogenesis and angiogenesis. We therefore decided to simulate µg using the Random Positioning Machine (RPM) that allows to culture cells in standard plates. RPM has been demonstrated to be a good µg simulator for plants, osteoblasts and T lymphocytes. Up to now, there are only few data about the behaviour of adherent cell cultures in RPM.

To optimize the RPM operative parameters, we chose as a model human monocytoid U937 cells that have been studied in simulated and real µg (Hatton et al., 1999; Maier 2004). On the basis of previous results, our aim was: a) to evaluate the best operative experimental conditions of RPM, b) to compare the results with those obtained in simulated (RWV) and real (spaceflight) µg conditions; c) to use RPM to grow HUVEC in order to confirm and, possibly, broaden the RWV results.

Rotating Wall Vessel

The RWV is a suspension culture vessel optimized to produce laminar flow and minimize mechanical stress on cell aggregates in culture. It provides fluid dynamic operating principles characterized by 1) solid body rotation about a horizontal axis that is characterized by colocalization of cells and aggregates of different sedimentation rates, optimally reduced fluid shear and turbulence, and three-dimensional spatial freedom; and 2) oxygenation by diffusion, excluding undissolved gases from the bioreactor (Hammond et al., 2001). The cylindrical culture vessel is filled with culture fluid and the cells or tissue particles are added. All air bubbles are removed from the culture vessel. Oxygenation is achieved through a gas permeable silicone rubber membrane. Since the Rotary Cell Culture System™ has no impellers, airlifts, bubbles, or agitators, tissue damage from impact and turbulence is decreased with shear stress and damage essentially insignificant.

Random Positioning Machine

The RPM is essentially a 3-axis clinostat, which creates a condition in which the weight vector is continually reoriented as in traditional clinorotation, but with increased directional randomization (Klaus, 2001). The instrument operates inside an incubator with controlled temperature and atmosphere (CO₂ percentage, relative humidity...). Standard consumable experiment hardware can be used, such as 6 wells multidishes sealed with a gas permeable membrane, in order to achieve a suitable gas exchange. More sophisticated hardware, derived from spaceflight experience, will enable automatic medium exchange, fixation, etc. Primary applications are cell and developmental biology and tissue engineering. Simulation of µg by the means of continuous random change of orientation of objects relative to the gravity's vector can generate effects comparable to those of true µg when the changes are faster than the object's response time to gravity. Thus, slow responsive living objects, are excellent candidates to be studied on RPM.

MATERIALS AND METHODS

HUVEC were obtained from the American Type Culture Collection (ATCC) and cultured in M199 containing 10% fetal calf serum, Endothelial Cell Growth Factor (150 mg/ml) and heparin (5 U/ml). The cell culture system was based on 2% gelatin-coated 6 wells multidishes. In order to achieve a suitable gas exchange, every dish was sealed with a gas permeable membrane (Breathe-Easy, Sigma) avoiding air bubbles formation. To be used in the RWV, HUVEC were seeded on beads (Cytodex 3, Sigma). Cells were subcultured using 0.05% trypsin 0.02% EDTA solution. All culture reagents were from Sigma. U937 cells have been cultured in RPM medium containing 10% calf serum. In order to assess the viability, cells have been counted every 48h after staining with Trypan Blue, using a Burker chamber. To simulate µg, we used the RWV bioreactor (Cellon) with 10 and 50 ml disposable vessels and the RPM facility at the Dutch Experiment Support Center (DESC, Amsterdam, NL) accommodated in a dedicated temperature controlled incubator capable of supplying a 5% CO₂/air mixture. To observe the cytoskeleton, immuno-fluorescence/confocal microscopy of cells fixed with paraformaldehyde and stained with fluoresceine isothiocyanate (FITC)- phalloidin has been performed.

RESULTS AND DISCUSSION

Cell proliferation

It is reported that µg affects cell growth. Here we evaluated the proliferation of U937 and HUVEC grown in...
the RWV and RPM systems. Figure 1 reports the duplication time of U937 and HUVEC. As expected on the basis of in flight experiments performed on the Shuttle, U937 in the RWV and in the RPM grew 40% slower than controls. On the contrary, HUVEC proliferated 50% faster than controls. These effects are reversible upon return to normal growth conditions. We conclude that µg simulated either by RWV or RPM leads to comparable results.

Figure 1. Duplication time of U937 and HUVEC cultured in RWV and RPM vs. static gravity controls. Standard Errors are <10%.

Cytoskeleton reorganization
Different cell types cultured in µg show cytoskeleton reorganization. We therefore stained HUVEC grown in simulated µg in the RPM and their ground controls with fluorescent phalloidin to visualize the cytoskeleton. Ground controls (A, C) show a well-organized cytoskeleton with abundant stress fibers organized into bundles. HUVEC grown in RPM (B) for 96 h show major modifications of cell shape, as previously observed in RWV experiments, with elongated extended podia (D), disorganized actin fibers and clusters. Cytoskeleton modifications are reversible upon return to normal growth conditions.

CONCLUSION
The similar U937 behavior in space-flight (Hatton et al., 1999) and simulated µg conditions supports RWV and RPM as good tools to simulate µg on earth. In addition, RWV and RPM exert similar effects on HUVEC behaviour. Our results indicate that both the systems work at similar simulation levels, thus allowing a wider spread of experiments.

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METHODS FOR THE CULTURE OF C. ELEGANS AND S. CEREVISIAE IN MICROGRAVITY

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To support the study of the effects of microgravity on biological systems, our group is developing and testing methods that allow the cultivation of C. elegans and S. cerevisiae in microgravity. Our aim is to develop the experimental means by which investigators may conduct peer reviewed biological experiments with C. elegans or S. cerevisiae in microgravity. Our protocols are aimed at enabling investigators to grow these organisms for extended periods during which samples may be subcultured, collected, preserved, frozen, and/or returned to earth for analysis. Data presented include characterization of the growth phenotype of these organisms in liquid medium in OptiCells™ (Biocrystal, LTD).

Subculture and sampling activities of C. elegans and S. cerevisiae in microgravity are more manageable when the organisms are grown in a closed liquid culture system. We have therefore examined the growth of C. elegans in C. elegans minimal medium (CeMM) (Lu et al. 1993, Szewczyk et al. 2003) utilizing Opticells™ as growth chambers. The Opticells™ are under investigation as a containment system for liquid C. elegans culture since they are amenable to flight subculture and sampling procedures. Our data show that C. elegans grow at a rate comparable to that in a standard culture flask, reach densities of up to 1 x 10⁶ worms/ml in liquid medium in Opticells™, and may be initiated with as few as 10 worms/ml (Figs 1 and 2). Similarly, S. cerevisiae cultured in standard YPD medium in OptiCells™ grow at a comparable rate to those in a static flask (Fig. 3). Note that the Opticells apparently mimic a static flask condition rather than a shaking flask condition. These results suggest that our culture conditions support the growth of C. elegans and S. cerevisiae in a manner comparable to conventional culturing methods.

Characterization of the required conditioned transport and storage of CeMM is important as it affects the hardware, power, and space requirements for the experiment. CeMM is known to contain components that are light and temperature sensitive and is therefore typically stored at 4 °C in the dark. To characterize the effect of storage at elevated temperature, we grew worms in medium that had been stored at room temperature in the dark for 10 months. As shown in Figure 4, this severely affected ability of the medium to support C. elegans growth.

Figure 1. Growth of C. elegans in OptiCells™ compared to flasks. Animals were inoculated 10 ml of CeMM in OptiCells™ or T75 vented flasks. Samples were incubated at 20 °C and worms were periodically counted. Error bars show the standard deviation from the mean. N = 3.

Figure 2. Growth of C. elegans in OptiCells™ with various starting densities. Animals were inoculated to a density of 10, 100, or 1000 worms/ml 10 ml of CeMM in OptiCells™ and incubated at 20 °C. Worms were periodically withdrawn and counted. Error bars show the standard deviation from the mean. N = 3.

Figure 3. Growth of S. cerevisiae in Opticells. Stationary phase S. cerevisiae BY4743 were diluted 1:1000 in YPD broth, transferred to shaker flasks or Opticells™ and incubated at 30 °C. The flasks were incubated either with shaking or statically. The Opticells™ were incubated statically with a 2mm space between replicate Opticells. Growth was monitored over time by measuring the optical density at 600 nm. Error bars depict the standard deviation from the mean. N = 3.

Characterization of the required conditioned transport and storage of CeMM is important as it affects the hardware, power, and space requirements for the experiment. CeMM is known to contain components that are light and temperature sensitive and is therefore typically stored at 4 °C in the dark. To characterize the effect of storage at elevated temperature, we grew worms in medium that had been stored at room temperature in the dark for 10 months. As shown in Figure 4, this severely affected ability of the medium to support C. elegans growth.
As expected, during growth testing of *S. cerevisiae* in YPD medium in OptiCells™, we observed the formation of large bubbles, CO₂, within the OptiCells™ (data not shown). Since bubbles are undesirable during flight experiments, and dextrose is a substrate converted to CO₂, we sought to mitigate bubble formation by reducing the dextrose in the YPD medium, which typically contains 2.0% dextrose. To this end, we grew samples in OptiCells™ in YPD medium modified to contain 2.0%, 1.5%, 1.0%, or 0.5% dextrose. With lower dextrose concentrations, we found a slightly reduced growth rate and final cell density (data not shown), yet a dramatic reduction in bubble formation (Fig. 5). This suggests that modulation of the glucose concentration in YPD medium may be used to reduce bubble formation in OptiCells™ by yeast.

*Figure 4.* Effect of CeMM storage temperature. Animals were inoculated to OptiCells™ containing 10 ml of CeMM that had been stored at either room temperature or at 4 °C in the dark for 10 months. Samples were incubated at 20 °C and worms were periodically withdrawn and counted. Error bars show the standard deviation from the mean. N = 3.

*S. cerevisiae* cells must be held in stasis during launch to allow controlled initiation of a flight experiment. We have examined methods that may allow us to prevent yeast growth yet maintain viability prior to experiment initiation. It is common practice to freeze cells long term in YPD containing 15% (v/v) glycerol. Although 15% (v/v) glycerol affects the growth rate of the cells, we found that lower concentrations of glycerol, such as 5% (v/v) do not significantly affect the *S. cerevisiae* growth rate (data not shown). We assessed the use of the lower glycerol concentration for preservation of *S. cerevisiae* at −20 °C. As shown in Figure 6, we found that 5% glycerol in YPD medium was sufficient to significantly improve the long term viability of *S. cerevisiae* cells at -20 °C. These data suggest that YPD supplemented with 5% glycerol may serve as an ideal freezing and growth medium for *S. cerevisiae* during flight experiments.

*Figure 5.* Gas bubble formation in OptiCells. *S. cerevisiae* BY4743 was grown in OptiCells in YPD with 0.5%, 1.0%, 1.5%, or 2.0% w/v dextrose. The bubble size is expressed as the ratio of distance to the bubble meniscus over the length of the OptiCell™ window. 0.5% dextrose did not produce measurable bubbles. Error bars depict the standard deviation from the mean. N = 3.

