ANALYSIS OF CELL PROLIFERATION IN NEWT *(Pleurodeles waltl)* TISSUE REGENERATION DURING SPACEFLIGHT IN FOTON M-2.

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ABSTRACT

Terrestrial organisms exposed to microgravity during spaceflight experience musculoskeletal degeneration. It is still not understood if longer-term exposures to microgravity induce degeneration in other tissues, and if these effects are also observed in neutrally buoyant aquatic organisms that may be pre-adapted to mechanical unloading. The "Regeneration" experiment conducted collaboratively between Russian and US scientists for 16 days in the Russian Foton M-2 spaceflight sought to test the hypothesis that microgravity alters the proliferation of cells in regenerating tail tissue of the newt *Pleurodeles waltl.* Our initial results indicate that we successfully delivered the proliferation marker 5-bromo-2'-deoxy Uridine (BrdU) during spaceflight, and that it was incorporated in the nuclei of cells in regenerating tissues. Cells in spaceflight tail regenerates proliferated at a slightly slower rate and were more undifferentiated than those in ground synchronous controls. In addition, the size of regenerating tails from spaceflight was smaller than synchronous controls. However, onboard temperature recordings show that the temperature in spaceflight was about 2°C lower than ground synchronous controls, possibly explaining the observed differences. Additional post-facto ground controls at matched temperatures will correctly determine the effects of spaceflight on regenerative cell proliferation in the newt.

I. INTRODUCTION

Degeneration of tissues from terrestrial organisms in microgravity is an established physiological effect of spaceflight (1). In bone and muscle this effect is attributed to reduced mechanical stimulation these tissues experience in space. Cellular mechanisms that mediate microgravity-associated tissue loss arc thought to involve decreased proliferation of tissue forming cells, such as osteoblasts, rather than increased activity of degenerative cells, such as osteoclasts. Conversely, increased mechanical stimulation of primary osteoblasts and bone marrow stromal cell cultures significantly increases proliferation92). As a whole, the current tissue, cellular, and molecular evidence, suggests gravity is an important source of biomechanical loading, responsible for activating kinase-mediated cell proliferation signalling pathways that in turn keep tissues healthy and regenerating. In terrestrial organisms, as the mechanical stimulus of gravity is diminished, mechanosensitivc tissues quickly lose a portion of their regenerative growth and begin to atrophy. An important hypothesis about this process is that if the effects we observe in bone and muscle are also occurring more slowly in other tissues and organs, then prolonged exposure to spaceflight will eventually also result in their degeneration. This will depend on the extent of mechanosensitivity of these tissues. In addition to asking which tissues are influenced by microgravity, it is also important to question the basic assumption that certain tissues will always degenerate in space due to mechanical unloading. We, and others have hypothesized that bone health of organisms living in aquatic habitats, such as the amphibian *Pleurodeles wall I* (figure 1), is not influenced by exposure to microgravity. in contrast to terrestrial animals. The basis for this hypothesis is the assertion that the neutrally buoyant aquatic environment, to which newts are evolutionary adapted to, is similar in mechanical unloading to microgravity.

Figure 1. The newt *Pleurodeles waltl* photographed underwater

To begin testing these hypotheses we used *Pleurodeles waltl* as a model organism for microgravity experimentation and examined regeneration of tail and lens of the newt. Surgically-induced tissue regeneration in the newt (figure 2) provides a model-process in which all cells are rapidly proliferating, and are readily quantified using standard cell and tissue biology methodology such as BrdU incorporation and immunodetection. In contrast, normal regeneration depends on the proliferation of rare somatic stem cell progenitors that often cannot be readily identified, and arc very low in abundance.

Pleurodeles walt has already been used successfully in 9 previous Russian space experiments (3-5), and it has proven useful for biological experimentation under the severe constraints of spaceflight. In parallel to the newt "Regeneration" experiment we, and others, also conducted the first spaceflight of the terrestrial gecko *Pachydactylus bibryonii* as a comparative model organism for regeneration studies of a terrestrial species similar in size, poikilothermia, body plan, and other features to the newt *Pleurodeles waltl.* The "Gecko" experiments in Foton M-2 describing its use as a spaceflight model organism are described elsewhere in this Journal. This paper describes the flight experimental condition for the newt "Regeneration" experiment. We present methodology for delivery of the nucleotide analog and proliferation marker 5-bromo-2'-deoxyuridine (BrdU) in-flight, as well as initial results from analysis of flight and ground control parameters, tail regenerative growth measurements, and cell cycle/DNA measurements in normal and regenerating tail tissues.

