Foton M-3

Russian-US Collaborative "Regeneration II" and "Gecko H"

Experiments Proposal

by

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NASA/US Science Team

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Introduction

The two major sources of concern for living organisms in space, especially outside of low earth orbit (LEO), are microgravity and space radiation effects, neither of which can be easily mitigated with current technology and engineering limitations. Given this fact, it is of key importance to understand the biological effects of microgravity and radiation, to investigate possible interactions during spaceflight, and to extend our understanding to chronic exposures prior to travel to and extended presence on the moon and Mars. The more obvious known effects of short exposure to microgravity in the absence of significant radiation include strong degenerative effects on bone. muscle, and possibly many other tissues, such as the immune system. Conversely, it is also well known that gravity loading of tissues, and artificial hypergravity in centrifuges, promotes tissue growth and cell proliferation, specifically via matrix-integrin-kinase signaling pathways. In Foton M-2 we focused on microgravity and initiated the investigations of its effects on regenerative cell proliferation in the newt, using a nucleotide analog marker bromo-deoxy uridine (BrdU) and other analyses of tissue growth, and tested the suitability of the gecko as a model organism for similar

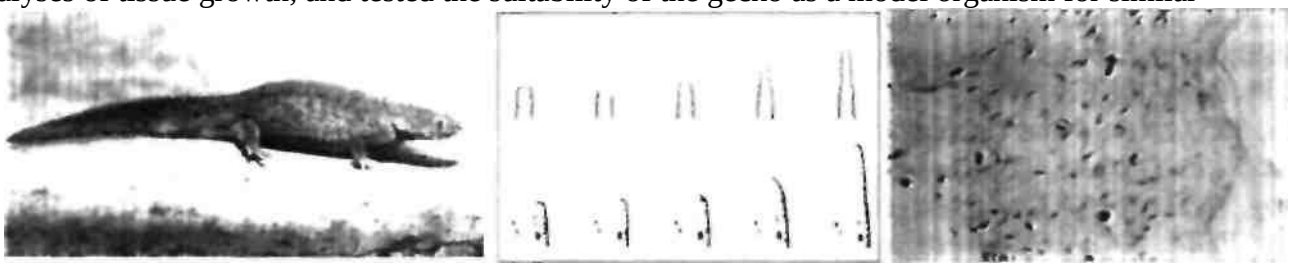

Figure 1 - The newt *Pleurodeles waltl* (left) and stages of tail regeneration after surgery (center). Tail surgery, and subsequent regeneration, is a model for the regenerative processes in vertebrate organisms, and mimics somatic stem cells rebuilding of bone, muscle, skin and other tissues. The image on the right shows BrdU positive nuclei incorporated in the regenerating tail of animals that were housed in the "Triton" spaceflight habitat with a water - soaked polyvinyl acetate (PVA) "carpet" containing BrdU delivered by osmotic pumps.

comparative studies. In Foton M-3 we hope to repeat the microgravity experiments performed in M-2 and extend the experiments to also address the existence of possible gravityradiation interactions.

Foton Vl-2 Working Hypothesis

In the Foton M-2 flight our working hypothesis was that microgravity creates an environment of low level mechanical loading for newt and gecko tissues, and that this change in loading alters the proliferation rates of somatic stem cells involved in tissue regeneration.

Foton M-2 Specific Aims

To test the working hypothesis above we proposed and conducted experiments to achieve the following objectives:

1) To measure cell proliferation in normal and regenerating tail/lens tissues by using incorporation into DNA of dividing cells of the nucleotide analog bromo-deoxy uridine (BrdU) (newt model organism only).
2) To measure cell cycle stage distribution in tissues of the newt and gecko by analyzing DNA content in nuclei using the DNA binding dye Hoechst 33457 (newt and gecko model organisms).
3) To measure bone mineral density and architecture using X-Ray micro computer tomography (microCT) (newt and gecko model organisms).
4) To measure apoptosis and cell cycle arrest markers (p53) in newt (newt and gecko model organisms).

5) To preserve frozen tissue samples for future gene array studies of microgravity-induced gene expression changes in the newt (newt model organism only).
6) To initiate the generation and sequencing of Expressed Sequence Tag (EST) libraries of mRNAs in the newt for future gene array studies of microgravity-induced gene expression changes in the newt (newt model organism only).

Foton M-2 Experiments Significance

The NASA/US experiments conducted in Foton M-2 were designed around pre-existing Russian experiments and flight constraints. Very few factors could be modified and the 11-month time frame to implement the NASA/Russian collaboration was extremely limited. Under these restrictive circumstances it is highly significant the NASA Ames scientists and flight support staff demonstrated the ability to successfully implement a scientific collaboration with Russia, adding a significant number of new and highly relevant investigations to the original goals of the Russian experiments. Specifically, the newt "regeneration" experiment was to be mostly limited to surgical removal of 2 cm of newt tail and also lens, to measure the magnitude of regenerating tissues in microgravity and to growth factor immunocytochemistry. The NASA participation in this experiment added great value to the original experiment by including the delivery of the nucleotide analog BrdU during the flight using simple osmotic pumping devices. This experimental enhancement allowed us to test the hypothesis that previous Russian results of increased tail regeneration in the newt were mediated by increased cell proliferation. Specifically both NASA and Russian investigators observed larger clusters of BrdU positive cells in spaceflight tissues, indicating that tail blastema in microgravity cells undergo cell division more frequently than in ground synchronous controls. Also highly significant are the additional investigations now being conducted of cell cycle progression in tissues, bone mineral density and architecture and genomic analysis of spaceflight tissues, none of which would have been conducted without NASA participation. Since only 7 months have elapsed since we started analyzing the more than 800 samples brought back to the US, all studies are still underway and no definitive results can be reported yet. However a small sample of bones examined by microCT shows that aquatic newts and terrestrial geckos appear to show opposite responses to microgravity with regard to bone tissue degeneration (subject to confirmation in larger sample). Given this fact, and other data such as the extensive Russian measurements on increased newt tail and lens regeneration in previous spaceflights, we are now inclined to speculate and hypothesize that amphibians like the newt may be pre-adapted to a microgravity environment that is in many ways similar to neutral buoyancy in water, and that during spaceflight they do not suffer the degenerative effects observed in terrestrial weight-bearing organisms such as the gecko. It is highly significant that we have for the first time performed a spaceflight comparative physiology experiment with two similar vertebrates one aquatic one terrestrial, and are measuring their respective responses. This avenue is particularly exciting from the scientific point of view because it opens the possibility that newts are a vertebrate model organism that may be pre-adapted to existence and survival in microgravity. This remarkable growth response of the newt to spaceflight makes it a pressing and significant reason to move forward with studies of the genomic changes in the newt tissue we preserved in Foton M-2 and hope to also obtain in Foton M-3.

