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EXPERIMENT K-6-03

GRAVITY AND SKELETAL GROWTH

PART I: GRAVITY AND SKELETEL GROWTH

PART II: MORPHOLOGY AND HISTOCHEMISTRY OF BONE CELLS AND VASCULATURE OF THE TIBIA

PART III: NUCLEAR VOLUME ANALYSIS OF OSTEOBLAST HISTOGENESIS IN PERIODONTAL LIGAMENT CELLS

PART IV: INTERVERTEBRAL DISC SWELLING PRESSURE ASSOCIATED WITH MICROGRAVITY

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EXPERIMENT K-6-03

PART I: GRAVITY AND SKELETAL GROWTH

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SUMMARY

Bone area, bone electrophysiology, bone vascularity, osteoblast morphology, and osteoblast histogenesis were studied in rats associated with Cosmos 1887. The results suggest that the synchronous animals were the only group with a significantly larger bone area than the basal group, that the bone electrical potential was more negative in flight than in the synchronous rats, that the endosteal osteoblasts from flight rats had greater numbers of transitional Golgi vesicles but no difference in the large Golgi saccules or the alkaline phosphatase activity, that the perioteal vasculature in the shaft of flight rats often showed very dense intraluminal deposits with adjacent degenerating osteocytes as well as lipid accumulations within the lumen of the vessels and sometimes degeneration of the vascular wall (this change was not present in the metaphyseal region of flight animals), and that the progenitor cells decreased in flight rats while the preosteoblasts increased compared to controls. Many of the results suggest that the animals were beginning to recover from the effects of spaceflight during the two day interval between landing and euthanasia; flight effects, such as the vascular changes, did not appear to recover.

INTRODUCTION

Experiments flown on Soviet Cosmos biosatellites suggest that growth in tibial diameter is suppressed in the long bones of growing rats (Morey and Baylink, 1978; Wronski and Morey, 1983). Further experimentation on Cosmos and Spacelab 3 (SL3) suggests that bone strength does not increase in these animals although the bone continues to grow in length (Spengler *et al.*, 1983; Shaw *et al.*, 1988). Strength tests of the humeri of flight rats on SL3 show that the difference from ground controls might be related not only to reduced bone diameter, but also to the composition and quality of new bone formed (Patterson-Buckendahl *et al.*, 1987). Also, a higher percentage of the total bone mineral has smaller crystals (which could be more soluble) in flight rats as compared to animals that remained on earth (Simmons *et al.*, 1986). Such data suggest that matrix turnover is altered during flight which in turn decreases the amount of bone added and either this defect alters the size of bone crystals formed or the flight environment somehow impedes crystal growth independent of matrix production; the suppressed bone formation may be a major factor in the failure of bone to increase in strength. These experiments were conducted in growing rats and suggest that gravity is important to normal growth and development of the mammalian skeletal system. The objectives of the Cosmos 1887 study were to continue the investigation of the effect of spaceflight on bone in growing rats by measuring bone area, bone electrophysiology, bone vascularity, osteoblast morphology, and ostoblast histogenesis.

MATERIALS AND METHODS

Specific pathogen-free, male, Wistar rats from the Institute for Experimental Endocrinology of the Slovakian Academy of Sciences were divided into 3 groups of 10 animals/group. The flight and synchronous groups were each housed in a single cage which had 10 nozzles for paste diet and 10 lixits for water. Fourteen gram boluses of food (55g/day/nozzle) were delivered every 6 hours beginning at 0200 each day. Water was provided ad libitum. The vivarium animals were housed similarly but were fed in a single bolus each day. The flight rats were launched on September 29, 1987, at 1550 hours and landed at 0703 hours on October 12, 1987. Problems associated with

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recovery of the craft were duplicated as closely as possible with the synchronous animals. The flight rats were killed 53.5-55.8 hours after landing; during the postflight time, they were transported from the landing site to the recovery site necessitating a 42hr fast and at least a 3 hour exposure to external temperatures ranging from -5 to -20C. Approximately 16 hrs before euthanization, the flight and synchronous rats were given 28 grams of food, and water ad libitum. The flight rats were 20 days older than the basals while the vivarium rats were 3 days older than the flight and the synchronous group was 3 days older than the vivarium group at the end of the experiment. At the end of each test period, rats were guillotined. One-half of each calvaria, 1/2 the proximal tibial metaphysis, and the tibial shaft were place in vials of 2% parformaldehyde in 0.1M cacodylate buffer, plus 0.5% glutaraldehyde, pH 7.4 at 4C for 48hr then rinsed 3 times with 0.1M cacodlylate buffer, pH 7.4 and shipped immersed in the buffer. The maxillae, with teeth, were fixed in phosphate buffered formalin, pH 7.0, stored and shipped at 4C. The thoracic vertebra was frozen and kept frozen during shipping. All samples arrived at this laboratory in excellent condition. The calvaria, maxilla, and tibial shaft were inadvertently placed in the freezer overnight causing potential freezing artifacts in these samples. The calvaria and proximal tibia were shipped at 4C by overnight mail to Dr. Doty for processing. The frozen vertebrae were shipped by overnight mail to Dr. Berretta for analysis. Dr. Roberts' samples were taken by car to San Francisco. All tissue arrived at their destination without incident.

The tibial shafts were dehydrated in ethyl ether and embedded undecalcified in polyester casting resin (Chemco, San Leandro, CA). The portion of the tibial shaft immediately proximal to the tibiofibular junction was sawed into 50 micron-thick cross sections with a Gillings-Hamco thin sectioning machine. Bone remaining in the blocks were shipped to Dr. Marc Grynpas, Toronto, Canada, (a collaborator with Dr. David Simmons) for further mineral analysis. Sections were mounted on slides and exposed to incident and polarized light.

The technique for measuring zeta potential has been described in detail (Berretta and Pollack, 1986). The vertebra were thawed, and wet ground in various solutions of NaCl using a diamond-tipped dental bur. The bone particles were homogenized for 10min and serially transferred twice for 15min of sedimentation per transfer to obtain a particle size of 5±2μm. A narrow size distribution of bone particles was desired so that light-scattering methods used in the zeta-potential measurements would yield histograms that could be interpreted as the percent of particles exhibiting a given zeta-potential value. Electrophoresis was used as the direct method of determining the zeta potential of bone particles. The bone particle velocity was measured in a fluid with a given ion concentration and pH while an electric field of known amplitude was applied. The ratio of the particle velocity to the electric field amplitude is defined as electrophoretic mobility (EPM) which, in turn, is proportional to the zeta potential. The direction of motion of the bone particles relative to the direction of the applied electric field was used to determine the polarity of the zeta potential. The PenKem System 3000 Automated Electrokinetic Analyzer (Pen Kem, Inc., Bedford Hills, NY) was used to measure EPM. In this instrument, a vertical, cylindrically focused beam of laser illumination traverses the stationary level (where net electroosmotic fluid velocity is zero) of a horizontally mounted cylindrical electrophoresis chamber 0.1 by 1.0cm. Light that is scattered 90° from the beam by the particles undergoing electrophoresis is focused through an objective onto the surface of a rotating radial grating. Pulses of light transmitted through the grating strike a photomultiplier tube whose output is analyzed. In the absence of current at the electrodes the particles in the chamber are stationary. Since the size of each particle is smaller than the width of each grating segment, the light scattered by each particle will be alternately blocked and then transmitted by the grating as the disk moves. Thus, the reference signal for the system corresponds to an output voltage from the photomultiplier tube that has a signal component at a frequency proportional to the speed of the disk. If a current is applied across the electrodes, an electric field will be produced that causes the particles in the chamber to move electrophoretically. The velocity of each particle produces a change in the tangential velocity of the image of that particle with respect to the grating. If the image of the particle is moving in the same direction as the grating, the image will cross fewer line pairs per unit time, producing a signal component for

that particle at a slightly lower frequency than the reference frequency. If the particle image moves in the opposite direction, the image will cross more line pairs per unit time and the signal will be at a higher frequency. Since there will normally be many particles in the field of view at any one time, each particle produces a signal component at a frequency shift related to its electrophoretic velocity. The mobility is thus determined by measuring the amount of the frequency shift with respect to the reference signal. This difference in frequency is proportional to the particle mobility, which is calculated directly on the basis of physical constants. When a population of particles

undergoing electrophoresis is examined, the frequency difference spectrum gives the EPM distribution of the population. A mobility histogram is then produced which shows the mobilities of various subpopulations of the bone particles studied. From this histogram, an average mobility can then be computed for the population. All solutions were prepared using distilled deionized water to which NaCl was added. The specific conductance of each sample was measured using a square wave current applied across palladium electrodes, with the resultant potential corrected for electrode polarization and joule heating. The NaCl concentrations corresponding to each conductivity value were determined from standard tables. The pH of each sample was measured automatically. Temperature was controlled by a water bath set at 20°C. The field strength used was 50±0.3V/cm. The EPM of human erythrocytes prepared using the method outlined by Seaman and Heard (Seaman and Heard, 1960) was used as a standard prior to each day's experiment. All specimens were allowed to equilibrate in their respective NaCl solutions at least 6 hr prior to testing. The average zeta potential was calculated from the average EPM using the Helmholtz-Smoluchowski equation:

$$\zeta = \frac{4\pi\mu\eta}{\varepsilon} \quad \text{or} \quad \zeta = \mu \times 12.873$$

where ζ is the zeta potential, μ the EPM, η the viscosity of the fluid, and ε the dielectric constant of the fluid