*Figure 6.* *S. cerevisiae* BY4743 survival in YPD + glycerol. 100 µl aliquots of stationary phase cells at 1 x 10⁵ cells/ml in YPD, or YPD + 3%, 10%, or 15% v/v glycerol were transferred to 1.5 ml microfuge tubes and placed at -20 °C. Samples were plated for viability over time. N = 3. Error bars depict the standard deviation from the mean.

The experiments described support the cultivation of *C. elegans* and *S. cerevisiae* in microgravity utilizing OptiCells™. In each instance the OptiCell(tm) containers provided growth rates similar to standard laboratory vessels. Our data suggest that OptiCells, coupled with the appropriate media, may provide a means to culture *C. elegans* and *S. cerevisiae* in a manner conducive to space flight experiments.

REFERENCES

Understanding the changes that occur in living organisms to bring about adaptation to the space environment is essential to support future plans for long-term missions to the Moon and Mars. The European Modular Cultivation System (EMCS), a life science research facility developed by the European Space Agency (ESA), will serve as a habitat for culturing multiple generations of *Drosophila melanogaster*. Two different Prototype Containers (I and II) were designed and developed at Ames Research Center to test the feasibility of culturing *Drosophila* specimens using the EMCS (Figures 1 and 2).

![Figure 1](image1.png)

**Figure 1.** EMCS Drosophila Prototype Containers and Food Compartments. A) Prototype I (PI) B) Prototype II (PII). PI has a fixed food reservoir while PII has a rotateable food cylinder.

![Figure 2](image2.png)

**Figure 2.** Fully assembled prototype containers with flies.

The objective of the study presented here was to optimize the biocompatibility of the prototype hardware. Fly behavior, humidity levels, and hardware mechanics were assessed. Modifications to the hardware were made as required to resolve any suboptimal performance issues. Flies were grown in containers using standard techniques for fly handling and videotaped using the video capabilities of the EMCS ERM camera system.

To determine the biocompatibility of PI and PII we tested both containers, as well as standard lab vial controls, with the indicated membranes or plugs for their ability to support *Drosophila* growth and survival (Figure 3). Wildtype flies were used in all experiments.

![Figure 3](image3.png)

**Figure 3.** Biocompatibility of PI, PII, and standard lab vials with Salad in a Bag or BreathEasy membranes, or standard cotton or buzz (cellulose acetate) plugs.

Neither Salad in a Bag nor BreathEasy membranes supported *Drosophila* growth but both cotton and buzz plugs did. Apparently, the diffusion rate of C02 and O2 through either membrane type is too low. Growth in PI and PII compared favorably with control standard lab vials with cotton or buzz plugs. We do not consider any differences in growth between cotton and buzz plugs consequential as only single replicates of the experiment were conducted. The reason behind this is that in the testing phase only one of each custom-made prototype container is routinely built so that any problems encountered can be resolved through design changes in the next generation of the container.

With the biocompatibility of PI and PII established, we next determined the effect of different starting population densities on progeny production in a single generation. The starting population size needs to strike something of a balance between generating a sufficient number of progeny that the results will be statistically significant, while at the same time not introducing the variable of overcrowding. As shown in Figure 4, starting population sizes of 15 males/30 females (15M/30F) produced greater numbers of progeny than 10M/20F. The lower number of progeny production in PII relative to PI is likely caused by first instar larval escape from PII. Based on our studies, 15M/30F is an appropriate number of starting adults.

Since multigenerational growth is a requirement for space experiments, we assessed the ability of PI and PII to support growth over four generations. These data are shown in Figure 5.
Figures 4 and 5: Comparison of parental density on progeny production in a single generation in PI and PII.

Figure 4. Comparison of parental density on progeny production in a single generation in PI and PII.

Figure 5. Ability of PI, PII, and control containers to support multigenerational Drosophila growth.

Over four generations, both PI and PII supported growth of larger fly populations than standard lab vials. This experiment demonstrates that PI and PII provide conditions sufficient for the growth of 200-250 Drosophila.

In the low gravity space environment where convection will not be occurring, we do not expect to observe significant changes in Drosophila growth rates or survivability because Drosophila has a relatively low metabolic rate that can be accommodated by exclusively diffusion-limited gas exchange. Also, the flight of adult Drosophila will promote air flow in the containers, and at least the adult Drosophila can move to regions where the O₂ and CO₂ are favorable if major gradients were to develop.

For many experiments, video imaging of Drosophila in PI and PII containers will be an essential assay for understanding the effects of altered gravity on fly behavior. The EMCS camera system is designed for this purpose. The EMCS camera has 18X optical zoom and 4X digital zoom. Initial results demonstrate that the EMCS video system was successful in imaging the behavioral parameters of the flies. Both analog video images and still digital images were captured. An example of a digital image is shown in Figure 6.

Summary

Two prototype containers (PI and PII) were developed for the purpose of supporting multigenerational growth of Drosophila on the International Space Station. Through population growth studies we determined that using cotton or buzz plugs allowed air circulation rates and humidity levels compatible with supporting growth of Drosophila. These containers support growth of 200-250 flies per generation. Population density studies showed that 30 females and 15 males is a good starting parental population size. The EMCS camera system successfully captures analog video images and still digital images.

Planned future studies include assessing the effects of different sterilization and packaging methods on food integrity when stored for extended periods. The growth of mutant strains of Drosophila will also be analyzed as mutant growth rates will likely differ from wildtype flies. In addition, the development of a sampling tool that will enable fly extraction from the prototype containers is planned.
NEUROPHYSIOLOGICAL LONG-TERM RECORDINGS IN SPACE: 
EXPERIMENTS SCORPI AND SCORPI-T 
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The International Space Station ISS offers the opportunity for physiological long-term observations in microgravity in awake animals. The 3-month period of experimentation favours heredity, development and physiological adaptation as scientific fields that can profit from those long-term exposures to microgravity. However, the most complicating problems are (i) the lack of proper animal habitats, and (ii) the available crew time. These facts dramatically decrease the types of experiments and, in particular, the animal species that can be used for long-term studies.

From the methodological point of view, automatic working recording devices are the most suitable techniques. Experience with neurophysiological long-term recordings based on ground studies, in particular in insects (cf. Miller, 1979), favours the study of central integration of neuronal, sensory and muscular activity of the organisms and, in particular, its adaptation to the microgravity environment. The lack of proper animal habitats on ISS favours the use of animals that are adapted to a life in extreme environmental conditions with rare food supply. Only those animals can be used that are adapted to a life in extreme environmental conditions and that are able to starve for long periods of their life due to limited access to food and water. Desert animals such as beetles and scorpions are species of first choice.

In 2001, an experiment with scorpions titled SCORPI was selected for flight on ISS mounted in the European research facility BIOLAB. Its main purpose is to analyse the adaptation of coordinating mechanisms between vegetative and sensorimotor activities to microgravity by means of neurophysiological recordings.

Among the basic coordinating principles of physiological mechanisms common in most organisms are biological clocks and their synchronization with external Zeitgebers such as the daily light-dark rhythm and other geophysical fields. In humans, desynchronisation of these rhythmic events during space flights (Gundel et al., 1997; Dinges, 2001) or microgravity simulation such as bed rest head-down tilt (Samel et al. 1993) can cause physiological, behavioral and psychological disturbances. Studies in animals and lower organisms revealed that circadian rhythms may be altered, but do not disappear during space flights of rhesus macaques (Fuller et al., 1996), the beetle Trigonoscelis gigas (Alpatov et al., 1994), Chlamydomonas and Neurospora (Ferraro et al., 1995).

The project SCORPI includes two important objectives, the hardware and the science objective. In particular, SCORPI has to demonstrate the reliability of a continuous neurophysiological multi-channel recording technique in the restrained animals during a long-term space flight. Furthermore, it has to analyze the integration of motor, neuronal and sensory signals and its long-term adaptation to microgravity with specific consideration of the biological clock system.

The first challenge for SCORPI was the development of a proper immobilization technique. A 3-point immobilization seems to be the most suitable one (Fig. 1), but a multi-point fixation at the base segment of each leg will be tested during the precursor space flight experiment SCORPI-T on the Russian satellite FOTON-M2 in 2005.

![Figure 1. A 3-point immobilization of scorpions for neurophysiological long-term recordings in space. Pedipalps and opisthosoma base are fixed by aluminium clamps (C). The tail is connected to a thread (TF) that restricts the extent of tail movements protecting the electrodes from damage by the sting. Electrodes are inserted at eye, leg, opisthosoma, and brain. Despite of this immobilization, animals catch prey actively that is a necessary condition for feeding. Animals survive in this harness for months.](image)

The second challenge was to develop the fully automated equipment that allow recordings of the visually elicited activity (electroretinogram, ERG), the muscular activity (electromyogram, EMG), the arousal of the brain (spontaneous cerebral electrical activity, SEA) and the heart beat frequency (electrocardiogram, ECG) (Fig. 2).

![Figure 2. Implantation sites of the electrodes for the neurophysiological multi-channel long-term recordings.](image)
The ERG is induced by short light pulses. Its basic shape is bipolar; in the case of rapid repetition of the stimulus, adaptation occurs (Fig. 3). For SCORPI, this is not critical because the individual test light stimuli are presented 10 to 20 min apart from each other. The parameters EMG, SEA and ECG are recorded during spontaneous activity. In general, during motor activity the motor signal (EMG) overwhelms all traces (Fig. 4); however, high- and low-pass filtering facilitates easy detection of all other activities such as ECG (Fig. 5) and SEA (Fig. 6, upper trace). For the low frequent signals such as ERG and ECG, low pass filtering is necessary while for the SEA, a specific filtering window is used.

Mounting of electrodes and the impact of animal movements on the reliability of the neurophysiological recording will be studied during the precursor flight on FOTON-M2 that lasts 2 weeks. Thus, the specific goals of SCORPI-T are to study critical aspects of the experiment SCORPI such as effects of animal locomotion on the implantation of the electrodes, or the impact of stress to the animals caused by launch vibrations, logistics and microgravity at all.

The techniques developed to date for immobilization and electrode implantation allow to record physiological parameters for several months. Thus it will be possible to study the stability of sensory, motor and neuronal integration in the long-term range in orbit and on ground. This technique can also be applied to long-term studies of ecological adaptation to extreme environmental conditions such as high temperatures, low humidity, or drought.