Foton M-2 Implementation and Progress Report

FOTON M-2 "Regeneration" and "Gecko" Experimental Methods Pre-flight Science and Technical Investigations

In the "Regeneration" experiment in June 2004 we agreed in principle with Russian investigators on hypotheses and pre-flight tests to be conducted to determine the feasibility of delivering BrdU during spaceflight using osmotic pumps. NASA and Russian investigators immediately initiated preliminary experiments during the summer and fall of 2004. This approach was successful and in early March 2005 NASA investigators traveled to Moscow and conducted final pre-flight tesrj jointly Specifically the joint experiments investigated surgical implantation of osmotic pumps. PVA carpet habitat delivery of Brdu, BrdU diffusion rates, dosage of Brdu. and incorporation/immunodetection of Brdu in newt tissues. After joint experiments in March 2005 the NASA and Russian teams conducted additional independent experiments virtually until the last possible day before pre-flight tail and eye surgeries. The final decision for the BrdU delivery method (osmotic pump in PVA carpet. Figure 3) was made about 3 weeks before the Foton M-2 spaceflight in late May 2005. The pre-flight joint experiments and respective timeline are shown in figure 2.

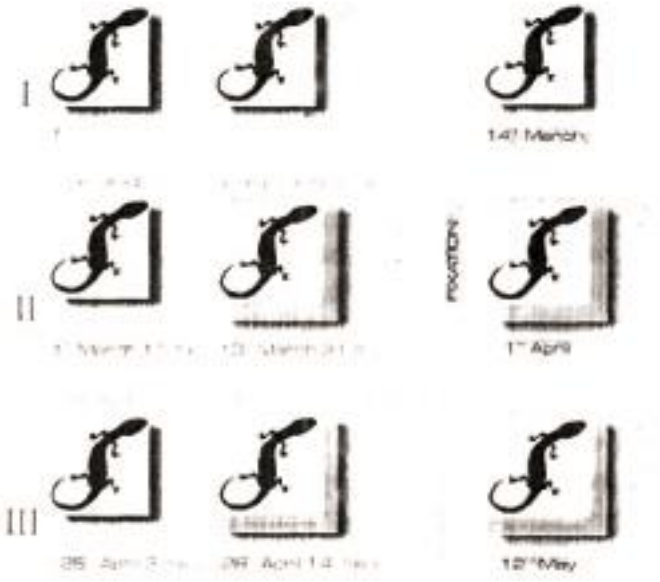

Figure 2 - Pre-Flight NASA/Russian Joint testing (Grigorian)

The "Gecko" experiment did not benefit from extensive flight experience and knowledge of spaceflight operations that Russian Investigators in "Regeneration" experiment had. This is due to the fact that the gecko had never been flown before and was a complete unknown. In addition during the fall of 2004. The gecko species was changed by Russian investigators, due to concerns about the suitability for spaceflight. This delayed pre-flight experiment development greatly. The method we had planned for delivering BrdU to geckos, namely in drinking water, proved impractical in Russian tests because the geckos did not drink consistently from the flight hardware water bottle. Ultimately the geckos were flown without a water supply, because of their ability to resist dehydration over prolonged periods, and no BrdU was delivered. Alternate BrdU delivery methods such as surgical implantation of osmotic pumps and water/BrdU misting were not explored at the time because there was no additional time to conduct the necessary ground experiments, and because this was the maiden voyage for the gecko as model organism. The Russian Investigators, having now proven the gecko as a spaceflight model, are looking forward to implementing the BrdU delivery system with NASA collaboration, and to jointly performing the necessary tests for use in Foton M-3.

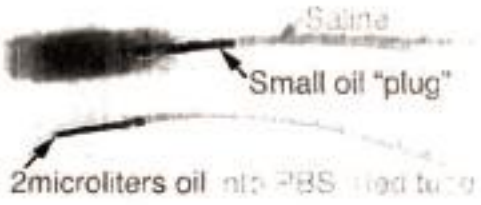

Figure 3 - Osmotic pumps for Brdu

Experimental Design for Spaceflight

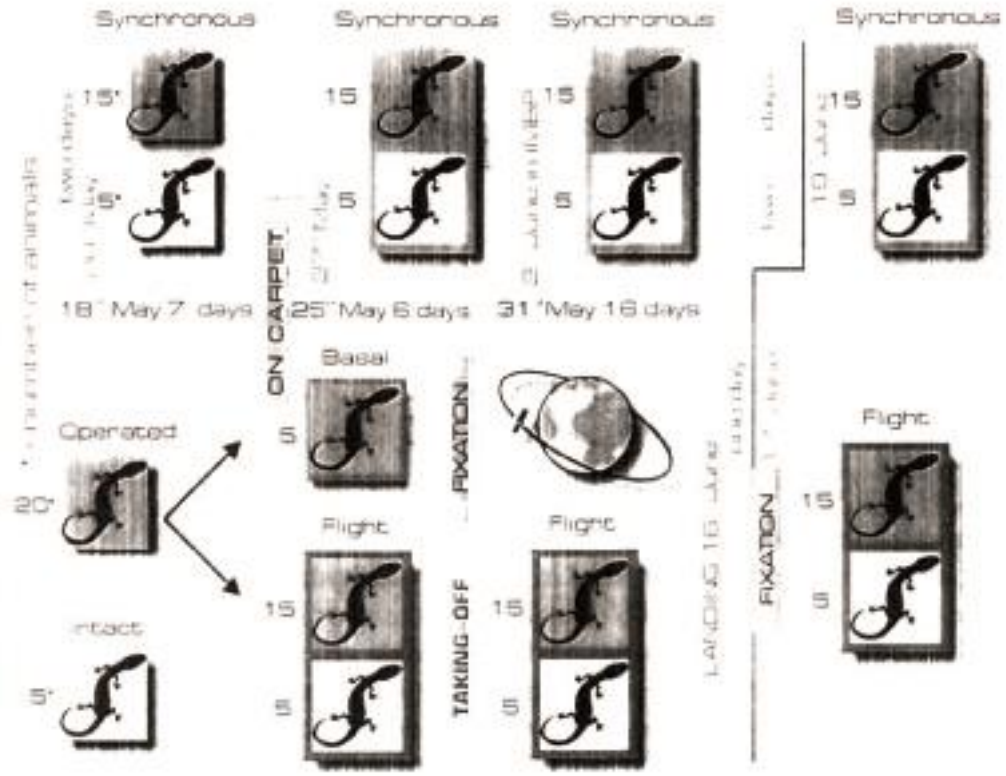

Figure 4) Regeneration Experimental design diagram for Basal. Syncronous and Flight groups (Grigorian)

After concluding the preliminary experiments, the final flight experiment design was agreed upon as diagrammed in Figure 4. In brief we included a flight group of 20 animals, a ground 48h -delayed "synchronous" control, and a basal control of 5 animals that were sacrificed at the time of launch. The basal control offered pre-flight tail regeneration growth data and a BrdU negative control, as well as other baseline data. In both the Flight synchronous and flight groups animals were operated 7 days prior to launch, and then 6 days prior to launch placed on a carpet with programmed miniature osmotic pumps loaded with BrdU. Finally 5 days prior to launch the sealed Triton habitat with 20 flight animals was sent from Moscow Koltsov Developmental Biology Institute to Baikonur. The identical Triton synchronous control habitat was sent to IBMP in Moscow for flight temperature simulation on a 48h delay. Once in orbit for 3 days, and presumably after the animals were adjusted to microgravity. the osmotic pumps initiated BrdU delivery after expending a delay layer of saline solution separated from concentrated BrdU by a 2 microliter mineral oil layer.