Techniques used by Dr. Doty in processing his tissues and the data are discussed in detail in Supplemental Report 1: Morphology and histochemistry of bone cells and vasculature of the tibia from Cosmos 1887. The techniques and dat from Dr. Roberts and his colleagues are discussed in detail in Supplemental Report 2: Nuclear volume analysis of osteoblast histogenesis in periodontal ligament cells of Cosmos-1887 rats.

RESULTS

The body weight of the basal group was 316+8.3(S.E.) on 9/24/87. The flight group weighed 303+2.4 on 10/14/87, while the vivarium group weighed 342+7.7 on 10/17/87 and the synchronous group weighed 349+5.8 on 10/20/87. The vivarium group gained an average of 1.13gm/day in the 23 days after the basal group was euthanitized while the synchronous group gained an average of 1.27gm/day in 26 days. The flight group lost an average of 13gm over a 20 days period compared to the basal group or -0.65gm/day. Further information on the weights of all groups before launch and following the flight period is essential to interpret the data.

Visual observations of tibial cross sections under brightfield or polarized light did not show any obvious differences between groups. Area and perimeter measurements (Table 1) showed no significant changes between any of the experimental groups. The only differences appeared in the periosteal perimeter and cross-sectional bone area at the tibiofibular junction in the basal rats as compared with the synchronous group ($p<0.05$).

The electrophysiology measurements are found in Table 2. The synchronous and vivarium control groups have very similar EPMs while the basal group is slightly less electronegative and the flight group is more electronegative than the controls. The summary histograms for the flight and synchronous groups are found in Figure 1.

DISCUSSION

Data from Cosmos 1887 are more difficult to interpret than previous missions due to unanticipated postflight problems and to the significant difference in mean body weight between the flight and the synchronous control group. Whether the weight difference reflects the six day age difference between the groups is not known. In preliminary experiments using normal, control animals, we found significant differences in bone strength in young, growing rats differing in age by only three days (approximately 235 vs 260gm). Thus, the older age of the control rats might bias some data toward significance. Also, the unanticipated postflight period (2.2-2.3 days) may have been sufficient for certain parameters to either recover or to begin recovery; distinguishing those parameters which are recovering and those which are not is extremely difficult.

The bone parameters including area and perimeter measurements at the tibiofibular junction show differences only between the synchronous and basal groups (Table 1). Data from the large rats in SL3 and previous Cosmos flights including Cosmos 782 and 1129 also showed no signficant changes in bone area between flight and synchronous controls; however, using a bone marker, a significant decrease in bone mineralization during flight was detected in these experiments. Only on Cosmos 936 was the bone area of the flight group significantly smaller than the synchronous group. Apparently the total bone area at the beginning of the flight period is much larger than the amount of bone formed in 1-3 weeks and can mask a mineralization defect unless bone markers are used.

Underlying Wolff's law on bone remodeling is the hypothesis that physical stresses applied to bone result in the formation of endogenous electrical potentials and that these potentials alter bone cell activity in a feedback manner to result in bone remodeling. Thus, any factor that can bring about a change in either sign or magnitude or both may alter these endogenous electrical potentials and change the balance between bone formation and bone resorption. The zeta potential is thought to be the surface charge at the bone-fluid interface which is related to streaming potentials. Streaming potentials are thought to occur when hydrated bone is mechanically deformed. The potentials have been determined to be of electrokinetic origin and can be quantified using the direct measurement of EPM which is proportional to the zeta potential. The electrical double layer at the bone-fluid interface within bone is different for some types of osteoporotic bone compared to normal bone and this difference can be quanitifed using zeta potentials characterized by EPM histograms (Berretta, *et al.*, 1987). The differences in the mean values of the histogram peaks may be only a manifestation of the disease or alternateively, this feature may indeed be responsible for the disease process. Systemic osteoporosis is characterized by EPM histograms with two or more peaks compared to controls which are single peaked (Berretta, *et al.*, 1987). The mean zeta potential for normal human bone in bone ECF was -3.6mV, while osteoporotic human bone was significantly less electronegative (-0.77mV). In rats, six months after castration, osteoporosis in the femur was indicated by double peaks and a significantly less negative mean EPM value. These data suggest that the more positive EPM values indicate net bone resorption; more negative values may be indicative of net bone formation.

In Cosmos 1887, the synchronous animals, which gained the most weight, had essentially normal zeta potential values (Table 2). The flight group has a more negative potential, indicating net bone formation, than all other groups. Interestingly, the potential in the flight rats is in the opposite direction of osteoporotic bone (that is, more negative rather than more positive mean EPM values). Whether the value reflects increased matrix synthesis during flight or during postflight recovery is not known. Data in this report by Roberts and coworkers suggest that osteoblastic histogenesis is

increased during the postflight period whereas a suppression of osteoblastic activity has been reported on past missions. The more negative potential in the space bone may also indicate that this bone defect may be theoretically reversible because it is of the same sign as normal bone; postmenopausal osteoporosis, which is opposite in sign to normal bone, is more difficult to return to the normal set point.

The time course and extent of bone changes during spaceflight must be understood before long duration spaceflight without compensating forces should be considered. A major concern is that adaptation to spaceflight might impair the ability to return to earth or other planets. Thus, we must understand what is happening to the mammalian skeleton during spaceflight. The Cosmos missions provide data for understanding skeletal adaptation in the growing rat to spaceflights lasting longer than one week.

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TABLE 1. COSMOS TIBIAL BONE PARAMETERS

	BASAL	FLIGHT	SYNCHRONOUS	VIVARIUM
Bone area	3.43±0.23	3.60±0.27	3.94±0.37*	3.75±0.34
Marrow area	0.79±0.19	0.77±0.16	0.84±0.17	0.79±0.17
Periosteal perimeter	7.93±0.20	7.97±0.29	8.38±0.29*	8.11±0.41
Marrow perimeter	3.37±0.13	3.32±0.31	3.45±0.34	3.36±0.37

Data are expressed as mean ± S.D.
* = significantly different from basal ($p<0.05$)
Area units are mm^2 while perimeter units are mm

TABLE 2. ZETA POTENTIAL MEASUREMENTS

GROUP	Mean electrophoretic mobility ($x10^{-8}$ µm-cm/V-s)	±SD	Mean zeta potential (mV)
Basal	-0.49±0.06		- 6.3
Flight	-1.20±0.04		-15.4
Synchronous	-0.56±0.35		- 7.2
Vivarium	-0.62±0.09		- 8.0

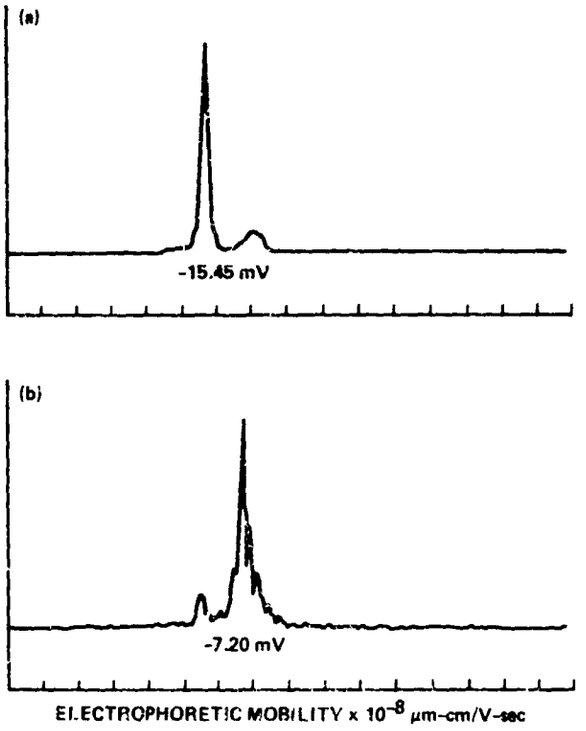

Figure 1. Summary histogram of zeta potentials. (a) Flight group; (b) synchronous group.