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Retinal degenerations can be promoted by many factors including ischemia, oxidative stress, and increased intraocular pressure. Damage to retinal neurons can be inhibited by neurotrophic factors such as PEDF, BDNF, and CNTF (Tombran-Tink and Barnstable, 2003). We present new evidence that the environment encountered in space shuttle flight can also disrupt normal retinal development and mimic stimuli that induce retinal degenerations on earth. Experimental evidence linking anomalies in visual perception with space flights has accumulated since the Apollo missions as have data suggesting pathological stimuli that disrupt retinal structure and function on earth are encountered in the space shuttle environment (Draeger 2000; Nicogossian et al., 1993). Orbital space flights cause physiological disturbances in humans including cephalad fluid shift, increased intraocular pressure, disruption of cardiovascular function and stress on the musculoskeletal system (Drummer 2000; Wang et al. 1996; LeBlanc et al. 2000).

Understanding the negative effects of space flight to the central nervous system (CNS) is of particular importance because, unlike most organs, the CNS has very little regenerative capacity. In this paper, we report the physiological effects of space travel on the retina of rodents in an NIH.R3 experiment at various stages of postnatal development during orbital flight on Mission STS-72, launched in 1996. Three groups of Sprague Dawley rats were analyzed in this experiment: Flight (housed in a modified Animal Enclosure Module [AEM]), Synchronous (housed identically to Flight litters) and Vivarium (standard colony housing). The rats were in flight for 9 days and sacrificed immediately after the shuttle returned to earth. Eyes from age and weight-matched female pups launched at post-natal days 5 (PN5), 8 (PN8), and 15 (PN15) were dissected, fixed in 10 % formalin, and retinal sections labeled with an antibody against rhodopsin or Hematoxylin and eosin for morphological and morphometric analysis of the retina.

The most striking difference among the space flight retinas and controls at all time points of rat neonatal development was the loss of outer segments of the rod photoreceptor neurons (Fig 1). The outer segments contain the visual pigment rhodopsin, which is essential to the visual transduction cascade for light detection. In addition, there was disruption of normal development of the inner plexiform layer (IPL) as evidenced by the decrease in thickness of the width of this structure as compared to ground controls (Fig 2). The IPL contains the synaptic connections that mediate information transfer from amacrine and bipolar neurons in the outer retina to the ganglion cell layer. There was a loss in the number of ganglion cells in the retina of flight animals as well, and those remaining appeared unhealthy.

Other abnormalities such as the detachment of the neural retina from the retinal pigment epithelium (RPE), loss of RPE cells or disruption of the RPE monolayer, and increased neovascularization were also associated with animals exposed to orbital space flight conditions (Fig 3).

Experimental studies have linked the perception of “light flashes” and hallucinations by astronauts in flight to the penetration of ionizing nuclei through the nervous tissue. This study has not allowed us to determine whether the effects we observed in the neonatal rodent retina are transient, reversible, or dependent on flight duration. Nor does it answer the question as to whether these changes are triggered by a warp in gravitational force, solar radiation, the impact of launching or reentry into the earth’s atmosphere, or fluctuations in oxygen level. It is also possible that only neonatal retinas and not well-developed adult retinas are affected by the hazards...
examined showed similar morphology, samples used were from animals that developed well, and there is no evidence that Vitamin A deficiency due to malnutrition causes thinning of the IPL, or promotes loss of ganglion cells and neovascularization in the retina. These changes are more likely related to earth-based degenerations caused by increased intraocular pressure and ischemia.

The impact on the neural retina and visual transduction cascades, however, highlights the importance of developing rigorous research to study how the eyes adapt or respond to various space related assaults since the duration of manned space journeys will significantly increase in the near future and could have irreversible adverse effects on human health and performance.

Knowledge of how this unusual environment affects molecular mechanisms and pathways of the CNS are key to accelerating development of appropriate physiological risk mitigation measures to remove biological barriers that could impede the astronauts’ ability to survive and function in future long-term human space exploration. Such studies could also lead to information that is important to similar earth-based retinal disorders.

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Cellular biotechnology experimentation on the International Space Station (ISS) sometimes requires that the collection of cells be preserved for long periods of time under adverse conditions. During Expedition 3, four different cell lines were grown using the Cellular Biotechnology Operations Support System (CBOSS), and were then preserved in either formalin or RNA later™ (Ambion) at refrigerat or temperatures. RNA later™ is marketed to preserve RNA in the refrigerator for 30 days. Cells treated with RNA later™ have previously been shown to contain antigenic proteins that can be visualized using Western blot analysis. These proteins appear to be stable for several months when stored in this RNA stabilizer at 4ºC. Antigenic protein can also be recovered from cells that have been processed using an RNAqueous® kit (Ambion) to remove RNA (1). Prior work demonstrated that mixed Müllerian ovarian tumor cells (LN1) (2, 3) were capable of being grown in rotating cell cultures that are analogs for microgravity (4). These cells grew on the ISS in the static bioreactor, Biological Specimen Temperature Controller (BSTC), and produced cytokines, although in reduced amounts compared with the ground controls (5).

In this set of experiments, LN1 cells grown on the ISS during Expedition 3 were examined for antigenic stability after removal of RNA. The cells were grown in 15 mL of media on Cytodex™ 3 beads (Amersham) in Teflon bags, stored for three months in 9mL of RNA later™ in the refrigerator, and RNA was extracted using an RNAqueous® kit. The RNA filtrate containing the protein was precipitated with a final volume of 5% trichloroacetic acid (TCA), washed in TCA, and suspended in buffer containing sodium dodecyl sulfate (SDS). Samples containing equal concentrations of protein as determined by the Bicinchoninic acid (BCA) method (6) were loaded onto SDS-polyacrylamide gels (7). After electrophoresis, gels stained with SYPRO Orange (Molecular Probes) were scanned for equal loading of protein. Further, gels were transferred by Western blot procedures (8, 9) to polyvinylidene fluoride membranes. Primary antibodies were as noted below. A horse-radish peroxidase secondary antibody was used and the Western blots were stained with an enhanced chemiluminescent ECL® Plus detection kit (Amersham) and scanned using a Storm™ 840 gel image analyzer (Amersham). ImageQuant™ TL software (Amersham) was used to quantify the densities of the protein bands.

The ground cell samples from day 3 (G3), day 9 (G9) and day 14 (G14) and flight cell samples from the same days (F3, F9 and F14) showed similar staining patterns over time with mouse antibodies to vimentin and epithelial membrane antigens, and rabbit antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Triplicate blots were made for each antibody. The graphs (Figures 1-3) show the average of the scanned pixel volumes for the stained protein band in each lane. A control using untreated cells was run on each blot and the pixel volumes normalized to the control value. Due to the normal variability of blots, the numbers were calculated as a percent of the control. Blots are shown below the corresponding bar on each graph.
These data demonstrate the presence of antigenic protein in the RNA-stabilized LN1 cells, even after long periods of time in refrigerator storage. The antigenic protein is recoverable even after the RNA has been removed from the cells by the filtration method. All three proteins examined here had similar profiles at different times in the flight and ground samples. Previously, proteins from human renal cortical epithelial (HRCE) cells had been shown to exhibit similar characteristics (1). This work further demonstrates that the technique can be generalized to other cell lines and might be a good way to preserve proteins for long term storage. Since this preservative is even more effective in protecting RNA when stored frozen, it seems likely that protein protection would be similarly increased in freezing conditions. Further work is needed to determine if freezing also protects the proteins for longer periods of time. In addition, a more comprehensive study should be undertaken to determine if this holds true over a large range of protein varieties and sizes.

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COUNTERMEASURE FOR SPACE FLIGHT EFFECTS ON IMMUNE SYSTEM: NUTRITIONAL NUCLEOTIDES
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Microgravity and its environment have adverse effects on the immune system. Abnormal immune responses observed in microgravity may pose serious consequences, especially for the recent directions of NASA for long-term space missions to Moon, Mars and deep Space exploration. The study of space flight immunology is limited due to relative inaccessibility, difficulty of performing experiments in space, and inadequate provisions in this area in the United States and Russian space programs (Taylor 1993). Microgravity and stress experienced during space flights results in immune system aberration (Taylor 1993). In ground-based mouse models for some of the microgravity effects on the human body, hindlimb unloading (HU) has been reported to cause abnormal cell proliferation and cytokine production (Armstrong et al., 1993, Chapes et al. 1993). In this report, we document that a nutritional nucleotide supplementation as studied in ground-based microgravity analogs, has potential to serve as a countermeasure for the immune dysfunction observed in space travel.

We employed two ground-based analogs, HU as an *in vivo* model and a clinostat bioreactor (BIO) as an *in vitro* cell culture model. We examined the effects of supplemental nutritional nucleotides on immune function in these analogs. BALB/c female mice (8-10 week old) were divided into the following two groups: control non-HU, and HU for 7 days. Mice were fed either control chow or chow supplemented with RNA or Uracil (referred as NT). Immune function was assessed by *in vivo* popliteal lymph node proliferation (PLN), and in *vitro* phytohemagglutinin (PHA)-stimulated proliferation of splenocytes and their cytokine production, (Kulkarni et al, 2002). PLN response was calculated as weight gain in allogeneic challenged lymph node versus weight gain in syngeneic challenged contralateral lymph node. Splenocytes were cultured *in vitro* in BIO with/without PHA and nucleoside/nucleotide mixture (referred as NS/NT). Cell proliferation was assessed by tritiated thymidine uptake assay by thymidine uptake in stimulated cultures versus unstimulated cultures. Cytokines released by cultured and stimulated cells were quantified by ELIZA method (Cayman Co.). PLN response was significantly suppressed in HU mice (P<0.05) and was restored by NT supplemented diets (Figure 1). Splenocytes from HU mice had decreased PHA stimulated proliferation and decreased IL-2 and IFN-γ cytokine levels (P<0.05) as well (Figure 1-A,B,C). These responses were restored by NT diets. In BIO cultures, PHA response was suppressed significantly, and NS/NT restored the proliferation response (Figure 2). Results in these figures are expressed as percent changes in groups. In both models, we documented that dietary and supplemental nucleotides (NT or NS/NT) restored several
immunological functions in mice including cytokine production, and decreased corticosteroid levels. Many of the parameters of cellular functions are shown in Figure 3. Similar effects are seen by NT supplementation in 1 G (Kulkarni et al., 1990) and in µG analogs. These observations clearly document the advantages and benefits of exogenous source of nucleotides in the form of nutritional NT supplementation. Observed decrease in corticosteroid levels in HU mice by NT supplementation may be one of the potential mechanisms for immune restoration observed in these mice.

Understanding of the mechanisms of immune restoration in microgravity analogs, including CNS mediated effects and their implications, is important in addressing the NASA’s CRP and CRL maps. Nucleotide metabolism is very important for cell cycle. Perturbations in nucleotide metabolism that lead to a variety of diseases are primarily in the de novo pathways. Supplemented nucleotides participate and contribute primarily in the salvage pathway and they are utilized by rapidly proliferating target cells (Kulkarni et al. 1990). As compared with the amounts of available nucleotides from the de novo synthesis and the endogenous salvage pathway, the levels of supplemental nucleotides in the experimental (Kulkarni et al., 1990, 2002) and clinical studies are much smaller (Bower et al. 1995, and Carver and Walker, 1995) and without any adverse or untoward effects. The amount of NT added was minimal in all the groups; however, the beneficial effect was seen only in the microgravity analogs of HU or BIO and not in any control groups where there was no demand or stress involved. Thus, in conclusion our studies presented in this report document the critical need of NT supplementation in physiological stress situations; such as space travel, where nutritional supplemental nucleotides have up-regulating and immunoprotective effects with potential as a countermeasure to the observed immune dysfunction in true microgravity.