In the Gecko experiment 5 animals were flown in microgravity with no experimental manipulations performed in this initial flight. A ground control of 5 animals was maintained at the IB.VIP. As with the newts in "Regeneration" the animals were sent to Baikonur from Moscow 5 days prior to launch.

Sample Recovery and transport to Moscow

Upon capsule reentry and landing the helicopter with the Foton M-2 recovery team was able to be at the landing site in 50 minutes, and removed the Triton habitat from the capsule 60 minutes after landing. IBMP investigators immediately removed the BrdU impregnated PVA blanket. replaced it with a PVA blanket containing only water, and cooled the "Regeneration" experiment to 4 degrees Celsius, to stop cell division and arrest BrdU incorporation, and transported it back to Moscow via commercial airline flight. All the spaceflight animals survived and arrived in Moscow at the Institute 30 hours post-landing. All the animal tissues were fixed and processed for experiments within the 5 hours following arrival in Moscow at the Koltsov Developmental Biology Institute by a joint NASA and Russian team.

The geckos were recovered on the same schedule described above for newts except that they were not cooled for transport to Moscow, since there was no BrdU incorporation to inhibit.

Sample transport to US

All sample tissues were packaged in Moscow either in dry ice or at 4 degrees Celsius in double insulated boxes and shipped via DHL-Danzas and Delta Airlines. NASA Ames flight logistics staff ensured samples were not exposed to X-rays at airports, expedited transit and pre-cleared shipments through USDA at the New York port of entry and customs in San Francisco. During transit, temperature and radiation exposure were monitored with onboard dosimetry and temperature recorders, and remained within normal limits.

Sample preparation for analysis

About 800 samples were generated from the "Regeneration" and "Gecko" experiments and were brought back to the US. Samples were either frozen or fixed in paraformaldehyde. Frozen samples are archived for future gene array analysis once an EST database for the newt is completed (effort underway). Fixed samples were either selected for analysis without further processing in the case of bone microCT. or embedded in paraffin or methacrylate plastic for sectioning and immunocytochemistry. Sectioning of processed tissue blocks and immunocytocfiemistry are underway and will take considerable effort to complete due to large sample size and staffing limitations.

FOTON M-2 "Regeneration" Experimental Preliminary Results

Results tail regeneration

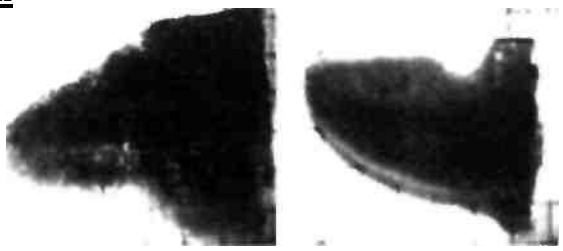

Figure 5 - Regenerating flight (left) and synchronous (right) Newt tails

Preliminary two dimensional measurements of regenerating tail suggests that spaceflight animals had an increase in size under microgravity conditions between 10-20% relative to synchronous ground controls with basal growth subtracted. However regeneration stage analysis shows no significant differences in morphology, suggesting that while more cell proliferation occurred, no additional tissue differentiation resulted from exposure to microgravity. More detailed three-dimensional volume-analysis of regenerative changes is underway. These results are consistent with an enhancement of regeneration by microgravity and inconsistent with degenerative losses observed in tissues of other terrestrial vertebrates in microgravity.

Results bone micro-CT

05-355, E. Almeida; microCT analysis of radius in newts flown in space (mean +*l*- SE).

	Baseline (n=2)	Synchronous Control (n=3)	Flight (n=3)	T-test P<**
Radius (cortical + cancellous)				
Length (mm)	3.6±0.01	3.1±0.1	3.5±0.2	0.114
Total Volume (mm3)	0.886±0.211	0.476±0.066	0.866±0.077	0.018
Bone Volume (mm3)	0.394±0,083	0.222±0.028	0.367±0.043	0.048
BV/TV	3.443±3.013	0.468±0.008	0.422±0.016	0.063
Proximal radius (cancellous)				
Total Volume (mm3)	0.046±0.0009	0.021±0.004	0.039±0.004	0.041
Bone Volume (mm3)	0.003±0.0009	0.002±0.001	0.005±0.002	0.238
BV/TV	0.063±0.020	0.089±0.024	0.129±0.046	0.485

Baseure radius in one newt cracked and very short data not included.

**Synchronous Control versus Flight only: Baseline not included in statistical analysis because of crack in radius of one specimen

Figure 6

Preliminary bone microCT results in the newt show significantly higher bone mineral volume to total bone volume ratios in cancellous bone of the proximal radius (Figure 6), consistent with either increased osteogenesis or decreased bone remodeling by osteoclasts. Results in the gecko are consistent with bone mineral loss, but are still too preliminary. Analysis is underway in collaboration with Dr. Russell Turner at the Oregon State University, Corvallis.

Results BrdU incorporation in newt tissues

Figure 7

Quantitative analysis of BrdU incorporation has been impacted by the fact that newt tissues have significant numbers of pigment cells that in light microscopy are difficult to distinguish from BrdU positive cells labeled with HRP amplification, especially given the low levels of BrdU present. Tissue with low pigmentation, such as the intestine, show clear BrdU incorporation in nuclei (Figure 7), and a 1.5 to 2-fold increase in microgravity regeneration. Regenerating newt tail, eylid and lens also show 1.5-fold increase in positive BrdU cells, in a cluster pattern. Because tail and eyelid are pigmented the numbers we report have a certain degree of uncertainty associated with the assay. We are now developing alternative methods for quantification of BrdU that distinguish false positives. including immunocytochemistry using strepavidin quantum Dots in the far red 655nm, and direct high resolution imaging of bromine in BrdU with Nano-Sims mass spectroscopy microscopy (in collaboration with Dr. Pett-Ridge at Lawrence Livermore National Laboratory). Preliminary results with Quantum Dot labeling are shown in figure 8 and suggest we will be able to readily distinguish between BrdU (Red), pigment cells (Brown), agaisnt a nuclear couterstain (blue). In addition to eliminating falsepositives, this method also has much greater resolution, as there is no signal amplification by HRP.