EXPERIMENT K-6-03

PART II: MORPHOLOGY AND HISTOCHEMISTRY OF BONE CELLS AND VASCULATURE OF THE TIBIA FROM COSMOS 1887

S.B. Doty

SUMMARY

Electron microscopy, light microscopy and enzyme histochemistry were used to study the effects of spaceflight on metaphyseal and cortical bone of the rat tibia. Cortical bone showed the most significant changes, which were reflected in changes in the endosteal osteoblast population and the vasculature near the periosteal surface. The osteoblasts demonstrated greater numbers of transitional Golgi vesicles following spaceflight, an effect possibly caused by a decreased cellular metabolic energy source. The vasculature showed accumulations of lipid and adjacent degenerated osteocytes. This result may be due to ischemia of bone or a developing fragility of the vessel walls as a result of spaceflight.

INTRODUCTION

A decrease in new bone formation during spaceflight (Morey and Baylink, 1978) occurs in weight-bearing bones and appears to be due to a reduction in new bone formation rather than increased bone resorption (Wronski and Morey, 1983). Therefore we have concentrated our studys on the bone forming cells, the osteoblasts, and more recently we have begun to investigate the vascular supply within bone matrix. It is the vascular supply which provides all the nutrients for collagen formation, including oxygen for the hydroxylation of proline to form hydroxyproline (Bornstein, 1974; Kruse and Bornstein, 1975), an important step for mature collagen fibril formation.

In a previous study of animals from Spacelab-3, the osteoblasts appeared to be slightly smaller in size following 7 days of flight (Wronski, et al, 1987). These osteoblasts were found along the endosteal surface of the diaphyseal bone and tended to have a more uniform size as compared to osteoblasts along trabeculae in the metaphyseal region of the long bones. Therefore we have concentrated much of our efforts on the cells found within the diaphyseal area of long bones following flight in Cosmos 1887.

Because the animals from spaceflight cannot undergo any experimental procedures while in space, we have applied electron and light microscopic and morphometric procedures to bone samples collected following spaceflight. We have applied histochemistry to enzyme activity along cell membranes (ie, alkaline phosphatase distribution), within various Golgi compartment (ie, NADPase and acid phosphatase) and within the lysosomal bodies (ie, acid lipid accumulation occurs in bone sites as a result of spaceflight [Jee, et al, 1983]). However lipid also accumulates in bone as a result of ischemia (Chryssanthou, 1978). Therefore we have begun to apply methods to localize lipid deposits in bone. And the relationships between vascular morphology, lipid accumulation, and osteocyte and osteoblast morphology have been studied in the weight-bearing long bones.

METHODS

These methods have been discussed in great detail previously (Doty, 1971; 1980). Tissue fixation was limited to the use of 2% paraformaldehyde plus 0.5% glutaraldehyde in 0.05M cacodylate buffer, pH 7.4, at 4°C for 18-24 hours. For histochemical purposes the tissues were decalcified in

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Tris buffered 10% EDTA, pH 7.4, following the aldehyde fixation. Following fixation and decalcification, 50µm thick selections were obtained with a vibratome, and sections incubated in the various media for alkaline or acid phosphatase, or NADPase (Smith, 1980). Tissues were embedded in LR White resin for light microscopy or Spurrs resin for electron microscopy. Staining for lipids was carried out on frozen sections of aldehyde fixed tissues or on vibrotome sections which were subsequently embedded material was thin sectioned and observed with a JOEL 100C microscope. Morphometry was carried out on light or electron micrographs using the Zeiss Interactive Data Analaysis System (ZIDAS). This system uses a dedicated computor and a hand held light pen to quantitate structures, determine areas, etc.

RESULTS

Light microscopy of trabecular and cortical bone and the included osteoblast populations showed no obvious morphological differences as a result of spaceflight. Attempts to quantitate osteoblast size were abandoned because of the extreme variation in cell size even within control groups. Thus any potential difference was hidden within the statistical variation. Initial data does indicate that, in the endosteal diaphyseal osteoblast population, osteoblast cell numbers were decreased by about 15%. Whether the variability reflects postexperiment changes or is the normal physiological state is not known. These measurements will have to be varified by the use of many more tissue sections before the stastics can be considered reliable.

Blood vessels within normal diaphyseal bone showed a typical appearance of endothelial cells enclosing a large open lumen. The endothelium was surrounded by pericytes and some undifferentiated cells (Figure 1). In large vascular spaces the undifferentiated cells were often replaced by osteoblasts and new bone formation was evident.

In diaphyseal bone from flight animals, blood vessels near the periosteal surface often showed very dense intraluminal deposits (Figures 2 & 3). The control tissues occasionally showed a similar pattern but in these instances a packing of intact red blood cells could be seen in the lumen. The deposits within blood vessels from flight animals showed no recognizable cell structure and were not red blood cells. In addition, near these vessels, darkly staining osteocytes were found. This unusual arrangement of intraluminal deposits and adjacent degenerating osteocytes were only seen along the periosteal portion of cortical bone.

Electron microscopy indicated rather normal morphology of the bone cells in the metaphyseal regions. In the diaphysis, the endosteal osteoblasts showed considerable variation in size, but no specific morphological change. The structural aspects of these osteoblasts were normal. In the periosteal region, however, many osteocytic lacunae were found devoid of osteocytes and sometimes filled with an osmiophilic substance (Figure 4). The blood vessels in this region showed endothelial cell disruption and lipid accumulations within the lumen of the vessels (Figure 5). In some cases there was cell debris within the lumen rather than lipid deposits but the degeneration of the vascular wall was evident. It was also apparent by electron microscopy that these osteocytic and vascular changes were confined to the periosteal portion of the diaphyseal bone. These results were never found in the metaphyseal region of the long bones.

The histochemistry of the NADPase activity indicated that this enzyme was localized to the intermediate Golgi saccules of the osteoblast and to the small vesicles and granules within the Golgi complex (Figure 6). All osteoblasts contained such activity within their Golgi regardless whether they were from flight or control animals. A quantitative count of the saccules and small vesicles associated with the Golgi showed that the number of saccules containing reaction product was similar for flight and control osteoblasts. Flight animals contained reactive saccules which averaged 11.3±6.1 saccules per cell, and the vivarium controls averaged 14.4±3.4 saccules per cell which contained reaction product. However when we counted the small vesicles (the transitory or intermediate vesicles) which bud off the saccules and which also contained NADPase activity, the

flight animals averaged 17.0±6.2 vesicles compared to the control average of 10.5±2.7. These reactive vesicles were found only in the Golgi region and are not found in any other area of the cytoplasm.

The histochemistry of alkaline phosphatase activity showed that the osteoblasts contained very high enzyme content along the external surface of the cell membrane (Figure 7). On very rare occasions, activity was also found within the large Golgi saccules prior to forming a secretory granule. Alkaline phosphatase activity was quite strong in the osteoblasts of the metaphyseal region such that we could not distinguish any differences in staining between flight and control animals. However in the diaphysis there was definite decrease in alkaline phosphatase associated with the vascular channels within diaphyseal bone. The vasculature of the flight animals were definitely less reactive than the control groups. This reaction was not limited to the osteoblasts within the vascular channels since the endothelial cells of the blood vessels also contained alkaline phosphatase activity (Figure 8). Therefore at the light microscope level of observation, the alkaline phosphatase activity associated with the vascular space is a combination of reactions from endothelial cells and perivascular cells.