Key words: nucleotide; nutrition; immunity; space travel

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The small G proteins of the Rho family (Rho GTPases) are key operators in the signaling arising from integrins and cell-matrix focal adhesions. They function as binary molecular switches that cycle between an inactive GDP-bound and an active GTP-bound form that in turn activates downstream effectors and a variety of signaling pathways. The best known Rho GTPases, RhoA, Rac1 and Cdc42 control the organization of the cytoskeleton and the focal adhesions-mediated transduction of exogenous and endogenous mechanical signals. Their localization at the cross-road between signaling, mechanical forces and the cytoskeleton along with reported effects of microgravity on cell shape and cytoskeletal arrangement led us to hypothesize that Rho GTPases might be involved in the reception and reaction of cells to gravity. To explore this hypothesis we created stable cell lines, SV40-transformed fibroblasts (WI-26), expressing a constitutively active form (QL) of RhoA, Rac1 or Cdc42 (manuscript in preparation). The reverse situation, i.e. suppression of the GTPases expression was obtained by transfecting small interfering RNA (siRNA) targeting each of these GTPases (Deroanne et al., 2003, 2005).

Experiments using these cells have been selected for the 2nd batch of experiments to be conducted in the Biolab facility on-board of the International Space Station. The deep impact of seric factors on the activity of Rho GTPases together with practical constraints related to the storage of frozen cells, their thawing and dilution on the ISS imposed a cryopreservation of the cells in serum-free conditions at -80°C. Here we describe the cryopreservative potential of various serum-free mediums that allow the persistence of the knock-down of the GTPases by siRNA after freeze-thawing of the cells.

WI-26 cells were transfected by siRNA targeting RhoA, Rac1 and Cdc42 using calcium phosphate precipitate. Naïve cells, cells treated with calcium phosphate alone or a with a scramble siRNA were used as control. After 16 hours the precipitate was washed-out and the cells kept for 48 hours in culture before cryopreservation procedure. Cells were detached from culture dish with trypsin, pelleted by centrifugation and suspended at 10^6 cells/ml in Dulbecco’s modified Eagle Medium (DMEM). Aliquots of cell suspension were pelleted, suspended in the indicated cryopreservative mediums, left at -20°C for 40 minutes and stored at -80°C. After the indicated periods of time, cells were thawed, diluted (1/20) in serum-free DMEM complemented or not by 1% bovine serum albumin (BSA), plated on collagen-coated wells and transferred at 37°C. After 4 hours the wells were washed twice with phosphate buffered saline and the number of attached cells was measured by the content of DNA assayed by fluorometry. The attachment of cells frozen in control cryopreservative medium (Medium A: fetal calf serum (FCS)/ dimethyl sulfoxide (DMSO) (95:5)) was taken as 100%.

In preliminary experiments, wild-type WI-26 cells were frozen in DMEM alone or supplemented with dextran, hydroxyethyl starch (HES), trehalose or DMSO as cryoprotective agents. After 11 days of storage at -80°C in DMEM alone about 25% of cells attached to the support. The addition of dextran (2 and 8%) and HES (2.5 and 5 %) resulted in a lower survival than DMEM alone, while trehalose (200 mM and 1 M) did not affect the cryoprotective effect of DMEM. As expected DMSO added some cryoprotective effect when present at 5%, and to a lesser extent at 10% (not illustrated).

In a second set of experiments, the cryoprotective potency of four mediums were tested on WI-26 (wild-type, expressing Rho GTPase QL or transfected with siRNA). The selected mediums were: DMEM/DMSO (95/5, = Medium B), Medium B containing 5% BSA (w:v, = Medium C), DMEM/ProFreeze (Cambrex)/DMSO (50/45/5, = Medium D) and Medium D containing 5% BSA (w:v, = Medium E). After 50 days at -80°C, cells were thawed and diluted (1/20) in DMEM containing 1% BSA before plating. Similar data (illustrated for WI-26 expressing Rac1 QL in Fig. 1) were obtained with the wild-type WI-26 and the various derived cells. They indicate that the rank of cryopreservative potency is Medium A = Medium C = Medium E > Medium D > Medium B.

**Fig. 1:** Cryopreservation of WI-26 cells in various mediums. WI-26 cells expressing constitutively active Rac1 were frozen at -80°C in various cryopreservative mediums. After 50 days they were thawed, plated onto collagen in serum-free DMEM, 1% BSA for 4 hours and the relative number of attached cells was measured. **A:** FCS/DMSO (95.5, = control medium); **B:** DMEM/DMSO (95.5); **C:** DMEM/DMSO (95.5) + BSA (5%, w:v); **D:** DMEM/ProFreeze/DMSO (50-45-5); **E:** DMEM/ProFreeze/ DMSO (50-45-5) + BSA (5%, w:v).
The cryoprotection offered by Medium C and E was further confronted to that of control medium for a variety of cell types (see Fig. 2). Data show that Medium E offered cryoprotection similar to or slightly better than control medium for the various tested cells except NIH3T3. Medium C was significantly less efficient.

![Fig. 2. Survival of several cell types after cryopreservation at –80°C for 50 days in Medium C and E. Data are expressed as the percentage (mean ± S.D) of attached cells as compared to control medium taken as 100%. MO-1, JO-1: human primary fibroblasts; MG-63: human osteosarcoma cells; A2058: human melanoma cells; ROS: rat osteosarcoma cells; NIH3T3: mouse transformed fibroblasts.](image)

Finally, the impact of freeze-thawing on the knock-down of the GTPases expression by siRNA was tested. WI-26 cells were transfected by siRNA as previously described (Deroanne et al., 2005) before freezing. After 50 days of storage, cells were thawed, harvested in DMEM containing 1% BSA, plated for 24 hours and then lyzed in Laemmli buffer. The expression of the GTPases was measured by Western blotting using antibodies specific for the three GTPases or against ERK1/2 as a control of protein loading. Transfection with the cognate siRNA reduced the expression of RhoA and Cdc42 by about 90% and that of Rac1 by about 70%, i.e. to levels identical to those observed in the cells before freezing, regardless of the cryopreservative medium used (illustrated for Rac1 and Cdc42 in cells frozen in medium C, Fig. 3).

In conclusion these data demonstrate the feasibility to store cells frozen in serum-free cryopreservative medium at –80°C on Earth and to plate them after dilution with serum-free medium on ISS. Moreover, they show that siRNA-directed knock-down of proteins is retained in these conditions.

**Fig. 3: Knock-down of Rho GTPases expression by siRNA is effective and retained after storage at –80°C. Cells were left untreated (1), mock-transfected (2) or transfected with siRNA against RhoA (3), Rac1 (4) or Cdc42 (5) or a scramble siRNA (6). After two days the cells were frozen in medium C for 50 days, thawed, plated and harvested after 24 hours. Total cell extracts were run on SDS-polyacrylamide gels and blotted to membranes probed with anti-Rac1, anti-Cdc42 or anti-ERK1,2 antibodies.**

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Testing of Saccharomyces cerevisiae Morphological Fixatives and Fixed Samples Stored at Ambient Temperature

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EMMYS-1, Effects of Microgravity on Model Yeast Specimen, is a yeast payload designed to investigate samples grown on-orbit for morphological and molecular analysis. Saccharomyces cerevisiae (yeast) is considered a model eukaryotic microorganism for space studies in part due to its small size, fully sequenced genome, availability of mutants covering most of the open reading frames and ability to be stored dry. Yeast is commonly used to study radiation effects, protein-protein interactions and cell regulation pathways.

Due to the constraints set by current payload scenarios, EMMYS-1 is being optimized for storage and operation at ambient temperature for a period of up to six months. Current testing includes determining the timeline of the experiment (activation and termination at either the beginning or ending of the flight scenario) and fixative-hardware compatibility with the goal of obtaining samples useful to the scientific community. To recommend the most appropriate flight scenario for optimal science return this study investigates hardware compatibility, fixative stability and staining capability.

S. cerevisiae, strain BY4743, were grown in Yeast-Peptone-Dextrose (YPD) at 30°C to an O.D. (λ=600 nm) of 1.5 to 1.7 for all experiments. Fixatives tested were formaldehyde, paraformaldehyde, glutaraldehyde and acetic acid based fixatives at various concentrations in either Phosphate Buffered Saline (PBS) or Cacodylic Acid (CA). The hardware being optimized for the EMMYS-1 flight experiment is BioServe Space Technologies’ Group Activation Pack (GAP), which allows for simultaneous activation of 8 Fluid Processing Apparatus’ (FPA), while maintaining three levels of containment.

To determine materials compatibility, septa from the FPAs were submerged in 5 ml of fixative and stored in the dark at ambient temperature (19 to 23°C). Observations were made on the appearance of the septa at regular time intervals. All fixatives, except glutaraldehyde and fixatives with acetic acid, showed no negative interactions with the septum after approximately 8 months of storage in the dark at ambient temperature (data not shown). Septa stored in glutaraldehyde and acetic acid based fixatives developed ridges on the surface.

To test chemical containment ability of the septa in the FPA, YPD was stored in one chamber with 12% formaldehyde in 30% acetic acid stored in the adjacent chamber at ambient temperature for up to nine months. The YPD was then extracted and a S. cerevisiae growth curve was obtained. There was no difference in growth curves between yeast grown at 22°C in YPD stored in FPAs with fixative when compared to those grown in YPD stored in FPAs without fixative (Figure 1).

To analyze the fixative effectiveness over a period of time, fixative ability to kill yeast within one minute exposure was tested (time determined empirically). Fixatives were freshly prepared and stored at either 4°C or 25°C. Aliquots were taken on a periodical basis, mixed with yeast in a 1 to 2 ratio and washed with YPD. Samples were allowed to recover for 30 minutes then plated onto YPD agar plates. Plates were incubated for two days and subsequently counted for colony forming units. 4% fixatives stored for one month at 25°C were not able to completely kill the yeast within the one minute (data not shown).

The samples stored up to one month, used for the fixative stability testing, were also used for staining capability. In addition, fixed samples stored at ambient temperature for up to 8 months were also tested. DAPI, Calcofluor, Mithramycin and Acridine Orange staining methods were performed based on protocols assembled by Hasek and Streiblova (1996). In this report, only DAPI results are presented; these results reflect those obtained for the other nuclear stains (Mithramycin and Acridine Orange). Percentage stained was determined by the number of properly stained yeast divided by the total number of yeast multiplied by 100.