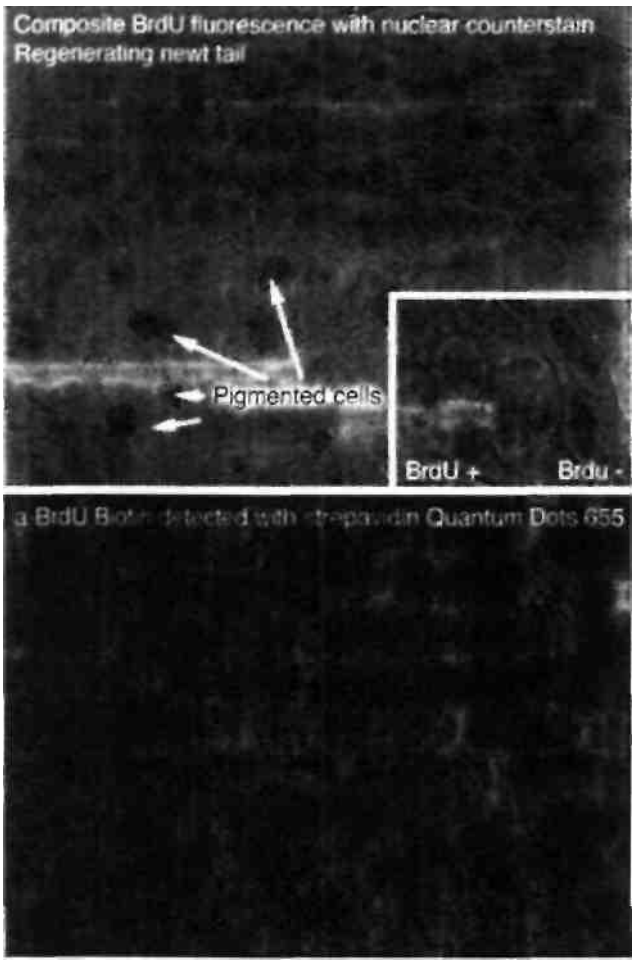

Figure 8. Ouantum Dot BrdU immunolabeling

Results Library Generation and Gene Array Analysis of Flight Tissues

Figure 9. Newt EST Clones

In response to the US-Russian agreement on Foton M-2 we have initiated efforts to generate and sequence newt EST libraries. (Figure 9), to create gene arrays that will allow us to perform genomic studies of archived frozen newt tissues from M-2. This activity is outside of the scope of the budget provided to the NASA PI, and up to now only libraries have been generated and tissues archived. However, a newt gene-sequencing proposal has been submitted to DOE and DARPA by a large consortium of NASA other US. Russian, Canadian, and Japanese regeneration investigators and headed by Dr. Almeida. The DARPA proposal is under consideration in the Regenerative Injury Repair Program, by John Mogford. Program manager and may be considered for a phase-2 submission in fall 2006. The DOE Joint Genome Institute CSP proposal was well-received, but considered out of scope of DOE mission. Current efforts are now focusing on other DOE/NASA inter-agency sequencing opportunities.

Publications

The preliminary results of Foton M2 "Regeneration" and "Gecko" experiments have been submitted/presented as abstracts at the Moscow Foton M-2 Preliminary Science Meeting at IMBP in October 2005, and at the upcoming Osaka ISGP and China COSPAR meetings. Short papers will also be published shortly in the proceedings of the ISGP meeting, in the Journal of Gravitational Physiology. Full-length publications will be prepared once all data is analyzed, likely in early 2007. Abstracts and a draft paper on the regeneration experiment are included in Appendix C.

Summary

The Foton M-2 collaboration was set and conducted in a highly accelerated time-frame and resulted in a mostly successful flight with significant foreseeable science results already being published in multiple venues. The success of the collaboration was due in large measure to great professionalism and collaborative spirit from our Russian counterparts, and also to very hard work from the part of the US/NASA team. Not all the initial goals were achieved, such as BrdU delivery in the "Gecko" experiment, and the limited time opportunity and resources to conduct pre-flight ground studies also prevented us from obtaining ideal results. In particular BrdU dosage appears low although detectable, posing challenges in exact quantification of proliferation. Recent experiments with alternative Quantum Dot BrdU detection methods appear to have resolved this problem. Foton M-2 pushed the limits of the feasible, and serves as important reference point for Foton M-3 planning. Finally, if repeated in Foton M-3, and in conjunction with previous Russian newt flight data, the current results will provide a rare set of well-replicated data on the effects of microgravity on amphibian tissue regeneration, providing a novel experimental insight into a possible physiological pre-adaptation of aquatic organisms to microgravity.

FOTON M-3 Proposal

Introduction

Our main objective in proposing to participate in Foton M-3 is to replicate the experiments conducted in Foton M-2, in a manner that resolves technical issues and difficulties encountered previously, and confirms or refutes, the current interpretation of results. In addition, we seek to extend the Foton M-2 microgravity experiments to include a radiation variable, and to address the important issue of the possible interaction between the two, a key question for ultimately enabling safe human exploration of the solar system.

The biological and medical effects of radiation are relatively well known for common forms of radiation on earth like medical X-rays and protons, but less well known for other forms of cosmic radiation such as heavy mass and energy (HZE) particles. Radiation has multiple effects on living tissues, including the induction of free radical formation and subsequent chemical attack of biomolecules, and more directly the induction of breaks in DNA, possibly leading to transformation and cancer. In the case of spaceflight radiation, until recently most efforts have focused almost exclusively on long-term cancer risks, but current thinking is also starting to focus on medium-term tissue degenerative conditions that are induced by radiation effects on the cell cycle of rapidly-dividing somatic stem cells. Because somatic stem cells involved in replenishing and regenerating human tissues are often found dividing, we hypothesize they are particularly sensitive to the combined effects of microgravity and radiation. In particular, both hindlimb unloading and hypergravity which are models for spaceflight gravity alterations, and radiation appear to act via common molecular p53 and Reactive Oxygen/Nitrogen (ROS/NOS) signaling pathways that could interact to arrest the cell cycle and induce rapid tissue degeneration. Ongoing ground studies from our laboratories with gamma rays and in the near future with HZE particle beams (Globus, Almeida, Vercoutere, Searby and Limoli - 2006 Moscow Space Radiation Meeting Abstract Included in Appendix C) are focusing on how the effects of radiation and hypergravity/hindlimb unloading might synergize through common p53 and reactive oxygen (ROS) pathways to alter the proliferative and tissue regenerative potential of bone marrow stromal cells that are precursors for osteoblasts, osteoclasts, and haematopoietic cells. In this context we are now proposing to conduct new Foton M-3 "Regeneration II" and "Gecko IF* experiments with