The alkaline phosphatase activity in the blood vessels of bone was located along the basement membrane surrounding the endothelial cells, and along adjacent cells (Figure 8). The pericytes and non-osteoblastic cells associated with the vessel were surrounded with alkaline phosphatase activity as were the osteoblasts adjacent to the vessels but aligned along the bone surface. In the flight animals, the alkaline phosphatase activity was significantly lessened compared to controls (Figures 9 & 10) around the vascular endothelium, and often appeared as an incomplete line of reaction compared to the complete reaction found in the controls.

In order to determine whether the vascular channels themselves had been affected by flight, we measured the area of these space in diaphyseal bone. The data collected shows that for flight animals, the average area of each vascular space was $762\pm157\mu^2$ whereas the same measurement in the simulated control group was $921\pm267\mu^2$. The difference was not highly significant because of the large standard deviation but does suggest that the vascular spaces were smaller within the diaphyseal bone of the flight animals. Measurements made of the numbers of vessels per area of bone also showed a difference:

Flight = 72.3±6.8 vessels per mm^2 of bone area.
Simulated controls = 49.0±8.8 vessels per mm^2 of bone area.

These results would indicate that more vascular space per area of bone existed in flight animals as compared to the simulated controls.

DISCUSSION

The decrease in new bone formation due to spaceflight has been documented (Morey and Baylink, 1978; Wronski and Morey, 1983) and most results suggest that the effect is on the bone forming cells, the osteoblasts. Bone resorption in weight-bearing bones appears to be functioning normally during hypogravity exposure (Vico, et al, 1987). Therefore the osteoblast has been the bone cell of interest for determining the effect of gravity on the skeleton. In addition, other studies have indicated that bone strength in reduced (Spengler, et al, 1983) and that a redistribution of bone crystal sizes occurs (Simmons, et al, 1986) as a result of spaceflight. So the structural integrity of the weight-bearing bone has been compromised in the hypogravity environment.

In our studies of the osteoblast cell size, the measurements were quite variable in different areas of the bone. Thus, these measurements were not usefull in this study. Similar measurements from osteoblasts flown on Spacelab-3 showed only about a 10% decrease in cell size following 8 days

of flight (Wronski, et al, 1987). These variations in size are also reflected in the alkaline phosphatase activity because the cells which are larger and more differentiated tend to have greater phosphatase activity (Doty, 1980). Therefore the large variation in cell size also produced considerable variation in alkaline phosphatase histochemistry. With careful timing of the appearance of the reaction product, it was estimated that the osteoblasts from the flight animals showed less reactivity than the various controls (data not shown) but the data were not always consistent. These observations were limited to the osteoblasts along the endosteal surface of the cortical bone.

The histochemical NADPase reaction has been used to study the activity of the intermediate saccules of the Golgi in ameloblasts (Smith, 1980). Because the Golgi saccules and the transitional vesicles were so specifically stained by the NADPase reaction, and are directly involved in collagen synthesis and secretion, we quantitated these structures in osteoblasts. The appearance of increased numbers of vesicles in cells from flight animals compared to controls was unexpected in that collagen synthesis must be altered during the reduced new bone formation induced by flight (Morey and Baylink, 1978; Wronski and Morey, 1983). However in other non-bone cell types, these vesicles have been shown to accumulate whenever the energy sources of the cell have been depleted (Saraste, et al, 1986). It is also interesting that in osteogenesis imperfecta, a collagen deficient disease of bone, we previously noted that these vesicles were present in unusually large numbers (Doty and Mathews, 1971). Thus the present data may be used to suggest that the condition of space flight creates an energy loss within the osteoblast and this could lead to reduced collagen synthesis. However, postflight readaptation complicates interpretation of these data.

The other area of bone physiology which appears greatly affected by spaceflight is the vasculature within bone matrix. In an earlier histochemistry study we showed that the blood vessels in bone contain a calcium-stimulated ATPase activity (Doty, 1985). The present study shows that there is a strong alkaline phosphatase activity as well, located between the endothelial cells and the pericytes and includes the basement membrane between the cells. However, as a result of space flight this activity was decreased. We cannot apply the calcium stimulated ATPase staining to samples from space because the enzyme is destroyed by the chemical fixation process, however it would be important to know if this activity is also reduced due to the hypogravity environment. Nevertheless these histochemical results indicate that the vessels within bone matrix contain enzyme activities which could be important in regulating ion transport between serum and bone matrix.

It was noted in this study that many of the vessels near the periosteal surface contained lipid inclusions and/or morphological signs of endothelial degeneration. Lipids can result in necrosis of bone (Jones, 1985) although the exact mechanism is unknown. Ischemia may also cause cell death and degeneration (Chryssanthou, 1978) and may occur with or without the appearance of lipid accumulation. So whether ischemia in bone and the presence of lipid are related events is not known at this time.

It was noteworthy that the vessels and osteocytes near the periosteal surface were most affected by space flight, as the periosteal surface receives less oxygenated blood than the bone next to marrow cavity (Lopez-Curto, et al, 1980).

We also noted that the vasculature cross-sections were more numerous in the compact bone from flight animals, even though the average cross sectional area per vessel was smaller than in the control bones. This smaller size could also be a factor in lipid being "trapped" within some of these vessels. And the increase in numbers of vessels may indicate a slower turnover of the whole skeleton, therefore not as much bone is produced between vessels during bone growth.

We have not determined whether a single factor is responsible for the decrease in bone formation as a result of hypogravity. But the evidence presented here would suggest that a vascular change may occur during flight which would then influence the bone forming ability of the osteoblasts in the weight-bearing bones. It is also possible however, that the vascular damage noted here could occur as an injury received to these fragile vessels during the impact and hypergravity forces of re-entry from space or during recovery following spaceflight. This possibility has not been investigated but needs to be considered for study in future space flights.

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Figure 1. An electron micrograph of a normal blood vessel within diaphyseal bone. Thin walled endothelial cells (arrows) surround an open lumen which contains two macrophages (MP). Perivascular or pericytic cells (PC) are situated between the endothelium and the bone matrix. An osteocyte (OC) is located in bone matrix adjacent to the vascular space. The bone matrix has been demineralized. Magnification = 5,600 X

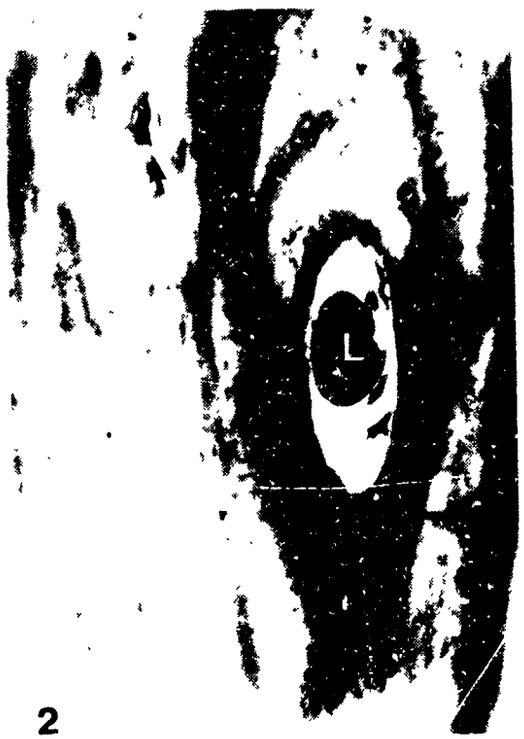

Figure 2. A light micrograph of a vascular space in bone filled with a large lipid (L) inclusion. Osteocytes are present in nearby matrix (arrows). This sample is from the diaphysis of a "flight" animal. Magnification = 1,200 X

Figure 3. A light micrograph of a normal vascular space from the tibia of a vivarium control rat. The lumen (L) of the vessel and the endothelial cells (arrows) can be seen; no lipid deposits are present. Magnification = 1,200 X

Figure 4. This electron micrograph shows a portion of the vascular space (VS) and an osteocyte (OC) from the periosteal region of a "flight" animal. Both structures are darkly stained and structurally degenerate, suggesting either a lipid accumulation or cell death. Magnification = 9,200 X

Figure 5. This electron micrograph shows the large lipid (L) inclusion which are found within the vascular spaces in diaphyseal vessels of the "flight" animals. Some structures resemble the remains of vascular endothelial cells (arrows) which have undergone degeneration. Magnification = 18,000 X