Figure 2. Tail regeneration is a model-process for the regeneration or tissues and organs during spaceflight. During Foton M-2 tail regenerates reached 4-5 mm of re-growth (insert shown on mm grid background) and exhibited complete epitheliazation and partial pigmentation

2. METHODS

2.1 *Pleurodeles waltl* tail regeneration model

To induce tail regeneration we used newts (10-12cm) from which the distal 2 cm of the tail was surgically removed under MS222 anaesthesia 13 days prior to launch. Basal (day 13) controls of stage I tail blastema regenerates (6) were taken at the time of launch and 27 days after surgery in both recovered flight and synchronous control animals. Tail regeneration in this experimental model can reach approximately stage II, characterized by complete epithelization of regenerated area and by a mixture of proliferating and differentiating cells.

2.2 "Triton" spaceflight habitat

Animals were maintained in the Russian "Triton" habitat, used in previous spaceflight experiments (figure 3). Briefly, the habitat consists of an aluminium box with a Plexiglas lid with a water-proof but oxygen/$C0_2$-pcrmeable membrane window (not shown in figure 2), and a polyvinyl acetate (PVA) sponge bottom. The PVA sponge absorbs and retains about 7 times its own weight in water, providing a wet environment for the newts during the flight. Only water is

provided during the flight. Newts however are adapted to periods of fasting during estivation, and remain healthy during the 22-day period of the flight experiment. During the "Regeneration" experiment one animal died in the synchronous control group. The loss was attributed to a hardware malfunction in which the newt was trapped and immobilized in a small space between the PVA carpet and the "Triton" habitat wall, due to a loose fastener.

Figure 3. "Triton" habitat being opened in Moscow, 30 h post flight landing.

2.3 Osmotic pump delivery of BrdU

BrdU delivery during spaceflight was accomplished using 24 miniature osmotic pumps in the PVA carpet (figure 4A). The pumps (Model 1002/14day -figure 4B), each with a capacity of 100 µl, were inserted in a lateral cut on the carpet 20mm in depth (figure 4A and 4C).

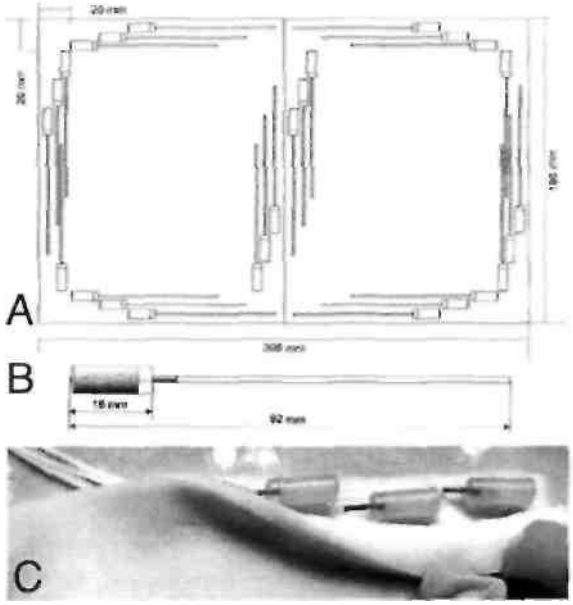

Figure 4. Osmotic pumps for BrdU delivery A) pump layout. B) pump with delay tubing, C) pump insertion in the PVA carpel

The delay tubing in miniature osmotic pumps contained 37µl of saline solution separated by a 3µl droplet of mineral oil. In sum, each pump delivered saline for the first 8 days after activation, and BrdU the following 12 days. Inflight BrdU delivery commenced 48h after launch and terminated about the time of landing. Because of the inclusion of the delay layer only 60µl of the total l00µl in each pump were delivered. The remaining 40иЛ of BrdU solution remained in the delay layer tubing. In total 1.44ml of 0.5mM BrdU solution was delivered. Pre-flight validation of this BrdU delivery method showed readily immunodctcctable BrdU in regenerating eyelid (figure 5), lens, tail and internal organs such as liver and intestine.

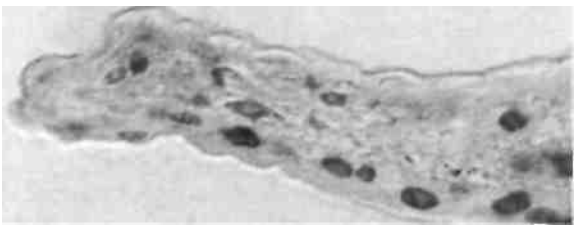

Figure 5. Regenerating eyelid nuclei labelled with BrdU delivered by osmotic pumps in pre-flight test (dark staining).