our Russian colleagues specifically investigating the effects of spaceflight microgravity and proton irradiation on the proliferation of cells and DNA damage in tissues of these animal models. This pair of vertebrate model organisms is particularly appropriate for testing the combined effects of gravity and radiation because they can both be induced to regenerate tissues, such as the tail: because of their reliability and ease of use under spaceflight constraints; and because one organism, the gecko, is terrestrial, and the other, the newt, is aquatic, providing different physiological responses to space. Finally, our Russian colleagues at Koltsov Developmental Biology Institute, and Institute for Bio-Medical Problems (IBMP) have already expressed their willingness and interest to conduct combined radiation and microgravity experiments, specifically at the Dubna synchrotron facility near Moscow. These experiments would test the effects of pre-irradiating the animals with protons, simulating a solar particle event, and then studying the effects of that event on the newt and gecko spaceflight tissue regeneration in tail/lens models. The specific radiation dose will be experimentally determined in pre-flight ground tests, but will be in general range of 0.1 to 3 Gy. The 3 Gy upper dose limit corresponds to the estimated exposure to astronaut skin inside the relatively well-shielded lunar command module if the 1972 solar particle event had occurred during an Apollo flight (J. Bailey, Biomedical Results of Apollo, Ch. 3) and is also the approximate cumulative expected dose for a Mars 2 type mission with a >300 day duration. The experimental design of ground prerradiation with protons closely (but not perfectly) simulates what would happen to a Moon or Mars crew exposed to a solar particle radiation event on a planetary surface with partial gravity, followed by a return to earth trip in microgravity. The scientific relevance of this experiment is the determination if the radiation damage to tissues synergistically affects regenerative processes in **a** more significant way when regeneration is also occurring in microgravity. In addition, cells from ground control and microgravity-exposed newts and geckos will be removed from the animals and irradiated post-flight to test whether irradiating cells already exposed to the deleterious effects of spaceflight are affected differently by a solar particle event.

Foton M-3 Working Hypothesis

Specifically our working hypothesis is that proton irradiation and microgravity regulate cell proliferation in regenerating tissues via common molecular mechanisms, including ROS/NOS and p53, and that these common mechanisms result in synergistic or additive results.

Foton M-3 Specific Aims

1) To replicate and improve the experiments enumerated above for Foton M-2 (E. Almeida Lead).
2) To extend the microgravity experiments performed in Foton M-2 to include a group of animals regenerating model tail and lens tissues following proton irradiation to simulate a solar particle event (N. Searby Lead).
3) To extend the experiments performed in Foton M-2 to include primary cell culture of tissues exposed to microgravity with and without proton irradiation post-flight (R. Globus Lead).
4) To extend the experiments performed in Foton M-2 to include an analysis of the feasibility to assess DN'A -damage in newt regenerating tissues (T. Straume Lead).
5) To leverage the utility of the M-2 experiment repetition, as a control for new proposed radiation/microgravity interaction experiments.

Foton M-3 Russian Teaming Arrangements

The initial Foton M-3 proposal we are submitting here, including the technical improvements,

and novel radiation experiments has been discussed with both the "Regeneration" Team lead by Dr. Mitashov, and by the "Gecko" team lead by Dr. Saveliev. Both Russian teams have agreed in principle to attempt conducting these experiments in collaboration with NASA. If US participation is approved, we will define in more detail with our Russian colleagues the specifics and feasibility of all aspects of the proposed experiments. Letters of collaboration from both Russian teams are included in Appendix B. In addition Dr. Eugene Ilyin at the IBMP has also supported and agreed to the proposed radiation experiment concept, and made available proton radiation resources via an existing IBMP/Dubna synchrotron agreement.

Foton M-3 US Teaming Arrangements

To extend the Foton M-3 spaceflight opportunity to as many investigators as possible, and to fully utilize the animal tissues derived from the flight, we have sought and obtained letters of collaboration from multiple NASA, Russian, and University investigators in the bone, radiation and tissue regeneration/proliferation fields. The list of collaborators and their respective proposed roles s included in Appendices A (Investigator Roles), and B (Letters of Collaboration).

Significance

The significance of the proposed experiments is both to provide validation and confidence on Foton M-2 microgravity tissue regeneration results, and to extend the experiment to provide fundamental knowledge of radiation effects in microgravity. The significance of discovering or disproving interactions between microgravity and radiation is enormous, as this may alter our current calculations about permissible doses of radiation, as well as future Mars and moon mission design and spacecraft shielding requirements. In addition, the focus on tissue regeneration, instead of long-term cancer-risks, is highly significant because any increases in tissue degeneration other than in that already established in bone and muscle may have severe implications for the ability of astronauts to perform physical and intellectual tasks during long-term space-travel. Finally, the proposal attempts to leverage a very limited resource, spaceflight biological experimentation, to multiple NASA and university investigators involved in basic scientific research, by strong collaboration and tissue sharing.

Foton M-3 Technical improvements proposed (newt and gecko)

Replacement of osmotic pumps with timed mechanical BrdU pumps

Inclusion of a misting or spray method for water/BrdU dispersion in the gecko habitat

Increase of BrdU dosage

Decrease of BrdU delivery time window

Include pre-flight injection of tetracycline to monitor bone-remodeling dynamics

Include in-flight co-delivery of tetracycline and BrdU to monitor bone-remodeling dynamics

Autonomous video monitoring of animal behavior in flight

Foton M-3 Pre-flight Science and Technical Investigations (newt and gecko)

Testing of timed mechanical pumps

Testing of tail surgeries in the gecko

Testing of BrdU dosage increases

Testing of BrdU incorporation and detection

Testing of autonomous video monitoring system

Testing of tetracycline incorporation and detection in bone

Ground Testing of solar event simulation/radiation dosage at Loma Linda Medical Center, CA and Dubna, Russia

Foton M-3 Preliminary Experimental Design for Spaceflight (newt)

Experimental design will be constrained by the M-2 design. The major addition and change will be the performance of animal irradiation with protons prior to launch. Tentatively, irradiation will

occur 1 day after tail and lens surgery and one day before the animals are sent to the launch site. In addition, experimental design will be changed, by reassigning half of the subjects, or 10 animals: in each synchronous (20 animal total), and flight (20 animal totals) groups for irradiation. Post-flight primary cell cultures will be prepared from control non-irradiated newt bones and liver, and irradiated with protons (or not in controls). This specific experiment will test the possibility that tissues exposed to microgravity are altered in such a way that modifies cell sensitivity to subsequent radiation damage. Finally we will analyze DNA repair and DNA damage patterns in newly regenerated tissues during microgravity exposure, to determine if repair mechanisms are gravity mechanosensitive. In these experiments we will use regenerating tissue interfaces with existing tissue, as an internal control for the role of regeneration on DNA damage and repair mechanisms.

Foton M-3 Preliminary Experimental Design for Spaceflight (gecko)

Experimental design will be altered to include both tail surgery and animal irradiation with protons. Animal number will be increased from current 5, to 6 smaller animals to include two groups of 3, one irradiated and one non-irradiated. If possible we will seek to double the number of geckos to 12, to obtain more significant data, however if this is not possible we will likely not conduct the radiation portion of the experiments due to lack of sufficient animals to obtain statistically significant data.

Summary

Foton M-3 offers a unique opportunity to repeat and enhance the Foton M-2 microgravity tissue regeneration experiments. Foton M-3 experiments will also offer new and important fundamental data to support or refute the hypothesis that microgravity and radiation interactions are of potential concern for long duration human spaceflight, especially in affecting the normal processes of tissue regeneration. The collaborative approach with our Russian colleagues, and among multiple NASA investigators proposed here for Foton M-3 experiments ensures the flight opportunity is fully utilized, and has the potential to generate significant results for advancing our basic understanding of the space environment.