Yeast fixed then stored at ambient temperature for one month before staining showed a dramatic decrease in the percent of DAPI stained yeast when compared with those stained 5, 7 and 10 days after fixation (Figure 2). No nuclear staining was observed in yeast fixed and stored at ambient for one month in 12% formaldehyde in 0.6M CA.

Figure 1. Results of the FPA chamber containment test. FPA A (8 months) and FPA B (9 months) represent growth curves of yeast in extracted YPD stored in FPAs that contained fixative in the fixative chamber. FPA Control represents growth curve of yeast in extracted YPD stored in FPAs that did not contain fixative in the fixative chamber. There is no significant difference between yeast grown in YPD stored with fixative in the FPA and the YPD stored alone in the FPA.
Figure 2. Percent of yeast stained with DAPI after being fixed and stored at ambient for 5 days, 7 days, 10 days and 1 month. There was a dramatic decrease of approximately 70 percent in yeast stored fixed for one month then stained. Data on yeast fixed for one month in 12% formaldehyde in 0.6M CA showed no stained cells.

Higher percentage fixatives stored at 25°C for one month showed significant (p<0.001) increase in yeast cells stained properly with DAPI compared to 4% fixatives (Figure 3). In general, yeast fixed with one month old formaldehyde based fixatives had a higher percentage of stained yeast compared to one month old paraformaldehyde based fixatives.

Figure 3. Percent yeast stained with DAPI seven days post fixation with fixatives stored one month at 25°C. There was a significant difference between the 4% formaldehyde and 4% paraformaldehyde with the higher percentage fixatives. *symbol represents significant difference (p<0.001) compared to other columns.

Yeast fixed with 12% formaldehyde based fixatives stored at ambient temperature over eight months showed no significant difference compared to fixative stored at 4°C for two weeks when stained with DAPI (Figure 4). In addition, viability tests using the fixatives stored at ambient temperature for eight months showed no viable yeast after one minute exposure (data not shown).

Figure 4. Percent of yeast stained with DAPI seven days post fixation. Fixatives used were two week old fixatives stored at 4°C (control) and those stored for eight months at ambient temperature. Yeast cells were fixed and stored at ambient for 7 days prior to staining with DAPI. There is no significant difference between the control and the eight month old fixatives.

In summary, the hardware compatibility tests determined that either formaldehyde or parafomaldehyde based fixatives (in PBS or CA) can be used for up to a 6 month ambient temperature flight scenario. Furthermore, the FPA can be stored in its inactivated form for the expected flight scenario since the growth curves analysis of the YPD media stored in FPA containing fixative in the fixative chamber suggest that the septum provides an adequate level of containment between the two chambers.

Viability tests suggest that higher concentration fixatives are more stable than lower concentrations. Yeast fixed in higher percentage fixative stained better than at lower percentages. Although both the 6 and 12% formaldehyde and parafomaldehyde fixatives performed well after one month at 25°C, it was observed that formaldehyde based fixatives have an overall higher percent of stained yeast. The results from the staining and viability analysis of 12% formaldehyde based fixatives stored at ambient temperature for over eight months demonstrate that the fixative will be stable for the expected six month ambient flight scenario and still allow for proper staining to occur. Since yeast fixed and stored at ambient show a decrease in the number of stained yeast over time, the flight scenario where incubation and fixation occurs at the beginning is not recommended.

These results demonstrate that the optimal flight scenario for EMMYS-1 will consist of 1) incubation and fixation just prior to return of payload and 2) 12% formaldehyde based fixative for morphological analysis. However, our results could not rule out either 6% formaldehyde or 6 and 12% paraformaldehyde for fixative purposes. Further studies will include testing of fixatives for antibody staining, Green Fluorescence Protein staining and Electron Microscopy analysis.

REFERENCE
POSSIBLE INVOLVEMENT OF FLOW DETECTION IN THE ACTIVATION OF OSTEOBLASTS

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One of the most potent mechanical stimulators for bone cells may be the interstitial fluid flow. Fluid flow induces various events such as Ca2+ signaling, c-fos expression, actin fiber reorganization, and appearances of bone markers in cultured cells (Chen et al., 2000). Fluid shear stress may distort cellular membrane and other structures, inducing cellular response. However, the nature of molecules that convert the mechanical strain into biological signals remains unclear.

The difficulty in identifying the mechanosensor molecules seems to lie, at least in part, in the lack of reliable methods for analysis. A contrast to regular cell culture experiments, that compare the effects of dilute substances, cellular responses to mechanical stimulations are often difficult to interpret. The mechanical stimulation may alter culture conditions drastically. It is extremely difficult to apply only mechanical stimulations, leaving all other conditions uniform. Excluding the effects of such environmental changes, induced secondarily by the operational procedure, is almost impossible. Thus, this study seeks to develop a simple method for applying mechanical stimulation with minimum secondary influences, especially from temperature changes during laboratory procedures.

We observed that conventional laboratory handling of culture flasks could stimulate the murine osteoblastic cell line MC3T3-E1. Fluid flow, generated either by manual rocking or by unplanned agitation of culture flasks, possibly induced c-fos expression to a far greater extent than that caused by intended stimulation such as centrifugation (Takaoki et al., 2004). Moreover, cooling of the culture fluid, due to handling at room temperature, induced similar response. To distinguish the effect of rocking from the influence of cooling, we successfully utilized small Styrofoam boxes and heat accumulators. With this simple system, it was shown that either fluid flow or cooling independently induced the responses (Figure 1). Although flow-cells are normally used for the purpose (Stevens and Frangos, 2003), this rocking method was suitable for the parallel analyses of responses in multiple flasks.

The response reached a peak 10 to 15 min. after the stimulation and settled to the background level within an hour. The second stimulation given 60 min. later resulted in a comparable response (Figure 2). In this study, we kept all cultures inside the incubator without any disturbance for at least 2 hours before rocking.

There was some possible mediation of the c-fos expression response by Ca2+ and the ERK signaling pathway. The addition of an ERK inhibitor, U0126 suppressed the response (Figure 3).

Fluid flow can be more efficiently detected when seen as a drag force against a projected structure than as a shear stress to a smooth surface. One such projection, primary cilium, served as a flow meter in kidney epithelial cells. A transient receptor potential (TRP) channel family protein, polycystin-2 (PC2) with polycystin-1, is one of the key molecules for this function. PC2 localized at the primary cilium distorted by the drag of flowing fluid are sent to neighbouring cells through gap junctions (Nauli et al., 2003). Whittfield (2003) discussed the possible involvement of similar mechanisms in bone cells. Interstitial fluid flow could be caused by the slightest distortion of bone tissue by a mechanical load. This flow could be easily detected by sensitive flow detecting primary cilia.
Primary cilia were found in the osteoblastic cell line MC3T3-E1. The cells also expressed PC2. These observations suggested that bone cells and kidney cells detected fluid flow through similar mechanisms. However, the localization of PC2 to the primary cilia was not as preferential as had been reported in kidney cells (Figure 4). This weak localization of PC2 in the primary cilia and the difference in reported flow sensitivities (Chen et al., 2000, Nauli et al., 2003) suggested the involvement of molecules other than those in kidney cells.

**Figure 3.** Suppression of c-fos response to fluid flow by U0126 37°C. Values are means and standard errors for 4 flasks.

**Figure 4.** Immunofluorescent staining of primary cilium (arrow) of an MC3T3-E1 cell. Immunostainings with anti-acetylated α-tubulin (AcTub) or anti-PC2 antibodies (right) are shown.

We observed that MC3T3-E1 cells expressed c-fos in cooling as well as in rocking of the culture flasks. This response to the cooling would not seem physiological, since we could find virtually no literature describing temperature dependencies in bone metabolism. Nevertheless, the response could be a factor observable only under culture conditions with a cell line. Detection of lowered temperature by PC2 or other TRP channel superfamily proteins could also be possible. Some TRP channel superfamily proteins have been shown to sense either cold, cool or hot temperatures (Clapham, 2003), while another member was assumed to sense mechanical stimuli (Corey et al., 2004). Most of these proteins are expressed in the respective neurons in a highly specific manner, but not in bone or kidney cells. However, we have not entirely excluded the possibilities of mechanosensing and temperature sensing by PC2, or contributions of other bone-specific TRP channel superfamily proteins.

The transient c-fos expression response could be induced by a variety of stimulations. These stimulations could be applied, either intentionally or unintentionally, to the cell culture during laboratory procedures. However, the effects of these stimulations might be considered trivial in regular experiments, where both reference controls and experimental groups undergo virtually identical environmental changes, and are treated side by side in a similar manner. On the other hand, cultures must usually be placed in separate locations when applying different mechanical stimulations. Therefore, it is extremely important to keep the environmental changes, such as temperature, humidity, and CO₂ concentration uniform among the groups. This is particularly true in space experiments, which must be precisely controlled to enable comparisons between flight and ground specimens.

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Astronauts and cosmonauts are exposed to a wide variety of different hazards while in space that include radiation, which presents one of the most critical barriers to long-term missions. A major deleterious effect directly associated with ionizing radiation is the production of reactive oxygen species (ROS) such as peroxides and hydroxyl radicals. The free radicals generated by ultraviolet (UV) or ionizing radiation can attack cellular lipids, proteins and DNA [1]. Endogenous free radical scavengers such as glutathione and the polyamines (e.g., spermidine and spermine) can inhibit the action of ROS. In particular, heme oxygenase-1 (HO-1), the enzyme involved in heme protein metabolism, can provide antioxidant protection through the production of the antioxidant bilirubin [2]. Furthermore, polyamines have been shown to indirectly increase HO-1 content and antioxidant protection[2]. The β2-adrenoceptor agonist clenbuterol has been shown to stimulate polyamine synthesis and by extension, might provide a margin of antioxidant protection through increasing HO-1 content. However, it is unclear whether the polyamines are acting as a tertiary messengers for antioxidant protection in the β2-adrenoceptor signal transduction pathway [3]. The purpose of this study was to study the role of the polyamine pathway in attenuating free radical-induced damage.

**Methods.** Cells/Culture Conditions: Human fetal retinal cells (Ret 205, an SV-40T immortalized cell line) were a gift from Dr. Kamla Dutt [4]. Stock monolayer cultures were fed DMEM/F12 (1:1) medium supplemented with: 10% FBS, NaHCO3, glutamine and gentamycin. Experiments were carried out in 12-well trays (1.5 mL media/well) when cells were at 75% confluency, they were differentiated using 8-bromo cAMP [4]. Treatment: All drugs were made up in phosphate buffered saline (PBS) and subsequently added to their appropriate wells (100 µL/mL). The vehicle control wells received just PBS in the same volume as the treatment and/or vehicle control. Exposure: Immediately before treatment (with vehicle, drugs or enzymes), cells were fed fresh DMEM/F12 media [with pH indicator] and incubated 24-hours prior to UVC exposure. Trays were placed on warmers (37°C) in the cell culture hood, uncovered and exposed to a UVC germicidal lamp for 30 min (398.1 J/cm2, measured through the use of a thermopile - the distance to source was 71 cm). All other sources of light were off during UVC irradiation, and both medium and cells were exposed to UVC. Following exposure, cells were incubated for an additional 24 hours prior to analysis. Analysis: Cell viability was determined 24 hours post exposure by the Trypan Blue dye exclusion method and cells counted on a hemacytometer. HO-1 content was measured by ELISA (StressXpress). Calculations: Cell viability was calculated as either the percent of irradiated control [%UV Control= (Experimental/Control)] or %Difference of Control [%Difference= (Experimental–UV Control)/UV Control]. With % of Control, the control is at 100%, while with % Difference of Control, the control is at 0%. Thus, any value less than the control would be a negative effect, meaning enhanced cell death, while any value greater than 0% would be protective.