Within sixty minutes post re-entry, the "Triton" habitat. was recovered and animals were immediately chilled to 4°C and removed from the BrdU soaked PVA sponge to avoid further BrdU incorporation on the ground at lg. Post-flight testing of the osmotic pumps showed no recoverable BrdU, (except for that in the tubing) indicating BrdU was delivered as planned.

2.4 Fixation, embedding and sectioning of tissues

For microscopy tissues from the newt were fixed in 4% paraformaldehyde in phosphate buffered salins at pH 7.4, dehydrated in an ethanol series and paraffin embedded. Sections 10 u.m thick were de-paraffinized with xylenes and rehydrated in cthanol gradients to water.

2.5 BrdU immunocytochemistry

For *in situ* BrdU immunostaining we used Zymed BrdU staining kits according to manufacturer instructions. For spaceflight samples we replaced colorimetric detection with a strepavidin Qdot 655 step for higher sensitivity and unambiguous detection relative to pigmented cells.

2.6 DNA quantification

For nuclear staining and cell cycle analysis we mounted de-paraffinized tissue sections in Aquamount anti-bleach mounting medium containing 1 μlg/ml of the DNA intercalating dye Hoechst 33342.

2.7 Image analysis

Nuclei in stained sections were imaged using an Olympus BX51 microscope with a 20X UplanApo lens NA0.7, a WUV filter block, and a grade 1 cooled CCD Spot RT camera from Diagnostic Instruments. Images of nuclei for DNA quantification were acquired with an exposure lime of 200ms, and fixed fluorescence excitation intensity aperture maximally closed in the microscope. Images were collected in TIFF format at 1600X1200 pixels with 4095 intensity levels and no gamma correction. Image sets were analyzed using CellProfiler software to identify nuclear outlines and integrated intensity as a measure of DNA content.

3. EXPERIMENTAL DESIGN AND LOGISTICS

To achieve the goals of comparing cell proliferation in ground controls and spaceflight tissues undergoing regeneration we used 3 experimental groups, basal, synchronous, and flight. Animals were kept in aquaria prior to flight and fed to satiation. Flight group animals were either surgically resected or left intact 7 days prior to delivery for transport to the launch site in Baikonur and 13 days prior to launch. Resections were performed to remove the distal 2cm of the tail and the lens in the eye. After surgeries animals were returned to aquaria until placement on the flight habitat 6 days for shipment to the launch site. The flight group included 15 resected and 5 intact control animals. A basal group of 5 resected animals was used as a reference for determination of regeneration stage at launch, and to provide a negative control for BrdU delivery. The ground synchronous control was initiated 48h after the flight group and contained 15 resected and 5 intact animals. The synchronous control was performed in a "Triton" habitat similar to the flight hardware described above and was incubated at the temperatures recorded and transmitted from the Foton M-2 satellite. Upon re-entry animals were recovered in 50min and flown to Moscow at

4°C, reaching laboratories 30h post-re-entry (figure 6).

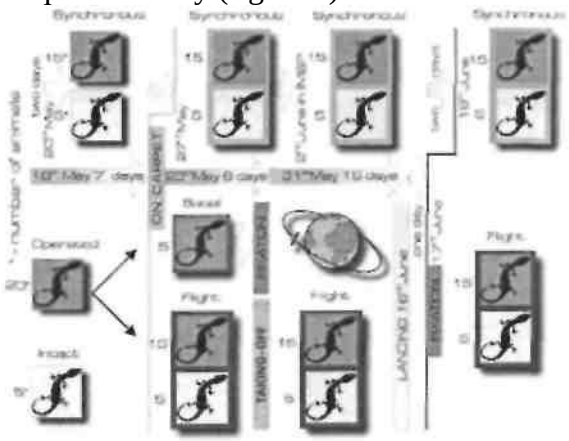

Figure 6. Regeneration experiment timeline for flight, basal and synchronous controls

4. RESULTS

4.1 Flight temperature recordings

Temperature in the Foton M-2 capsule was measured and transmitted to ground tracking stations during spaceflight and used to set the temperature of the ground 48h delay synchronous controls. This measurement however was not made at the location of the "Triton" habitat, and could be different from that experienced by the newts. To control for this possibility we included a miniature temperature recording data logger (ACR Systems JR-1000) within the habitat (see Figure 3, upper right corner). Post-flight analysis of habitat temperature data (Figure 7 left chart), in comparison to the data used lo simulate the flight control, shows that on average the animals in flight experienced a temperature about 2°C lower than controlled for in the synchronous group. In addition synchronous control animals were not cooled pre- and post-flight as the flight group.