Appendix A

M-3 Investigator Roles

NASA/US Team

Eduardo Almeida, Ph.D., NASA and University of California San Francisco, Principal Investigator

- Dr. Almeida will conduct the experiments measuring cell proliferation in regenerating tissues of the newt and gecko, and will coordinate the scientific collaboration with all the other NASA/US and Russian co-investigators.

Jonathan Phillips, Ph.D., NASA Post-Doctoral Fellow, Co-Investigator

- Dr. Phillips in conjunction with Dr. Almeida will conduct genomic studies of microgravity effects on tissue regeneration, including the creation of expressed sequence tag (EST) libraries from newt regenerating tissues.

Esther Hill, Ph.D., NASA Co-Investigator

- Dr. Hill in conjunction with Dr. Almeida will conduct the histological and immunocytochemical analysis of the newt and gecko tissue samples for BrdU label incorporation.

Ruth Globus, Ph.D., NASA and University of California San Francisco, Co-Investigator - Experiment Lead

- Dr. Globus in conjunction with Dr. Almeida will conduct a post-flight newt somatic stem cell proliferation experiment with and without proton irradiation to determine if exposure to

microgravity sensitizes cells for proton radiation exposure.

Hisataka Kondo, Ph.D., NASA Post-Doctoral Fellow, Co-Investigator

- Dr. Kondo in conjunction with Dr. Globus will perform post-flight newt primary cell culture and proton irradiation experiments.

Nancy Searby, Ph.D., Scientist, NASA Co-Investigator - Experiment Lead

- Dr. Searby in conjunction with Dr. Almeida will conduct pre-flight irradiation with protons of a portion of the flight newts to determine the possible interactions between microgravity and radiation exposure on tissue regeneration.

Tore Straume, Ph.D., Scientist, NASA Co-Investigator - Experiment Lead

- Dr. Straume in conjunction with Dr. Almeida will conduct experiments to assess the feasibility of measuring radiation/DNA damage effects in microgravity.

Hami Teal, Ph.D., Scientist, NASA Co-Investigator

- Dr. Teal in conjunction with Dr. Straume will conduct experiments to assess the feasibility of measuring radiation/DNA damage effects in microgravity.

Russell Turner, Ph.D., Oregon State University, Corvallis

- Dr. Turner will perform bone Micro-CT analysis of newts and geckos, as well as Tetracycline incorporation analysis to estimate bone remodeling in proton irradiation/microgravity exposed bones.

Russian Team

Victor Mitashov. Ph.D., Russian Principal Investigator "Regeneration"
Eleanora Grigorian, Ph.D., Russian Co-Investigator "Regeneration"
Elena Domaratskayaya, Ph.D., Russian Co-Investigator "Regeneration"
Sergei Saveliev, Ph.D., Russian Principal Investigator "Gecko"
Victoria Gulimova, Ph.D., Russian Co-Investigator "Gecko"

Appendix B

Letters of Collaboration

From Victor Mitashov "Regeneration" Russian PI

In response to the NASA PI request to modify the "Foton M-3 Regeneration" experiment to include proton irradiation of 10 (of 20) animals, the Russian PI, Victor Mitashov, responded he and his co-Is agreed to participate and the they also had IBMP agreement from Dr. Eugene Ilyin, including access to the Proton Irradiation facility at Dubna (near Moscow) with which IBMP has a use agreement.

From: "Mitashov" <nitashov@proxima.idb.ac.ru>
To: "Eduardo Almeida" <ealmeida@mail.arc.nasa.gov>
Subject: Re: VERY URGENT - Joint Experiment Proposal for Foton M-3
Date: Thu, 9 Mar 2006 16:21:56 +0300

Dear Eduardo,

Thank you for your letter. I discussed your proposal with Nora, Elena, Murad and Eugene Ilyin. We are agree to participate in the M3 flight experiment. E. Ilyin supported us also I think we will discuss with you all derails in Osaka. We are ready to send a paper for Osaka meeting Galina Tverskaya for translation. E-mail addresses are

<mailto:nora@proxima.idb.ac.ru>nora@proxima.idb.ac.ru
<mailto:edoma@proxima.idbac.ru>edoma@proxima.idb.ac.ru
<mailto:mitashov@proxima.idb ac.ru>mitashov@proxima.idb.ac.ru

Thanks, Victor.

From Victoria Gulimova and Sergei Saveliev "Gecko" Russian Investigators
From Victoria Gulimova
To: ealmeida@mail.arc.nasa.gov
Subject: Foton-M3 experiment

Dear Eduardo,

We have carefully read your letter and discussed it with all of the appropriate specialists. In order to materialize your proposals we have to solve a number of problems.

Our greatest wish for the Foton-M3 experiment is the possibility of video recording. This will finally solve the problem of floatmg-vs-contact of the animals. If you plan to participate in the experiment we together can suggest the most compact and least consuming model of camcorder. Since the USA is the leader in this field, may be you could search for the equipment at NASA.

Also we must take into account the necessity of orbital control (intact animals present on the satellite together with experimental ones). The group of 10 animals is not enough to study both effects of proton radiation and tail regeneration Prof. Saveliev considers it optimal to use 5 animals for orbital control and 5 animals for study of proton radiation effects. In this case the Brdu method would come in nicely. Still you must think of the device to sprinkle Brdu inside of the box. This can cause problems, for the energy source must be considered Also humidity increases the threat of fungus diseases. If you find our comments acceptable, our cooperation in the frames of Foton-M3 will be possible and we would be quite happy about that.

Sincerely yours,

Victona

Date: Tue, 21 Mar 2006 11:24:06 - 0800

To: ealmeida@mail.arc.nasa.gov

From: Ruth Globus <Ruth.K.Globus@nasa.gov>

Subject: M3 project

Dear Eduardo,

I am delighted to have the opportunity to work with you again on a Foton flight as a coinvestigator. According to our prior discussions, I will be responsible, along with Dr. Hisataka Kondo, for cell culture tests on the M3 flight in the 2006-2007 time frame, with financial support for our work provided by NASA. As you know, our current research on radiation effects on murine bone marrow stromal cells is progressing nicely, and puts us in an ideal position to extend our assays to the newt in both preparatory ground-based and spaceflight experiments. I am looking forwarded to a productive collaboration on the M3 project.