**Results.** Cells were pre-treated with the β2-adrenoceptor agonist clenbuterol (Cb, 10^{-7}M) and the antioxidant enzymes catalase (CAT, 10 units/mL) and superoxide dismutase (SOD, 10 units/mL) and incubated for 24-hrs before exposure to UVC radiation (exposure time: 30 min). Treatment with Cb, CAT and SOD significantly increased the rate of cell survival: Cb 2.13-fold (±0.34), CAT 2.61-fold (±0.26) and SOD 3.00-fold (±0.15), respectively, when compared to the UV control (1.0 ±0.05) (Figure 1). The combination of CAT or SOD with Cb produced significantly higher (p< 0.05) levels of cell viability than in those treated with CAT or SOD alone. Data expressed as mean ± S.D. To establish that Cb was acting through the β2-adrenoceptor in providing a measure of protection against UVC-induced oxidative damage, the β2-adrenoceptor antagonist ICI-118551 (ICI, 10^{-6} M) was used to competitively inhibit Cb. When treating cells with both ICI and Cb, the cells were initially treated with ICI (10^{-6} M) and one hour later the cells received Cb (10^{-7} M). All wells...
received the same final volume of treatment and/or vehicle control (Figure 2). Cell viability, measured as the percent difference from the irradiated control (set to 0% difference, see methods). Treatment with ICI alone showed a small but significant (p<0.05) agonist effect but ICI significantly (p<0.01) attenuated Cb′s protective effect in the combination treated controls, but HO-1 was significantly (p<0.001) higher than the non-treated UV Controls. HO-1 content in the UV control group was significantly less than in the non-exposed control group. In the Cb + ICI group, HO-1 levels were not significantly different from the Cb-treated group. However, DFMO significantly inhibited Cb′s effect on HO-1 content, thereby showing that the polyamines were acting, in part, as tertiary messengers in Cb-induced antioxidant activity (Figure 4).

Discussion and Conclusion. Exposure of the media to UVC radiation results in the production of ROS such as peroxides and hydroxyl radicals. Following exposure to UVC radiation, cells showed significant levels of cell death. Treatment with the antioxidant enzymes CAT and SOD significantly increased cell viability, suggesting ROS involvement. Likewise, treatment with the β2-adrenoceptor agonist Clenbuterol (Cb) or the ODC substrate Ornithine (Orn) significantly increased cell viability in exposed cells. In contrast, the ODC inhibitor DFMO increased the level of retinal cell death and when combined with Cb or Ornithine, it attenuated their protection. This suggests that polyamines mediate Cb/Orn-induced antioxidant protection. Previous studies conducted in our laboratory showed that Cb increased skeletal muscle polyamine concentrations while reducing atrophy, thus further supporting the protective role of polyamines [3]. In addition, the ability of Cb to act through the polyamine spermine (as an NO donor) in stimulating HO-1 activity and the subsequent production of bilirubin results in increased antioxidant protection and cell viability [2]. Thus, with increased protection provided by both elevated polyamine and bilirubin levels, the magnitude of UV-induced cell death is reduced. This study confirms that Cb acts through polyamines and the antioxidant enzyme HO-1 to protect against UV-induced oxidative damage. This work was supported by NASA Grant NCC9-112 and NIH Grants 1R25RR17694 and 5P20RR011104.

References.
ERYTHROPOIETIN AND IL-3 RECEPTOR CELL SURFACE EXPRESSION IS DECREASED UNDER CONDITIONS THAT MODEL SOME ASPECTS OF MICROGRAVITY

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Human and experimental animals in space develop the anemia of space flight (Tavassoli, M., 1982; Udden et al., 1995). Studies have shown that erythropoietin (Epo) is implicated in this microgravity-induced abnormality (Udden et al., 1995). The status of the Epo receptor (EpoR) in microgravity, however, is unknown. The Rotary Cell Culture System (RCCS), based on NASA rotating wall vessel (RWV) technology and manufactured by Synthecon Inc., is an in vitro culture system. Within this system individual cells, to a certain extent, experience an environment with similarities to true microgravity (Gao et al., 1997; Battle et al., 1999). BaF3 cells stably expressing the transfected human Epo receptor (BaF3-EpoR cells) were cultured in RPMI-1640 supplemented with 5% fetal bovine serum and either 1 unit/mL of recombinant human Epo (rhEpo) or 5 ng of recombinant mouse interleukin-3/mL (rmIL-3) in either the RCCS or in 175 cm² standard tissue culture flasks (control) at 37 °C for 48 hours. Cells were then harvested by centrifugation, incubated with 125I-labeled rhEpo or 125I-labeled rmIL-3, and the bound/free ligands were separated by centrifuging the cells through a serum cushion according to published methods (Yonekura, 1991). The radioactivity of the cell pellet was quantified by gamma scintillation spectrometry.

Starting from 0.1 nM of 125I-Epo (the lowest concentration tested) upward, the specific binding of radiolabeled ligand to flask-cultured cells ( []) was significantly greater than that to RCCS-cultured cells ( □) (p < 0.01) (Figure 1). At 3.2 nM (the highest concentration tested), the bound radioactivity (surface EpoR density) of RCCS-cultured cells was only 56.6% of that of the flask-cultured counterpart. The non-specific binding of radiolabeled Epo to the flask- ( ▲) and RCCS ( △)-cultured cells were similar.

Figure 1. The binding curves of 125I-Epo to BaF3-EpoR cells grown in flasks ( ▲) and RCCS ( △). Each point shown represents the mean of triplicates ± standard errors (S.E.).

To determine the specificity of the RCCS effect on EpoR, the abundance of IL-3 receptor was also studied. Similarly, 125I-IL-3 binding to RCCS-cultured cells was significantly reduced compared to that to flask-cultured cells (p < 0.01) (Figure 2). This result suggests that culture in the modeled (simulated) microgravity environment of the RCCS has a generalized effect on cell surface growth factor receptors.

Cell viability from both flask- and RCCS-cultured cells was assayed by trypan blue dye exclusion. Both exhibited cell viabilities of greater than 90% (p > 0.05) (Figure 3). This result excludes the possibility that the effect of RCCS culture on cell surface receptors is due to mechanical damage to the cells or other intrinsic factors, such as toxicity.

Figure 2. The binding curves of 125I-IL-3 to BaF3-EpoR cells grown in flasks ( ▲) and RCCS ( △). Each point shown represents the mean of triplicates ± S.E.

Figure 3. Histogram of survival rates of BaF3-EpoR cells grown in RCCS and flasks. Each bar shown represents the mean of triplicates ± S.E.
The possibility of nutrient depletion as a factor contributing to the reduction of cell surface Epo receptors was excluded by the following study. Cells were cultured in RCCS with either 1 (\textbullet) or 2 (\textcircled{2}) mg/mL of glucose in the cell culture medium. No changes were observed in both specific binding (SB) and non-specific binding (NSB) between the two glucose levels (Figure 4).

![Figure 4. The histograms of \textsuperscript{125}I-Epo binding to Ba/F3-EpoR cells grown in Low (\textbullet) and high (\textcircled{2}) glucose in RCCS. Each bar shown represents the mean of triplicates \pm S.E.](image)

This is the first study showing possible microgravity effects on the EpoR and the potential involvement of the EpoR in the anemia of space flight. This study also suggests that the expression of other cell surface growth factor receptors may be affected by gravity.

REFERENCES


Materials for cell wall growth are delivered to the wall by secretory vesicles, and recent studies have highlighted the role of secretion in plant growth generally and plant gravitropic growth specifically. Funke and Edelmann (2000) show that the growth of graviresponding nodes of *Tradescantia* is controlled by auxin-induced secretion and subsequent infiltration of wall materials that promotes wall extension. Similarly, Zhang and Hasenstein (2000) demonstrate that Brefeldin A, a potent inhibitor of the delivery of secretory vesicles from the trans-Golgi to the plasma membrane in plant cells (Driouich et al, 1993), blocks the asymmetric delivery of expansins to the cell wall of graviresponding maize roots. They further show that this effect is correlated with a delay in the graviresponse. The data of Morris and Robinson (1998) reveal that Brefeldin A also blocks actin-dependent cycling of PIN proteins, which serve as important auxin efflux facilitators, to the plasma membrane, and they strongly suggest that other auxin transport inhibitors may work by interfering with membrane-trafficking processes.

Annexins are a multi-gene, multifunctional family of calcium-dependent membrane-binding proteins found in most eukaryotic cells. Annexins appear to be important targets of Ca^{2+} action in plant cells and are proposed to be key participants in the process of secretion of newly synthesized plasma membrane and wall materials during growth and development. We have identified eight different members of the annexin gene family in *Arabidopsis*. Using in situ RNA localization, we found that two of these annexins, AnnAt1 and AnnAt2, exhibited mostly distinct localization patterns with highest expression levels in secretory cell types in young *Arabidopsis* seedlings (Clark et al., 2001).

More recently, we have obtained immunolocalization data for these two individual annexins in conjunction with autoradiography data of ^3H^-galactose incorporation in young *Arabidopsis* seedlings (Clark et al., 2004). For the immunolocalization experiments we used monospecific anti-annexin antibodies raised against divergent 31-mer peptides from AnnAt1 and AnnAt2. These peptides show only 35% amino acid identity. As a marker for polysaccharide secretion, we used ^3H^-galactose, which is taken up by seedlings, converted into more complex polysaccharides and detected by immunocytochemistry. We observed strong similarities between this pattern of secretion and the individual protein localization patterns for these two annexins in most cases.

In the present study, we applied these same techniques to determine the localization pattern for these two individual annexins and surveyed secretion activity in graviresponding hypocotyls and roots in young *Arabidopsis* seedlings. We also examined hypocotyl and root growth for several T-DNA mutants of these two annexins in the dark.

Figure 1. Autoradiography of ^3H^-galactose incorporation and annexin immunostaining in young Arabidopsis shoots that have been gravistimulated, showing (A) asymmetric deposition of labeled polysaccharides in mainly epidermal cells of the hypocotyl (arrows), and (B) asymmetric anti-AnnAt2 staining of the hypocotyl epidermis that corresponds to the faster growing side (arrows).