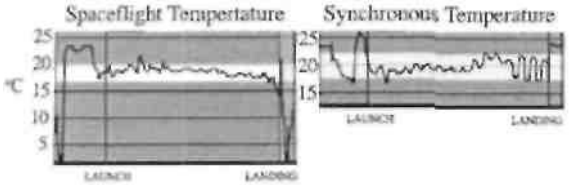

Figure 7. Spaceflight and synchronous temperature recordings

4.2 Tail regeneration in flight and synchronous groups

The image analysis of area of tail regenerates in flight and synchronous animals was compared and found not to be significantly different in size, although the basal-subtracted mean for the flight group (54,409 pixels) was 19% lower than synchronous control (64,628 pixels). Differentiation of muscle fibers in regenerating *Pleurodels waltl* tail appears delayed under spaceflight conditions. Specifically flight tail regenerates (figure 8A) are thinner than synchronous controls and do not contain elongated muscle nuclei seen in controls (figure 8B)

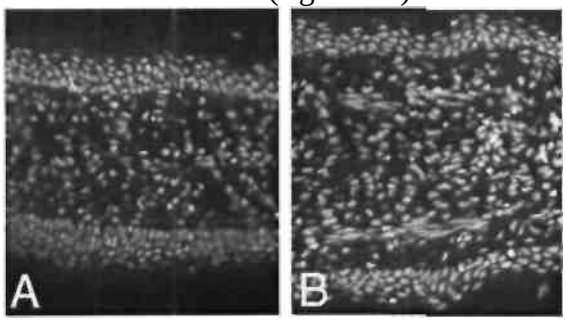

Figure 8. Spaceflight and synchronous tail nuclei (DNA stain)

4.3 Cell cycle analysis

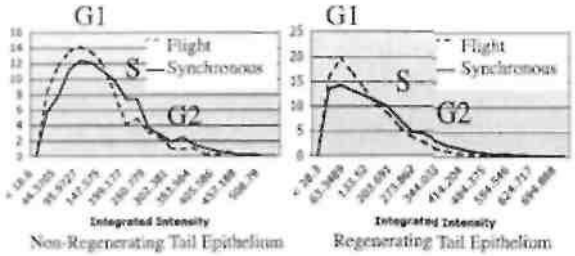

Figure 9. Cell cycle in normal and regenerating tail epithelia

DNA content measurements of spaceflight nuclei in tail epithelia show a small upward shift in Gl suggesting more cells were arrested in the cell cycle prior to undergoing DNA synthesis and mitotic division (figure 9). Both non-regenerating (left) and regenerating (right) tail epithelia show the same Gl shift during spaceflight.

4.4 BrdU incorporation during spaceflight

Rapidly dividing cells in regenerating tail epithelia show broad BrdU incorporation during spaceflight (figure 10). The epithelial tip region of the regenerating tail incorporated BrdU in about 45% of the cells countcrstained with hematoxylin. In addition BrdU label is also observed in internal organ tissue from spaceflight animals such as liver, intestine, and muscle, but in less than 1% of cells. Comparisons of BrdU incorporation in spaceflight and synchronous controls will be performed after temperature-corrected controls are completed.

Figure 10. Spaceflight regenerating tail sections stained for hematoxylin (left) and BrdU (right).

5. CONCLUSIONS

Initial results from the "Regeneration" experiment conducted during the Foton M-2 spaceflight show successful delivery of the proliferation marker BrdU to animals in-flight, and detection of cell division in microgravity. Posi-facto analysis of in-situ temperature recordings and tail regeneration data, however, shows higher temperatures in the ground synchronous control were not adequately simulated during the initial experiments, and may have influenced tissue regeneration rates. Additional temperature-corrected synchronous controls, and a mechanically unloaded aquarium control, arc underway to complement the data presented here, and allow the performance of valid proliferation comparisons.

This work was supported in part by NASA Foton M-2 PI grant to E.Almeida, USRA Bioastronautics and FSB Research Fellowship to J. Phillips, and by MCB and Russian Foundation for Basic Research Grants (project nos. 04-04-48044 and 05-04-48502) to V. Mitashov.

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