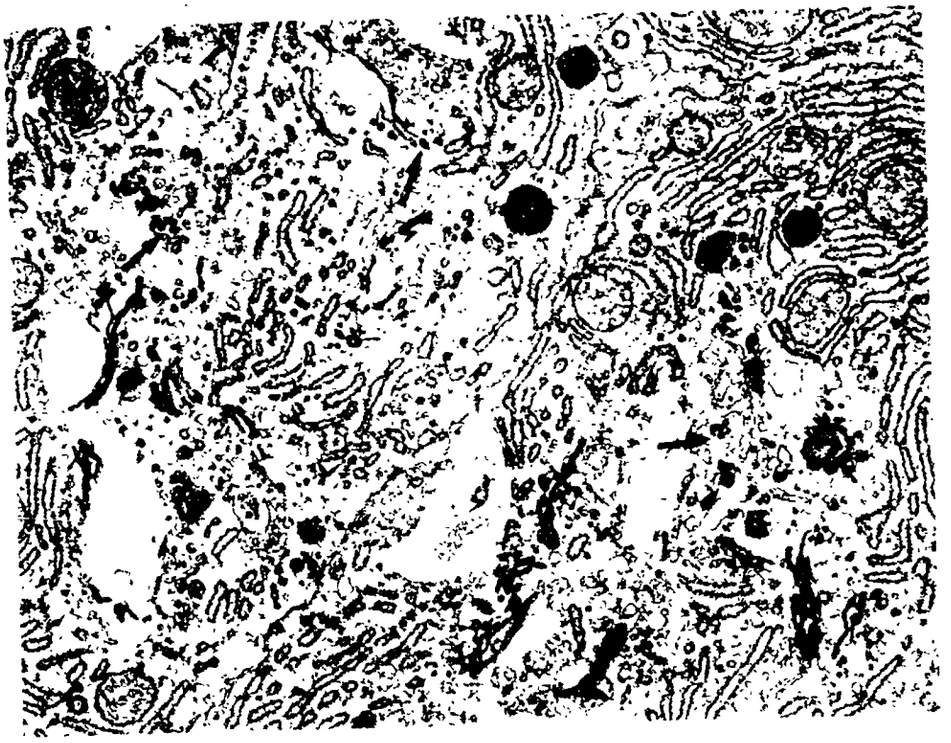

Figure 6. An electron micrograph of the Golgi region of a single osteoblast which has reacted for NADPase activity. The small transitory vesicles (arrows) were found to be more numerous in osteoblasts from "flight" animals. Magnification = 16,000 X

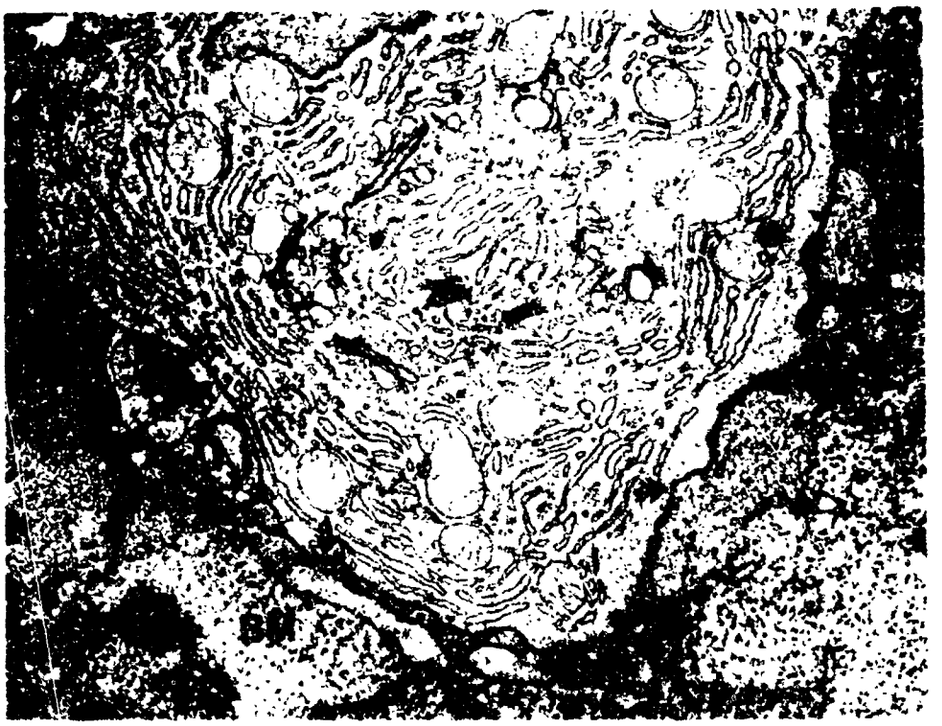

Figure 7. An electron micrograph which shows the alkaline phosphatase reaction (arrows) along the external cell membrane of a single osteoblast. Enzyme reaction can also be seen in the underlying bone matrix (BM). Magnification = 16,000 X

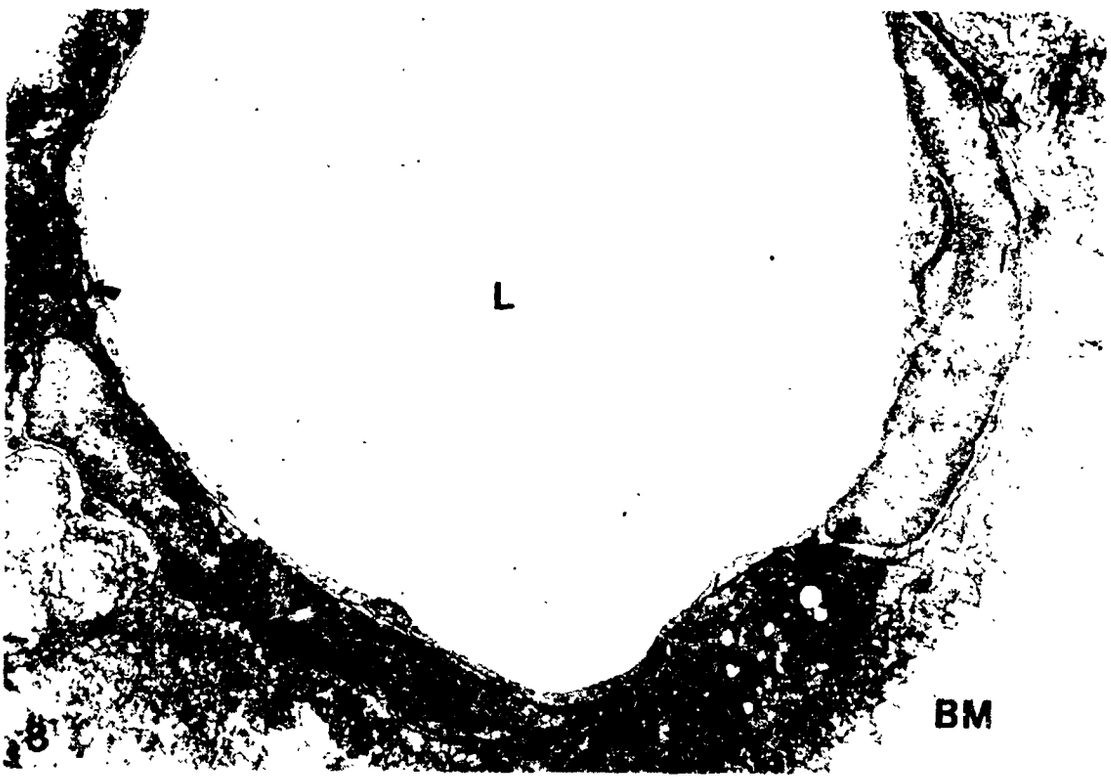

Figure 8. An electron micrograph of the alkaline phosphatase activity (arrows) distributed among the endothelial cells and pericytes of a blood vessel in bone. L = lumen of blood vessel; BM = demineralized bone matrix; Magnification = 10,000 X

Figure 9. A light micrograph showing the alkaline phosphatase reaction (arrows) among the blood vessels of normal bone. BM = bone matrix. Magnification = 700 X

Figure 10. A preparation identical to Figure 9, except the bone is from a "flight" animal. Note that the intensity of the reaction is less than the control and some areas (arrows) show a definite reduction or loss of enzyme activity. Magnification = 700 X

EXPERIMENT K-6-03

PART III: NUCLEAR VOLUME ANALYSIS OF OSTEOBLAST HISTOGENESIS IN PERIODONTAL LIGAMENT CELLS OF COSMOS 1887 RATS

L.P. Garetto, W. Eugene Roberts, and M. R. Gonsalves

SUMMARY

Periodontal ligament (**PDL**), the osteogenic interface between tooth and bone, was morphometrically analyzed in rats subjected to 12.5 days of weightlessness and a ~55h recovery period at 1 G. Compared to synchronous controls, this treatment resulted in a 40% decrease in less differentiated osteoblast progenitor cells, a 42% increase in preosteoblasts (immediate precursors to osteoblasts) and increased numbers of **PDL** fibroblast-like cells within 25μm of the bone surface. These results are consistent with a post-flight osteogenic response in **PDL** adjacent to previously resting or resorbing alveolar bone surfaces. This osteogenic response occurred despite physiological stress in the flight animals that resulted in a highly significant ($p \geq 0.001$) increase in adrenal weight. The data suggest that following spaceflight there is a strong and rapid recovery mechanism for osteoblast differentiation that is not suppressed by physiological stress.