Figure 2. Autoradiography of ^3H^-galactose incorporation and annexin immunostaining in young Arabidopsis roots that have been gravistimulated, showing (A) asymmetric deposition of labeled polysaccharides in mainly epidermal cells of the root (arrows), and (B) asymmetric anti-AnnAt1 staining of the root epidermis that corresponds to the faster growing side (arrows).

Figure 1 shows asymmetric polysaccharide secretion as demonstrated by ^3H^-galactose incorporation and asymmetric localization of AnnAt2 in epidermal cells of gravistimulated hypocotyls. In gravistimulated roots, we also observed asymmetric polysaccharide secretion as demonstrated by ^3H^-galactose incorporation and correlated this secretion asymmetry with an asymmetric localization of AnnAt1 in epidermal cells (Figure 2). In both cases, we observed an asymmetric distribution pattern for an individual annexin and for labeled polysaccharides which were almost identical for the faster growing side. The asymmetric ^3H^-galactose incorporation and annexin immunostain shown in gravistimulated seedlings was repeated several times to insure that sectional differences were not the source of the asymmetries. Additionally, control non-stimulated
seedlings were run in parallel and showed an even distribution of ³H-galactose incorporation and annexin immunostain in the epidermal cells on both sides of hypocotyls and roots, respectively.

In order to clarify the function of individual Arabidopsis annexins we have obtained T-DNA knockouts for these two annexins. In initial experiments, an annexin T-DNA knockout mutant for AnnAt2, annA2-1, shows inhibited hypocotyl growth in 3.5-day-old dark grown seedlings (Figure 3a) and annexin T-DNA knockout mutants for AnnAt1, annAt1-1 and annAt1-2, show inhibited root growth in 7-day old dark grown seedlings (Figure 3b).

We have presented autoradiographic and immunolocalization data linking two different annexins with the enhanced secretion that accompanies asymmetric growth during gravitropism in Arabidopsis. Specifically, there is a correlation with asymmetric secretion and differential localization of AnnAt1 in gravistimulated roots and AnnAt2 in gravistimulated hypocotyls. Additionally, we have shown that T-DNA mutants for AnnAt1 exhibit inhibited growth in roots and a T-DNA mutant of AnnAt2 exhibits inhibited growth in hypocotyls. Taken together these results suggest that these two annexins may play a role in differential growth during gravitropism.

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REFERENCES


PHOTOTAXIS AND AEROTAXIS IN A CALCIFYING ALGA
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INTRODUCTION

The coccolithophore, *Pleurochrysis carterae*, is a unicellular marine alga, with an outer covering of scales known as coccoliths, instead of a cell wall (Figure 1; 1). Coccoliths are composed of calcium carbonate (CaCO₃) in the form of calcite, and are formed intracellularly in the Golgi cisternae in a very precise and well defined process before being positioned outside the cell membrane (1). Because this is an excellent model of calcification in a single cell, we proposed *P. carterae* for flight on the International Space Station, and spent several years in experiment development prior to having the experiment deselected from flight. In addition to being a model for calcification, *P. carterae* was to have been used to address questions of reproduction, cellular polarity, secretion, and gravitaxis in the microgravity environment (2-4). Requiring only low levels of light and capable of being stored for over 70 days in the cold and dark before use, *P. carterae* remains an excellent choice for space flight studies (5,6). Additional descriptions of the proposed experiment and various educational links regarding this alga can be found at (7).

In preparation for the gravitaxis portion of the spaceflight experiment, we spent considerable time assessing gravitaxis in *P. carterae* at 1G, using the Wintrack 2000 software. In these studies, we determined that cells of *Pleurochrysis carterae* do not start to move in a measurably oriented manner until a certain cell density is reached, concomitant with the start of bioconvection (convection due to movement of microorganisms.) The bioconvective process begins with the accumulation of cells at the surface of the medium, which in turn requires that the cells swim up (8). The objective of the current study was to determine if taxes other than gravito- or gyrotaxis, specifically photo- and aerotaxis, were the cause of surface accumulation of cells. Because our deselection was due to lack of videomicroscopy, macroscopic methods were used in these experiments.

MATERIALS AND METHODS

Cells from a stock cell culture of *P. carterae*, Plymouth strain 136, a swimming strain, were expanded in F/2 medium (18°C), then divided into 4 t-flasks. After 24 hours, cultures were observed for accumulations of cells in the region of the air bubble. For phototaxis, box covers, with only a small opening at the bottom of one side, were placed over the t-flasks as an obstruction of the light source. Cells in these cultures did not accumulate in any region of the culture, and cultures did not grow due to the lack of light. The t-flasks were then placed horizontally in the incubator with the boxes covering all but the top 25% of the flask. The air bubble was located in the dark region of the flask. Flasks were incubated overnight, and observed for accumulation of cells in particular regions of the flask.

RESULTS AND DISCUSSION

During the initial culture of the cells to expand cell numbers, a green halo of cells surrounded the air bubble in each flask, demonstrating aerotaxis. The first phototaxis experiment used boxes that completely covered the flask, with only a small opening for light. This arrangement did not support cell growth as after the incubation period, no accumulations of cells were seen. This agrees with previous experiments in which we

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**Figure 1:** Scanning electron micrograph showing coccoliths on the surface of *P. carterae*. Photo by Dr. Mary Marsh.

**Figure 2:** T-flask from phototaxis experiment. Cells are visible as a cloud in the medium, and are located in the left hand side of the flask which was exposed to light during culture in the incubator.
demonstrated that no increase in cell number is seen in cultures of *P. carterae* kept in the dark.

In the partially covered t-flasks, cells accumulated in the portion of the flask that was not covered, demonstrating phototaxis (Figure 2). The bubbles in these flasks were carefully sequestered in the dark region, so that the aerotaxic response would not interfere with the phototaxic response. The lack of accumulations around the air bubble in these cultures demonstrates that the cell’s response to light is stronger than the response to the presence of air. Similar results were seen in experiments with *Euglena gracilis*, a fresh water photosynthetic alga (9).

These simple experiments demonstrate that macroscopic methods can be used to observe movement of populations of algal cells, thus obviating the need for microscopic observations to see overall cell accumulations. These observations, which can be captured automatically on videotape in several of the current cell culture systems developed for ISS, can be used to determine if bioconvection occurs in space. Although bioconvection, according to theory, should not occur in a microgravity environment, there may be enough g disturbances on ISS to begin the process, which might be then continued by swimming action of the organisms themselves.

The results in these studies were definitely density dependent—at lower densities the cloud of cells would not be visible to the naked eye. At higher densities, bioconvection would occur.

Finally, such results must be taken into account when designing space flight experiments—for example, the light source should be perpendicular to the gravity vector in the culture system to ensure that the cells are responding to gravity and not to light. Alternatively, during g-response experiments, an infrared source of light can be used.

**CONCLUSIONS**

From this experiment we conclude that *P. carterae* is aerotaxic and phototaxis, and that the phototaxic response is stronger than the aerotaxis response. In the ocean, *P. carterae* is found near the surface of the water, due probably to phototaxis and aerotaxis, not gravitaxis or gyrotaxis. Future experiments will determine the response to air during the nonphotosynthetic period of the light:dark cycle.

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**REFERENCES**


CO-EXPRESSIOIN AND HORMONAL REGULATION OF GENES IN RESPONSE TO GRAVITY AND MECHANICAL STIMULATION IN THE *ARABIDOPSIS* ROOT APEX.
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Plant roots direct their growth in response to gravity, light, and mechanical stimuli. Spatial changes in the rates of cell elongation and division are responsible for the directional growth. Local changes in hormone concentration and/or sensitivity have been shown to be part of the signal transduction and response mechanisms that result in those tropic growth responses. Part of most hormone regulated mechanisms are regulation of transcription and transcript stability. We have focussed our analysis of whole genome microarrays on the differential regulation of transcript abundance changes during the first hour after gravity and mechanical stimulation with respect to the involvement of hormones – especially auxins and brassinosteroids.

Root apices from 7-day-old etiolated *Arabidopsis* seedlings were harvested and analyzed for relative changes in transcript levels in response to gravistimulation and mechanical stimulation using the *Arabidopsis* ATH1 GeneChip (Affymetrix). In a time course experiment, approximately 150 root tips were harvested before (0 time point) and 2, 5, 15, 30, 60 min after 135° reorientation by pouring RNAlater (Ambion) onto the plates and cutting off the root apex (~7.5 mm). Mechanical stress control seedlings (0, 2, 5, 15, 30, 60 min) were moved horizontally for 5 sec without changing their orientation towards the vector of gravity and were processed in an identical manner. Total RNA was extracted and purified (RNeasy column, Qiagen), amplified and hybridized to microarrays using standard protocols (Kimbrough et al. 2004).

From 24,000 transcripts analyzed, we found 1730 with significant changes in abundance at two consecutive time points after reorientation (gravity) and 1691 transcripts regulated after mechanical stimulation (mechanical). While both stresses had 1641 regulated transcripts in common (Figure 1), 65 genes were gravity-specific up regulated and 26 were specifically regulated by the mechanical stimulus. Of those regulated by mechanical stimulus, 16 showed an increase in abundance and 10 transcripts showed a decrease compared to the vertical control. Of the transcripts regulated by both stimuli, 897 showed an increase in abundance and 744 a decrease (Kimbrough et al. 2004). Many of the stimuli specific genes exhibited co-regulation throughout the time course (Figure 2).

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**Figure 1:** Genes regulated (up or down) by both stimuli were classified into functional groups. The number represents the percentage of genes in each functional group from all genes regulated under both stimuli.

**Figure 2:** Intensities of red and green reflect degree of up or down regulation, respectively, compared to vertical control (0).
Hormones including, but not limited to auxin, cytokinins and brassinosteroids are known to be mediators regulating gene expression changes in plants. Previously identified genes whose expression levels change upon treatment with cytokinins (Rashotte et al., 2003), brassinosteroids and/or auxin (Goda et al., 2002) were analyzed to determine their transcript abundance profile throughout gravity and mechanical stimulation (Figure 3). We observed many hormone regulated genes that were also regulated throughout gravity and mechanical stimulation in a similar fashion. In some cases, transcripts showed distinct differences in their temporal profiles. A specific example of this is the cytokinin regulated gene for a disease resistance protein (At4g11190, see Figure 3C). This gene showed up-regulation from 2 through 15 minutes during gravity stimulation but was not up-regulated until 30 minutes in mechanical stimulation. This suggests the regulation of this gene is controlled through a similar pathway but at different times in gravity or mechanical stimulation. The xyloglucan endoglucosyl transferase gene (TCH4) was observed to be transiently up-regulated throughout both mechanical and gravity stimulation and was previously shown to be up-regulated upon treatment of cytokinins and the combined treatment of BR and auxin. This analysis can reveal which genes are regulated by gravity/mechanical stimulation through certain hormones but it cannot determine local or spatial effects of hormones. It is probable that these hormones affect gene expression through gravity and mechanical regulated genes in specific cell types in the root or preferentially on the upper or lower side of the root to produce gravitropic curvature. Currently, we are analyzing the expression profile of certain genes in mutants affected in hormonal response. The results will allow us to identify whether genes regulated by gravity/mechanical stimulation and hormones are regulated through parallel pathways or if they are controlled in a single linear pathway.