Sincerely,
Ruth

Ruth Globus, Ph.D.
Scientist
Bone and Signaling Laboratory
and
Science Manager
Center for Gravitational Biology Research
Life Sciences Division
MS 236-7 (Building 236, Room 221)
NASA Ames Research Center
Moffett Field, CA. 940350-1000
phone: (650)604-5247
fax: (650)604-3159
new email: Ruth.K.Globus@NASA.gov

From: "HISATAKA KONDO" <hkondo@arc.nasa.gov>
Subject: Request
To: ealmeida@mail.arc.nasa.gov <ealmeida@mail.arc.nasa.gov>
Date: Wed, 15 Mar 2006 21:28:22 -0800
Dear, Eduardo.
I'd like to colaborate with you on FOTON M3 project.
I'm thinking of specifically analysing cell proliferation before and after proton irradiated cells.
Hisataka Kondo D.D.S., Ph.D.
****
NASA Ames Research Center
Bone and Signaling Laboratory
Mail Stop236-7
Moffett Field, CA9035-1000
tel: 650-604-6390
e-mail: HKondo@arc.NASA.gov
****

Date: Tue. 21 Mar 2006 16:51:58-0800
To: ealmeida@mail.arc.nasa.gov
From: Hami Teal <hteal@maiI.arc.nasa.gov>
Subject: FOTON M3
Cc: tstraume@mail.arc.nasa.gov

Dear Dr. Almeida,

Tore Straume and I greatly look forward in collaborating with you on the FOTON M3 newt project. We agree to participate in evaluating the feasibility of studying DNA damage in regenerating tissues.

Sincerely,
Tore Straume
Hami Teal

Tore Straume, Ph.D.
Chief Scientist
Life Sciences Division
MS 239-11
NASA Ames Research Center
Moffett Field, CA 94035
Tel: 650-604-3943
Fax: 650-604-3954
Email: tstraume@mail.arc.nasa.sjov

Hami E. Teal, Ph.D.
NASA Ames Research Center
M/S 236-5
Moffett Field, CA 94035
Bldg. 236, Rm. 207
650.604.1102 (Tel)
650.604.3159 (Fax)
hteal@ mail.arc.nasa.gov

To: ealmeida@mail.arc.nasa.gov
From: Nancy Searby <Nancy.D.Searby@nasa.gov>
Subject: Foton M3 flight

Dear Eduardo,

I look forward to our collaboration on the Foton M3 newt project. I believe that repeating the M2 experiment is critical. Asking the additional question about the influence of irradiation on the newt spaceflight-induced proliferative changes will yield some exciting new results.

Sincerely,
Nancy

From: Jonathan Phillips <jphillips@arc.nasa.gov>
Subject: Foton M3
To: Eduardo.A.Almeida@nasa.gov

Dear Dr. Almeida:

In support of your ongoing progress with the Foton series of life sciences missions, I would be

pleased to continue collaborating with you. I offer my assistance with developing genome-focused experiments to study the influence of the spaceflight environment on newt tissues.

Best Regards,

Jonathan A. Phillips, Ph.D.

To: Eduardo.A.Almeida@nasa.gov

From: esther hill <ehill@mail.arc.nasa.gov>

Subject: M3 Collaboration

Cc: awray@mail.arc.nasa.gov. Kathleen Hinds <khinds@mail.arc.nasa.gov>

I would be very happy to collaborate with you on the Newt experiment for the M3 FOTON mission, and look forward to contributing my expertise in bone and bone histology to this effort.

Esther

Appendix C

Publications

Abstracts 27th Annual International Gravitational Physiology Meeting 23-28

April, 2006, Osaka, Japan

ANALYSIS OF CELL PROLIFERATION IN NEWT TISSUE REGENERATION USING BRDU-INCORPORATION DURING SPACEFLIGHT IN FOTON M-2. [12]R. GLOBUS, [1]N. SEARBY, [1]W. VFRCOUTERE, [1]F. MORFY-HOLTON, [3]U. IWANIEC, [3]R. TURNER, [4]N. GRIGORYAN, [4]F. DOMARATSKAYA, [4]V. POPLINSKAYA, [5]M. TAIRBEKOV, [4]V. MITASHOV, [12]F. A. C. ALMEIDA

[1]NASA Ames Research Center, Moffett Field, California. [2]University of California San Francisco, California, USA. [3]Oregon State University Corvallis, Oregon USA. [4]Koltsov Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia. [5]Institute for Biomedical Problems, Russian Academy of Sciences, Moscow, Russia.

Impaired human bone, muscle and other tissue regeneration in an altered gravity and radiation space environment is a key scientific question that should be understood and resolved, before human beings travel to other planets in the solar system. To begin elucidating this problem multiple Russian satellite experiments have been conducted using the newt model-system for tissue regeneration *(Pleurodeles wattt).* Specifically experiments by Mitashov *el at* have repeatedly shown increases in lens and tail regeneration in space, following surgical injury. The joint collaborative Russian-US experiment "Regeneration" in Foton-M2 sought to rigorously test the hypothesis that previously observed increases of tissue regeneration during spaceflight were due to increased cell proliferation. To test this hypothesis under the constraints of spaceflight operations we developed a method to delay the delivery of the cell proliferation marker and nucleotide analog bromodeoxyuridine (BrdU) until newts were in orbit for-Win and adjusted to the space environment. Marker delivery was accomplished with miniature osmotic pumps from Al/a Inc., programmed with a BrdU-free delay layer separated from the marker by an oil droplet. This methodology allowed BrdU delivery and incorporation selectively during spaceflight. Newts in the Foton VI-2 capsule "Triton" habitat were recovered Ih after reentry and landing, removed from BrdU-conlaining water, and immediately cooled to 4^0C to avoid additional marker incorporation nitial independent data analysis by Russian and US teams of tail lens, liver and intestine, indicate that the experiment was successful delivering the BrdU marker at the correct time, and support the hypothesis that increased tissue regeneration in space is caused by a twofold increase in cells observed proliferating. (Supported by NASA and the Institute for Biomedical Problems, Moscow, Russia)

COMPARATIVE EFFECTS OF SPACEFLIGHT ON A TERRESTRIAL GECKO AND AN AQUATIC NEWT IN FOTON M-2 [1]N. SEARBY, [12]R. GLOBUS, [1]W. VERCOUTERE, [1]F. MOREY-HOLTON, [3]U. IWANIEC, [4]R. TURNER, [4]N. GRIGORYAN, [4]V. MITASHOV. [5]V. I. GULIMOVA, [5]S. V. SAVELIEV, [6]M. TAIRBEKOV, [12]F. A. C. ALMEIDA [1]NASA Ames Research Center, Moffett Field, California; [2]Universily of California San Francisco, California, USA; [3]Oregon State University, Corvallis, Oregon, USA; [4]Koltsov Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia; [5]Institute of Human Morphology, Moscow, Russia; [6]Institute for Biomedical Problems Russian Academy of Sciences, Moscow, Russia;