INTRODUCTION

Bone is a mechanically sensitive tissue that is particularly responsive to gravitational factors. An understanding of changes in bone mass is important since it functions not only as a structural support, but also as a metabolic source of calcium (Morey-Holton *et al.*, 1988). Previous studies have shown that microgravity associated with spaceflight results in marked musculoskeletal changes including muscle atrophy (Riley *et al.*, 1985) and suppression of bone formation (Morey *et al.*, 1978). The reduction in bone formation appears to occur as a generalized, systemic phenomenon since both the weightbearing axial skeleton (Jee *et al.*, 1983) and non-weightbearing bones (Simmons *et al.*, 1983) are similarly affected.

At least one aspect of the suppression of bone formation during spaceflight is an inhibition of osteoblast production (Roberts *et al.*, 1981; Roberts *et al.*, 1987). The periodontal ligament (**PDL**), a well defined cell kinetic model for assessing the proliferation and differentiation of the cells associated with osteoblast histogenesis (Roberts *et al.*, 1981), was used to study the effect of microgravity on osteoblast production. Roberts and Morey (1985) have identified distinct, fibroblast-like, cellular compartments within the **PDL** that comprise progressively more differentiated osteoblast precursor cells. These compartments have been classified as 1) **A**, less-differentiated, self-perpetuating precursor cells; 2) **A'**, committed osteoprogenitors that are derived from **A** cells and are the source of preosteoblasts; 3) **C**, G_1 stage preosteoblasts; and 4) **D**, G_2 stage preosteoblasts which undergo mitosis and form mature osteoblasts as shown in Figure 1.

Previous spaceflight data has suggested that the population of the less differentiated osteoblast progenitor cells (**A+A'**) increases (Roberts *et al.*, 1981; Roberts *et al.*, 1987) while the number of preosteoblast cell types (**C+D**) is reduced (Roberts *et al.*, 1987). Recovery of osteoblast precursor differentiation following spaceflight has been sparsely investigated. Analysis of rat **PDL** from the Cosmos 1129 flight revealed that all cell populations were identical to synchronous control animals by 6 d post-flight and remained so at 29 d post-flight (Roberts *et al.*, 1981). Twelve hours following the landing of the 7 d, Space Lab-3 (**SL-3**) flight, there were no residual changes in the

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cell population of young rats; the only significant difference was an increase in the A+A' category of older animals. Assuming 7 d of spaceflight is sufficient to suppress osteoblast differentiation, these data suggest that 12 h of recovery at 1 G is sufficient to return the osteogenic compartments to near normal.

The present study of the **PDL** from the Cosmos 1187 flight allows evaluation of 12.5 Days of spaceflight and 55 h of recovery under somewhat stressful conditions. The data indicate that a return to normal gravity resulted in a very strong compensatory replenishment of osteogenic capacity that occurred despite post-flight physiological stresses.

METHODS

Ten male (specific pathogen free) Czechoslovakian-Wistar rats were housed in a single cage aboard the Cosmos 1887 satellite. A paste diet was provided in 14g boluses at 0200, 0800, 1400, 2000h each day through ten nozzles in the cage. Water was provided *ad lib* via ten lixits. The rats were maintained on a light cycle with the lights on from 0800-2400 h. The flight group (F; born 7/1/87) was launched on 9/29/87 and remained in space until 10/12/87. The total duration of the microgravity environment was 12.5d (Fig. 2). Rats were last fed in flight on 10/12/87 at 0200 h but due to difficulties upon re-entry were not fed again until 42 h later, which was approximately 11-13h prior to sacrifice. As a result of the re-entry problems and distant landing location in Siberia, the animals were subjected not only to disruption of their feeding schedule, but also disruption of the light cycle and a decreased capsule temperature. Furthermore, it was necessary to transport the animals for about 10h by bus, airplane and van to reach the sacrifice/dissection area. Because of these post-flight recovery problems, sacrifice (at 105d of age) was delayed a total of~53-55h following re-exposure to 1 G.

Three groups of rats were used as controls for the flight animals (Fig. 2). Ten basal controls rats were housed in similar cages and placed on a paste diet for 14d with sacrifice occurring 5d prior to the actual flight (at 85d of age). The temperature and lighting conditions were maintained similar to the inflight conditions. An equal number of animals served as vivarium controls (108d of age at sacrifice). These rats were kept in similar environmental conditions to the flight animals, but were fed all of their food at a single feeding. Finally, a synchronous control (S) group (10 rats) was maintained in flight-type cages on a paste diet. These rats were subjected to similar launch forces, vibration, lighting regimen and temperature as flight rats. After their simulated flight, the S group was deprived of food for 42h and their sacrifice was delayed for 53-55h to mimic the F group conditions. Re-entry G forces, vibration and post-flight transportation conditions were not simulated for this group. Synchronous controls were sacrificed 6d after flight animals and were 111d of age.

At sacrifice, the maxillae from five animals in each group were removed and immersed in neutral buffered formalin for 48h at 4°C. Fresh fixative was added and the samples were maintained at 4°C during shipment to the NASA Ames Research Center, Moffett Field, CA. The samples were prepared for demineralized histological sectioning in the midsagittal plane of the mesial root of the first molar. Details of the histological methods are published (Roberts *et al.*, 1987). Briefly, 3µm sections of methyl methacrylate embedded maxillary halves were stained with hematoxylin and eosin. Nuclear volume analysis of the cells in the midroot area of the **PDL** was performed as described by (Roberts *et al.*, 1982). Only **PDL** samples with a resorbing (scalloped with occasional osteoclasts) or resting (no morphological evidence of active resorption or formation) alveolar bone margin were selected for analysis. At some point during transport or storage prior to reaching the authors' laboratory, the samples appear to have been frozen, resulting in varying degrees of artifact within the tissue. Because of this, only four animals from each group could be evaluated. It was necessary to select areas within the **PDL** where cells were not morphologically disrupted. Following ocular micrometer measurement of the major (a) and minor (b) nuclear dimensions at x1000, the nuclear volume for the cells were calculated as previously described

(Roberts *et al.*, 1982) according to the formula for a prolate spheroid: Volume = $4/3\pi ab^2$. Each cell was classified as **L, A+A', B, C,** or **D** type according to the following nuclear size categories : <40, 40-79, 80-119, 120-169 and ≥170μm^3, respectively (Roberts *et al.*, 1985). Data from each category is expressed as group means ± SE. Statistical analysis of differences between groups was performed using the two-tailed Student t-test with $p \leq 0.05$ considered as statistically significant.

RESULTS

Body Weights. At sacrifice, the body weight (mean±SE) of the animals in the flight group (302.2±2.4g) was significantly less than that for vivarium (342.0±7.7g) and synchronous controls (349.0±5 g). The terminal weight of the basal controls (316.0 ± 8.3 g) was significantly lower than the synchronous controls and reached a near statistical difference from the vivarium group. The effect of the ~42h of fasting on the synchronous control and flight groups was not measured.