REFERENCES


The alkali metal lithium is known to produce a number of effects in plants and animals. Lithium salts have been an important tool in dissecting the intracellular pathways of signal transduction systems. Lithium induces an increase in diacylglycerol (DAG) in animal cell culture; the mechanism of this effect is not fully understood. Brami et al. (1993) suggest that lithium may alter the degradation of phospholipids by acting on phospholipase C or D. In the second messenger signal transduction system phosphatidylinositol-4, 5-disphosphate (PIP2) is converted into phopholipase C (PLC).

Lithium can alter the second messenger signal transduction system by altering enzyme activity. Myo-inositol-1-phosphatase (I-1-P) converts D-glucose-6-phosphate to myo-inositol via the intermediate L-myo-inositol-1-phosphate. This enzyme can hydrolyze both the D- and L-enantiomers of I-1-P. Lithium inhibits myo-inositol-1-phosphatase activity in both plants and animals although plant tissue is less sensitive to the effects. In pollen of lily, 50 mM of lithium is required to produce 50% inhibition of the enzyme (Gumber et al., 1984).

It has been reported that lithium salts increase inositol phosphate levels in animal tissue but that lithium has no effect on inositol phosphate levels in plants. Morse et al. (1987) found no evidence that lithium enhances the recovery of inositol phosphates in the leaf pulvina of the legume *Samanea saman* during the night movement of the leaves. Additionally they found that inositol is not the rate-limiting factor in the biosynthesis of phosphatidylinositol in *Samanea saman*.

Lithium salts alter ethylene biosynthesis in mung beans (*Vigna radiata*) (Lee and Kang, 1987). Ethylene inhibits plant growth and is produced through the conversion of methionine to s-adenosylmethionine to aminocyclopropane carboxylic acid to ethylene. Lithium ions may alter ethylene biosynthesis by effecting the conversion of ACC to ethylene.

Lithium inhibits thigmomorphogenesis in *Bryonia dioica* (Boyer et al., 1983) and *Bidens pilosus* (Desbiez et al., 1981). Rubbing the internodes of *Bryonia* decreased the height of the plants by inhibiting cell division and elongation. Pricking one cotyledon on a *Bidens pilosus* plant inhibits the growth of the hypocotyl within one hour. Lithium treatment of the plants reduced the amount of inhibition which was observed. Thus it was concluded that lithium inhibition of thigmomorphogenetic responses resulted from lithium inhibition of the effect of ethylene formed in the mechanical stimulation of the plant tissues.

**Plant Material:** Grains of maize (*Zea mays* L., Pioneer 3343) were soaked overnight in running tapwater and placed between wet paper towels on opaque plastic trays. The trays were placed in a vertical position with the grains aligned along the vertical axis. The seeds were germinated at 30 C and used when the primary root is 1-1.5 cm in length.

**Measurement of Elongation:** A computer-based root auxanometer system (Mulkey et al, 1982b) was used to determine the effects of lithium on elongation of intact primary roots of maize. Roots were placed into the auxanometer chamber containing half-strength Meyer's solution.

**Isolation of Membrane and Cytoplasmic Fractions.** One-cm root tip segments (at least 3 g of roots) were ground on ice with homogenization buffer containing 50 mM MES-NaOH, pH 7.0, 5 mM MgCl2, 0.5 mM DTT, 0.25 M Sucrose, 5 mM EDTA, and 0.5 mM PMSF using a mortar and pestle (1:2 w/v). The homogenate was filtered through Miracloth and centrifuged for 10 min at 7,000 g to discard nuclei and cell walls. The supernatant was collected, centrifuged for 30 min at 100,000 g for 30 min; and the supernatant was used for procedures requiring cytoplasmic fractions. The pellet was suspended in homogenization buffer at the same volume and centrifuged again at 100,000 g for 30 min. The final pellet was suspended in homogenization buffer (1 ml/5 g initial fresh weight of root), and used for procedures requiring membrane fractions. The protein content of fractions was determined by BCA Protein Assay kit.

**Protein Phosphorylation.** *In vitro* protein phosphorylation assays were conducted at 30C in a total reaction volume of 100 µl containing 50 mM MES-NaOH (pH 7.0), 0.5 mM DTT, 5 mM MgCl2, 0.2 mM EGTA and 200 µg of membranous or cytoplasmic proteins. Ten µl of 1% Triton X100 was added 15 min before phosphorylation of the membrane fractions. The reaction was initiated by adding 0.037 Bq [gamma-32P]-ATP. The reaction was terminated after 1 min by adding the same volume (100 µl) of electrophoresis sample buffer and by boiling the reaction mixture for 5 min. The reaction mixture of membrane protein was centrifuged at 10,000g for 5 min and the supernatant was used for analyses by SDS-PAGE. The reaction mixture of cytoplasmic protein was used without further centrifugation.

**Results:** Figure 1 illustrates the effect of 1 mM lithium chloride on response of roots to 0.1 nM auxin. The solid line indicates that 1 mM lithium is added at 60
min and 0.1 nM auxin is added at 120 min. Note the 30-35% increase in elongation rate after the addition of auxin. The dash line illustrates the effect of adding IAA to roots at 120 min without previous exposure to lithium. The solid line indicates that 1 mM lithium is added at 60 min and 1.0 µM auxin is added at 120 min. The dash line illustrates the effect of adding IAA to roots at 120 min without previous exposure to lithium. This treatment produces a very strong inhibition (90-95% of the control rate) of elongation. These results are similar to the interactions observed between ethylene biosynthesis inhibitors and auxin previously reported Mulkey et al. (1982a, 1982b).

Figure 3 and 4 show preliminary data on the effects of lithium and auxin on protein phosphorylation pattern. In the first lane of each panel is the control, the fourth lane is 1 mM Lithium, lane G is 1 mM lithium and 1 µM auxin, and lane K is 1 mM lithium and 0.1 nM auxin.

Differences in the protein phosphorylation pattern in both fractions are evident. Phosphorylation of membrane polypeptides in the 14 to 22 kDa range increased under conditions that promote elongation and were suppressed when elongation was inhibited. These effects are not due to an ion effect but appear to be a lithium specific response. This preliminary data suggests that at least part of the effects observed with lithium and auxin treatment may be mediated through a second messenger system.

REFERENCES


Light and gravity give clues about time and space which plants use to direct their growth. Roots grow towards the vector of gravity (positive gravitropism) to access water and nutrients in the soil and to provide stability. Their response to light depends on its wavelength and intensity. Roots grow towards red light (positive phototropism) and away from blue and white light (negative phototropism) (Kiss et al. 2003). Plants respond to these signals via differential cell elongation which results in directional growth. Light and gravity are simultaneous stimuli so plants must respond to both at the same time and integrate them into a single response. Our hypothesis is that integration of light and gravity responses in roots can be observed at the level of gene expression. One of the fastest known signal transduction elements described for gravity and light responses is inositol-1,4,5-triphosphate (InsP$_3$). Cellular levels of InsP$_3$ increase within 15s to 30s in response to stimulation by gravity or light, respectively (Morse et al. 1987; Perera et al. 1999). We therefore analyzed light induced changes in transcript abundances of genes known to respond specifically to gravity in roots of wild type and transgenic plants dampened in their gravity-induced signaling (Kimbrough et al. 2004; Perera et al., in preparation). Several of the genes that exhibited gravity specific transcript abundance changes in wild type plants were not up-regulated in response to gravity stimulation in the transgenic plants (Salinas-Mondragon et al. in preparation). This indicates that the gravity-induced regulation of these genes is mediated by and dependent on InsP$_3$. This makes the transcript abundance of these genes a quantifiable marker for InsP$_3$-regulation and integration of other environmental stimuli.

In all the experiments we used 7-day-old dark-grown wild type and transgenic Arabidopsis seedlings (t2-8). Light regulation of the transcript abundance changes for specific genes was analyzed using exposure to directional light (seedlings were approximately 50 cm from the source of light) for 1 hour of incandescent light (2 µmol $s^{-1}m^{-2}$ or approximately 100 lux). The interaction of light and gravity induced transcriptional regulation was investigated using combined stimulation: Dark-grown plants which were gravity stimulated either in the dark or after 15min or 1h of light exposure and compared to vertical controls (Fig 1). Plants were harvested by adding RNA later (Ambion) and the root apices (ca. 7 mm) separated. RNA extraction (Qiagen), cDNA synthesis (Ambion) and Real Time PCR (Sybr Green) were carried out as described by Kimbrough et al. 2004.

Our first experiment was to perform Real Time PCR to confirm our microarray data (Kimbrough et al. 2004), comparing the wild type and our transgenic (t2-8). We found that gravity induces fast and transient changes in transcript abundance of specific genes as shown by microarray experiments of Arabidopsis root apices in comparison to mechanical stress (Kimbrough et al. 2004). For some nuclear genes, these transcriptional changes occur within one minute as shown by real-time PCR analysis. Interestingly the transcripts that were fast and transiently up regulated by gravity stimulation in the dark, did not show any significant changes in the transgenic plants with dampened InsP$_3$-signaling (Fig. 2).

This suggests that gravity induced changes in transcript abundance of at least some genes are InsP$_3$-mediated. Five of the fastest gravity specific genes, that we previously selected from the microarray data (At5g48010, AtPEN1; At2g16005, expressed protein; At4g11310, cysteine protease; At5g38020, SAMt-homologue; At4g23670, major latex related protein), also show a fast and transient up regulation in the wild type, under directional incandescent light. The levels of these transcripts in the transgenic (t2-8) plants were not up-regulated in response to light stimulation. (Fig. 3).
A sequential combination of gravity and light stimulation and the subsequent analysis of transcript abundances showed that a short period of light (15 min) has a synergistic effect on gravity induced transcriptional regulation (Fig. 4). This effect is transient and mediated by InsP₃ because no transcriptional changes for those genes were detected in the transgenic plants (t2-8).

Figure 2. Transcript abundance of At2g16005 increases more than 3-fold in gravity stimulated root apices in the dark within 1 min. after reorientation but showed no up-regulation in the same time-span in gravity stimulated transgenic (t2-8) plants.

Figure 3. Light induces changes in transcript abundance in the same genes that were identified as fast and transiently up-regulated genes after gravity stimulation in wild type plants.

CONCLUSION

Gravity stimulation induces fast and transient increases in transcript abundance in specific genes in Arabidopsis root apices in the dark. When light-grown plants were gravity stimulated, the abundance of these transcripts did not change. However, a short light stimulus transiently enhanced gravity induced transcriptional changes. Using transgenic plants dampened in their InsP₃-mediated signaling, by over-expression of inositol polyphosphate 5-

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