Decreased human tissue regeneration in an altered-grav ity and radiation space environment is an important problem that we seek to understand, using appropriate animal models, before humans travel to other planets in the solar system. In this context the Institute of Human Morphology in Moscow tested the gecko *Pachydactylm hibrtmiL* in Foton M-2 as a modelorganism for a terrestrial vertebrate exposure to the space environment. It is thought that *Pachydacrilus sp.* may have reduced problems from notation in microgravitiy given that its toes have adhesive setae, allowing

the animal lo walk in the habitat walls in space. Initial spaceflight results indicate *Pachydaciilus sp.* can be used as a practical animal model for long-term space Right. Additionally, this new terrestrial model-organism can also provide valuable comparative physiology data in conjunction with the aquatic (newt) *Pleurodeles* wa/r/also flown in Foton M-2 and in many other Russian space-biology missions. Both organisms share many features, including general body plan, si/.e. poikilothermia and tissue regenerative ability. A major difference, however, is that *Pleurodeles* is primarily aquatic, and mostly free from gravity mechanical loading, due to neutral buoyancy in water, while *Pachydactytus sp.* is terrestrial experiencing full gravity mechanical loading and stimulation. Our working hypothesis is that a terrestrial organism such as *Pachydactytus* will experience tissue degenerative losses in space when it loses gravity mechano-stimulation while *Pleurodeles sp.,* an organism already pre-adapied lo an aquatic neutral-buoyancy env ironment. in part similar to nicrograv ity may not. Preliminary micro computer tomography anal) sis of bone in the limbs of both species, indicate .hat while the newt *Pleurodeles sp.* maintained or gained radius cancellous bone volume during Foton M-2. the gecko *Pachydactylus sp.* lost cancellous bone in the humerus. (Supported by NASA and the Institute for Biomedical Problems, Moscow, Russia)

COSPAR Meeting Abstract

Experiment aboard Russian satellite "Foton M2" in 2005: new approaches for study on stimulating effect of space flight on cell proliferation and regeneration in Urodela

E. Grigoryan (1), E. Almeida (2), E. Domaratskaya (1), V. Poplinskaya (1), M. Tairbekov (3), K. Aleinikova (1), V. Mitashov (1)

(1) Koltsov Institute of Developmental Biology. Russian Academy of Sciences, Moscow, Russia: (2) NASA Ames Research Center, Moffett Field. California. USA, (3) Institute for Biomedical Problems. Russian Academy of Sciences, Moscow, Russia
(nora@proxima.idb.ac.ru / Phone: +7-495-1350052)

A study on space flight effect upon processes of regeneration is due to the necessity to know their characteristics in animals and human exposed to space and earth conditions shortly after flight. Several experiments on the newts performed earlier aboard Russian biosatellites showed that the rate of organ and tissue regeneration in space was greater than that on the ground. Space flight effect stimulating regeneration was enduring and apparent not only just after flight but long time later as well. This observation found support in studies simulated physiological weightlessness by means of fast-rotating clinostat. It was shown also that the higher rate of

regeneration was associated with enhanced cell proliferation. For instance, we found that the number of cells in S-phase in regenerating tissues was significantly greater in space-flown animals than in the ground controls. However, it was unclear whether cell proliferation stimulation was induced by micro-"g" per se or by conditions of hyper-"g" during launching and re-adaptation on the earth. Molecular mechanisms underlying the change also remained obscure. These issues were addressed by the joint Russian-USA experiment "Regeneration" performed on Foton-M2 in 2005. In 16- day flight we used two well-known models of regeneration: lens regeneration after lensectomy and tail regeneration after amputation in adult newts Pleurodeles wait (Urodela). In order to evaluate cell proliferative activity in time limits of microgravity influence the original method for in-flight delivering DN'A precursor BrdU was developed for the first time. Our preliminary results showed that during the flight the number of DNA synthesizing cells in the regenerating eyes and tails significantly increased. These data together with those obtained earlier suggest that the cell proliferation and, consequently, the regeneration rates increase in response to the accumulated effect of all changes of gravity during and after flight. For better understanding of molecular mechanisms of stimulating effect of space flight upon regeneration we studied an expression of bFGF in regenerated tissues of flown and control animals. It was found earlier that bFGF is one of the important proteins regulating cell proliferation and differentiation during regeneration in vertebrates. Using immuno-histochemical methods after flight we observed bFGF expression higher and steadier in tail and lens regenerates of flown animals than in control ones. In particular, cells of tail spinal cord, chord, skin, muscles and cells of new formed lens epithelium demonstrated the maintenance of bFGF expression in newts exposed to space while those cells of control animals lost it. In addition, the expression of two proteins of generalized stress (HS70, HS90) in regenerating tissues of space-flown newts and ground controls was examined. It was found that studied stress proteins had the different pattern of expression in flown animals in comparison with the control. Therefore, the data obtained in experiment aboard Foton M-2 is the part of the reason for the accelerating effect of space flight upon regeneration in lower vertebrates.

Moscow Space Radiation Meeting June 2006 Abstract

Shared oxidative pathway in response to gravity-dependent loading and gamma irradiation of bone marrow-derived skeletal cell progenitors.

H. Kondo, C. Limoli, N.D. Searby, E.A. Almeida, D.J. Loftus, W. Vercoutere, D. Hilton, R.K. Globus

Department of Radiation Oncology, University of California, Irvine U.S.A.; Life Sciences Division NASA Ames Research Center, Moffett Field, U.S.A.

Astronauts are exposed to radiation during space travel under conditions of greatly diminished

weight bearing activity. However, we know little about how gravity-dependent loading affects tissue sensitivity to radiation. We hypothesize that gravity-dependent loading and irradiation share common molecular signaling pathways in bone cell progenitors, such as generation of reactive oxygen species (ROS), and these pathways consequently impact skeletal health. To begin to address this, progenitor cells with potential to differentiate into either bone-forming osteoblasts or bone-resorbing osteoclasts were extracted from bone marrow, and cells either were centrifuged to simulate increased gravity loading (5-180 min at 5 to 50 times gravity (g), day 2 or 4) or were exposed to a single dose of Ce-137 gamma irradiation (1-5 Gy at 1 Gy/min on day 3-4). Bone marrow cells from 6-12 wk old, male C57BL6/J mice were grown in alpha-MEM supplemented with 15 % 'etal bovine serum, and with factors that either promote osteoblast or osteoclast differentiation. Production of ROS was assayed by flow cytometry using the fluorogenic dye, CM-H2DCFDA, nitric oxide (NO) production was assessed by measuring the stable product, nitrite, in conditioned medium by the Greiss reaction, and cell numbers were assessed by manual cell counting or by measuring DNA content (CyQuant). Osteoblastogenesis was estimated by production of mineralized matrix (Alizarin Red staining), and osteoclastogenesis was estimated by counting Tartrate-Resistant Acid Phosphatase (TRAP)-positive cells. Transient centrifugation was a potent stimulus to bone marrow stromal cells, increasing production of ROS, nitrite, cell number, and formation of mineralized matrix by osteoblastic cells. Similarly, centrifugation increased the numbers of TRAP-positive osteoclastic cells. Radiation also caused dose- and time-dependent increases in ROS by bone marrow stromal cells, but reduced cell number and osteoblast differentiation. In summary, both gravity-dependent loading by centrifugation and radiation stimulated ROS production. Centrifugation increased numbers of osteoblasts and osteoclasts (indicators of increased bone turnover) and enhanced osteoblast differentiation, whereas radiation decreased cell number and osteoblast differentiation. We conclude that gravity-dependent loading and radiation both stimulate production of ROS yet differentially affect critical cell functions such as growth and differentiation.