Cell Population Kinetics. A comparison of the **PDL** cell populations from the three control groups showed significant differences in only the **L** and **A+A'** categories (Fig. 3). The basal control rats had fewer **A+A'** cells than did both the synchronous and vivarium controls. Although the sample of **L**-type cells was small (< 10% of the population), the synchronous control group had nearly twice the number of these cells compared to any other group. Because the environment and handling of the synchronous control group most closely simulated that of the flight group, all further comparisons were made between these two groups.

Spaceflight followed by a ~55 h recovery period resulted in a 40% decrease in the **A+A'** cell population (**F**-17.75±2.53 vs. **S**-29.25±3.17; $p \leq 0.05$)and a 42% increase in the **C+D** cells (**F**-50.26±4.09 vs. **S**-31.25± 3.35; $p \leq 0.05$) compared to synchronous controls (Fig. 4). The increase in the **C+D** cell category was significant primarily because of the **D** cell population. The mean of the flight group **C** cell population tended to be higher that its corresponding control but did not reach statistical significance. The total cell density of fibroblast-like cells along the **PDL**/bone interface was also increased by 39% (**F**-0.24±0.01 vs. **S**-0.14 ±0.02 cells/100μm; $p \leq 0.05$) in the flight group compared to synchronous controls (Fig. 5). The differences in the flight group occurred despite a degree of physiological stress that was highly significant compared to synchronous controls, as indicated by the increased adrenal weights in the flight animals (Fig. 6). The adrenal weights of the flight animals were higher than that of the control group despite the reduced body weights in the flight group.

DISCUSSION

Previous spaceflight data have demonstrated that microgravity induces a block in the **A' --> C** conversion (a mechanical-stress dependent step) of osteoblast histogenesis (Roberts *et al.*, 1981). This block results in an increase in the **A+A'** cells and a decrease in the more differentiated **C+D** cells. Assuming a similar flight effect, results from the analysis of **PDL** in rats flown on Cosmos 1187 show that a recovery of preosteoblast formation occurred within ~55h following return to a 1 G environment. **L** cells are a subclassification of fibroblast-like cells with very small nuclei. This type cell is commonly seen lining inactive surfaces of trabecular bone. The significance of this population of cells in **PDL** is unknown. Unfortunately, the increase numbers of **L** cells in synchronous controls is uninterpretable at this time.

There has been little opportunity, however, to study recovery from the effects of microgravity. The length of time needed to restore osteoblast histogenesis following spaceflight is not known. Young rats flown on **SL-3** apparently recovered their ability to form preosteoblasts within 12h after returning from 7d of spaceflight. However, older rats on the same mission still exhibited a significant increase in the **A+A'** cell compartment suggesting an age related delay in recovery of

osteogenic differentiation (Roberts *et al.*, 1987). In contrast, numbers of osteoblast precursor cells in **PDL** of rats flown for 18.5d aboard Cosmos 1129 were recovered 6d after return to earth (Roberts *et al.*, 1981). In the present study, the large increase in **C+D** cells and the decrease in the **A+A'** cells at ~55h post-flight indicate that return to 1 G strongly stimulated the osteogenic activity of the **PDL** following 12.5d in space.

The strong post-flight recovery of preosteoblast differentiation (Fig. 4) is similar to the osteogenic response when **PDL** is subjected to orthodontic force (Roberts *et al.*, 1982). Placement of a 0.5mm latex elastic between the maxillary first and second molars induces a stimulation of differentiation in the osteogenic cells of the area studied in the **PDL**. Cell kinetic data following orthodontic stimulus shows a burst in differentiation of preosteoblasts (i.e. an increase in **C+D** cells) at 10h after insertion of the latex elastic. A second wave of preosteoblast formation occurs 40 h later, at approximately 50h post orthodontic stimulus (Roberts *et al.*, 1982).

With respect to the present study, it follows that the increase in the **C+D** cells seen ~55 h after spaceflight is probably the second wave of preosteoblast differentiation stimulated by return to a 1 G environ-ment. Support for this conclusion is the significant increase in cell density at the alveolar bone surface of the **PDL**. Roberts and Chase (1981) previously reported similar kinetics of cell migration toward the bone surface during osteogenic induction. The decrease in **A + A'**, and increase in **C + D** cells and increase in cell density at the bone surface suggest a strong osteogenic response in **PDL** post-flight. The strength of the osteogenic recovery following spaceflight is striking. It persists despite an array of physiological stresses endured by flight animals following return to .. Physiological stress is thought to result in a increase in the less differentiated osteoblast progenitor cells (**A+A'**) and a decrease in the more differentiated precursor cells (**C+D**; Fielder *et al.*, 1986); i.e. a pattern opposite to that presently observed.

Physiological stress was probably a contributing factor to the considerable difference in weights between the flight and synchronous control groups. A similar effect of spaceflight was seen in the data from the Cosmos 1129 flight. Flight animals sacrificed 6d following re-entry actually lost weight during this period (Wronski *et al.*, 1981) The inability of the flight rats to regain weight at the same rate as the synchronous controls may be associated with physiological stress during spaceflight, and upon return to a gravitational environment. Stresses such as the disruption of the circadian light cycle, wide fluctuations in capsule temperature and the lack of food for 42h post-flight are expected to reduce preosteoblast production. Enhanced osteoblast histogenesis under stressful conditions post-flight highlights the strength of the compensatory recovery of osteoblast histogenesis.

The three control groups had significant differences in only the **L** and **A+A'** cell categories (Fig. 3). These data are difficult to interpret because the basal control rats were about 3wk younger at sacrifice than animals in the other control groups. The synchronous control group was subjected to conditions intended to simulate the physiological stresses endured by the flight group except for the reentry G forces and post-flight transportation conditions. While not showing statistically significant changes, the differences in this group compared to the other controls was in the direction one would expect for physiologically stressed animals.

The reason for the lack of significant stress effects in cells of **PDL** from synchronous control rats (as judged by the lack of difference in adrenal weights; data not shown) is not clear. These data may mean that flight animals were exposed to a longer duration, more chronic stress, i.e. spaceflight *per se* is stressful. Considering that the flight animals were probably chronically stressed, the persistence of the post-flight osteogenic response was remarkable.

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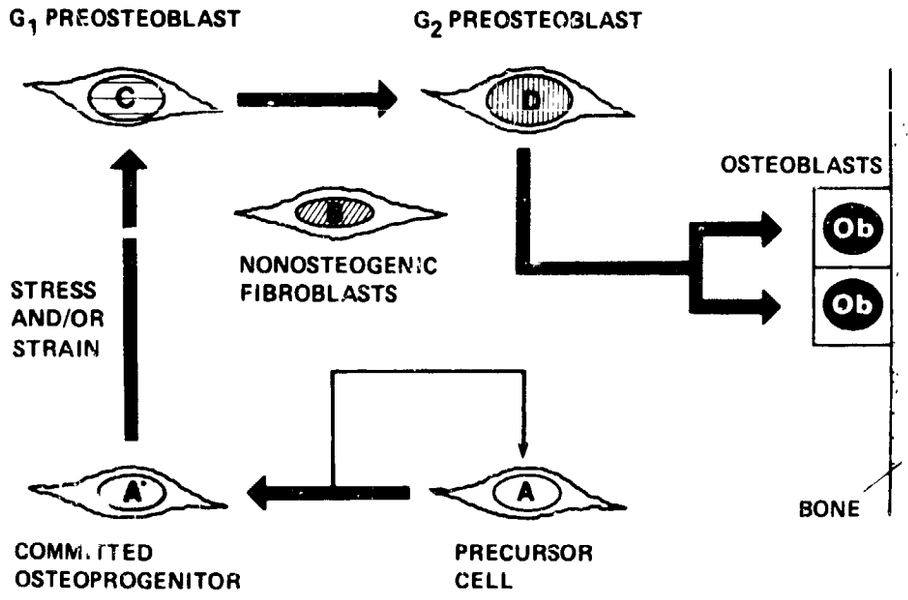

Figure 1. G_1 and G_2 stage preosteoblasts undergo mitosis and form mature osteoblasts.

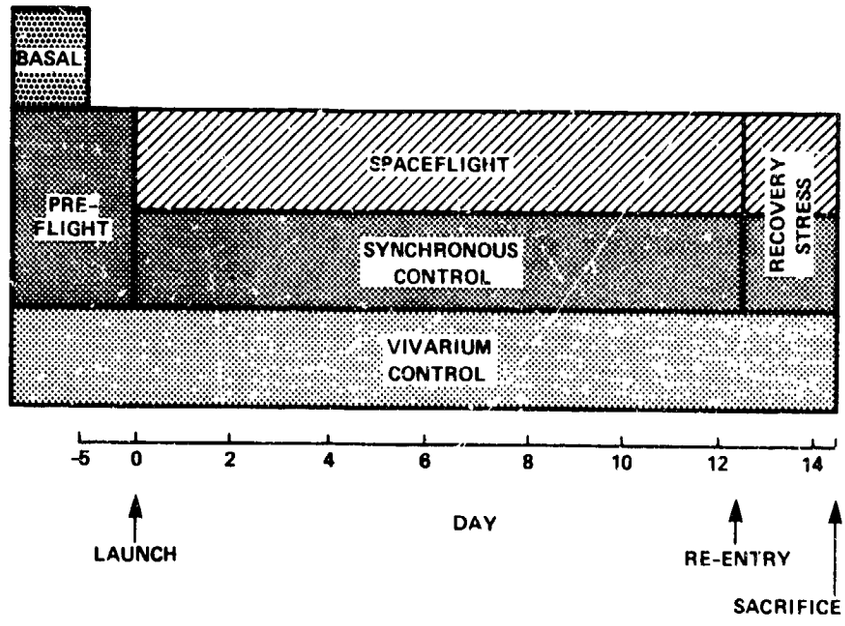

Figure 2. Diagramatic representation of flight and control groups of Cosmos 1887. The basal control group was sacrificed prior to flight. See description in Methods.

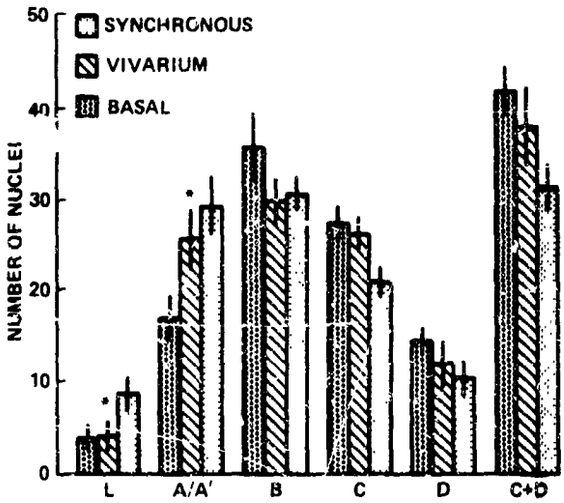

Figure 3. Comparison of control group PDL cell populations. * indicates a significant difference ($p \leq 0.05$).

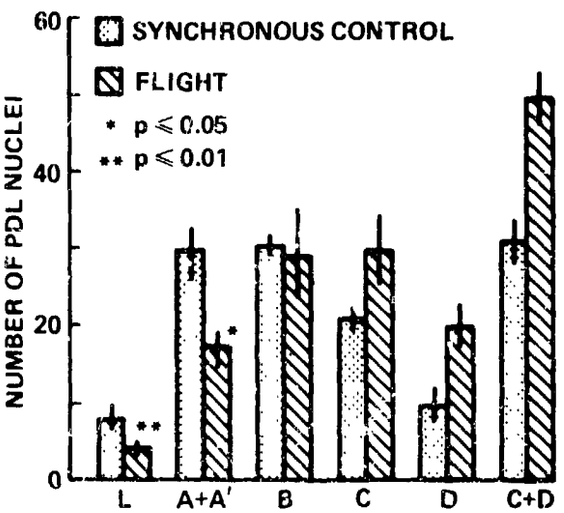

Figure 4. Comparison of synchronous control and flight PDL cell populations.

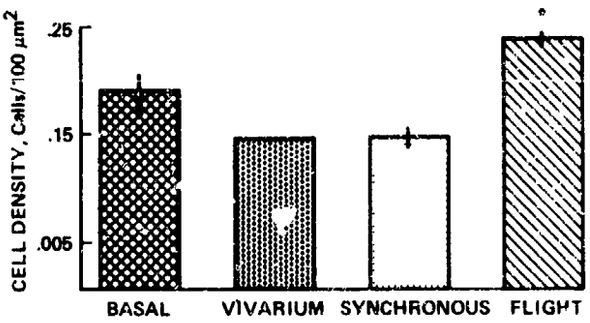

Figure 5. Comparison of the cell densities within 25 μm of the PDL/bone interface. Total nuclei were counted in this region without regard to their size classification. * indicates a difference from the synchronous control group significant at $p \leq 0.01$.

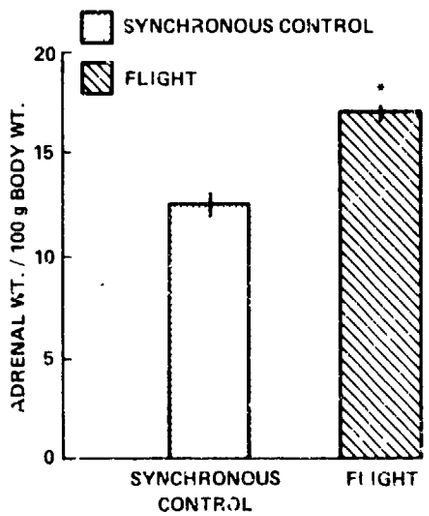

Figure 6. Comparison of adrenal weight/100 g body weight. * indicates a difference from the control group significant at $p \leq 0.001$.

EXPERIMENT K-6-03

PART V: INTERVERTEBRAL DISC SWELLING PRESSURE ASSOCIATED WITH MICROGRAVITY ON COSMOS 1887

A. R. Hargens, S.A. Gott, B. Rydevik, and G. Durnova

SUMMARY

Swelling pressures within discs from Cosmos 1887 rats exposed to 12.5 days of microgravity were compared to those in two groups of ground-based control rats. Swelling pressures were not significantly different between the three groups, probably because rats aboard Cosmos 1887 were re-exposed to normal gravity for over 50 hours prior to tissue harvesting.

INTRODUCTION

The back pain experienced by space travelers during exposure to microgravity may be caused by spinal lengthening due to swelling of their discs, and subsequent stretching of anterior and/or posterior spinal ligaments. This hypothesis may be supported by the observation that the human spine lengthens about 4-6 cm during microgravity (Thornton et al., 1977). To examine fluid movement into discs of animals exposed to actual microgravity, we compared equilibrium swelling pressure of nucleus pulposus from rats exposed to 12 days of microgravity to that of two groups of ground-based control rats. Recently, we developed a new compression-type osmometer that allows direct measurement of nucleus pulposus swelling pressure in samples as small as 5-10 mm^3 (Glover, et al., 1987) Subsequently, we determined that these swelling pressures depend on environmental conditions and species (Gott, et al., 1987, Gott, et al., 1988). Other work has documented that pooling of lumbar discs from the rat spine allows sufficient disc material for direct measurement of swelling pressure in this species (Gott, unpublished observations). Therefore, studies of Cosmos rats allowed testing of the hypothesis that microgravity causes fluid uptake and decreased swelling pressure within the intervertebral disc of flight rats as compared to ground-based, control rats.

METHODS

Isolated spines were frozen and transported to NASA-Ames Research Center. Lumbar discs were incised by scalpel and nucleus pulposus was pooled for direct measurement of equilibrium swelling pressure for each rat. Data from each group (flight group versus two groups of ground controls) were compared using paired t tests. Statistical significance was set at $p < .05$. All measurements were completed within ten days after we received the isolated spines.

RESULTS

No significant differences were found in the swelling pressures between the flight and control groups of rat nucleus pulposus. Swelling pressures ranged between 622 to 690 mmHg (Table 1).

DISCUSSION

Because of the extended period between the time that the flight rats returned to Earth and the time of death (53-56 hours), we conclude that the flight animals already were fully readapted to normal gravity in terms of fluid movement into and out of their intervertebral discs. We hope that we will

be given an opportunity to participate in tissue analysis of future Cosmos missions so that our hypothesis can be resolved definitively.

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TABLE 1

COSMOS 1887 RATS

SWELLING PRESSURES OF LUMBAR DISCS
(mm Hg ± S.E.)

Cosmos Flight Rats (N = 5)	690 ± 45
Synchronous Controls (N = 5)	675 ± 32
Vivarium Controls (N = 5)	622 ± 25

No significant difference between groups.