Microgravity generated by space flight has little effect on the growth and development of chick embryonic bone

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Abstract Seven days’ space flight of fertilized chicken eggs pre-incubated for 7 and 10 days on earth caused no differences in the morphology of osteoblasts, osteoclasts, and osteocytes of humerus and tibia from those of control embryos. Bone-resorbing and forming activities of the femur were not different between control and flight groups. As a consequence, calcium and phosphorus contents of the femora between control and flight groups were not changed. Alkaline phosphatase activity of 3 different regions (resting cartilage, growth cartilage, and cortical bone) of tibia showed no significant difference between control and flight groups. No significant difference of gene expressions of hepatocyte growth factor and receptors of fibroblast growth factor was observed in perichondrium, trabecula, and skeletal muscles and tendons of hind limbs between control and flight groups. Unlike the results of previous space flight experiments in which young growing mammals were used, these morphological and biochemical results indicate that microgravity has little effect on bone metabolism of the chick embryo.

Key words: microgravity, bone morphology, bone resorption, alkaline phosphatase, FGF receptors

Introduction

It is well known that the bone mineral of astronaut decreases during orbital flight (9). Histomorphological studies showed that tibia and lumbar vertebrae of rats after 13 days of space flight contained a lower number of osteoblasts (3), indicating that microgravity inhibits bone neoformation. In addition, a certain contribution to bone loss was also made by enhanced bone resorption because an increased number of osteoclasts was observed (3). In simulated experiments of microgravity such as tail suspension of rats (18) and headdown bed rest of humans (2), the stimulation of bone resorption was also observed. Therefore, microgravity greatly affects the bone metabolism of adult animals. However, very few studies have been done about the effect of microgravity on the growth and development of embryonic and fetal bone. In order to confirm and analyze the effect of microgravity on bone loss observed in previous space flight experiments and also to investigate the effect of low gravity on the embryonic or fetal bone metabolism, we loaded thirty fertilized chicken eggs pre-incubated for 0, 7, and 10 days (10 eggs each) onto the space shuttle Endeavor.
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that was launched on September 12, 1992.

After a 7 days’ space flight, only one of the 0-day-old embryos survived. The reason why most of these embryos died during space flight was reported earlier (14). All ten of the 7-day-old and 9 of the ten 10-day-old embryos survived. Half of the 7-day- and 10-day-old eggs were morphologically and biochemically examined for the determination of bone-forming and -resorbing activity immediately after the space flight. The remaining eggs were further incubated on earth until hatching to know whether the effect of microgravity remained for a long time.

Morphological examination of humerus and tibiae were carried out by light and electron microscopy. Bone-resorbing and bone-forming activities were determined by the organ culture of the femur bones. Alkaline phosphatase activity of tibiae was also determined. Members of the fibroblast growth factor (FGF) family are important factors for chondrogenesis and osteogenesis (5, 19). Therefore, gene expression of FGF receptors (FGFR) in the tarsometatarsus was determined by in situ hybridization.

**Materials and Methods**

**Materials**

Fertilized eggs were obtained from APS Co. Ltd., Pennsylvania, U. S. A.

**Methods**

**Experimental schedule**

Fertilized eggs incubated for 0, 7, and 10 days at 37 °C on earth were subjected to microgravity for 7 days achieved by space flight (flight group ; F). Two types of controls were employed. The eggs in the ground control group (GC) were incubated on the ground in the same type of incubator as used in space. The eggs in the other control group (C) were incubated in a standard commercially available incubator. F7 and F10 indicate eggs pre-incubated for 7 and 10 days, respectively, on earth followed by the 7 days’ space flight. Half of the eggs of each group were analyzed immediately after flight. The rest of them were incubated further on earth until hatching. These groups were classified as HF7 and HF10. The same expression was employed for GC and C groups.

**Light microscopical examination of humerus and tibia**

The left humerus and the distal half of tibiae in each group (F, GC and C groups) were fixed with 10% buffered formalin for 10-16 hr at 4 °C. To identify osteoid tissues in decalcified sections, we treated the humerus with 0.5 % cyanuric chloride, and then decalcified it with 0.5 M EDTA by the method of Yoshiki (20). The specimens were next dehydrated through a graded series of ethanol and xylene, and subsequently embedded in paraffin. Thin sections were stained with hematoxylin and eosin to identify osteoid tissues (20). The sections were also stained with Alcian blue to examine possible changes in the cartilaginous matrix.

The fixed tibiae were washed in cold phosphate buffer solution for 10 hr at 4 °C. They were dehydrated through a graded series of ethanol and xylene, and subsequently embedded in JB-4 (Polyscience, Inc., Warrington, Pa, U.S.A.) to prepare undecalcified sections. The polymerization of JB-4 was carried out at 4 °C to prevent the temperature from rising during the polymerization. Serial sections (4μm) were cut with a Reichert-Jung Autocut microtome. These sections were stained with the following stains: hematoxylin and eosin, Alcian blue, alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP), and von Kossa.

**Electron microscopical examination of humerus and tibia**

The proximal half of the left tibia was cut into small pieces and fixed in a mixture of 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 10-16 hr at 4 °C. The samples were then post-fixed for 30 min at room temperature in 2% osmium in the same buffer. After the fixation, they were rinsed in the buffer, dehydrated by passage through a graded series of ethanol, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate before observation with a Hitachi H-300 electron microscope.
**Determination of bone-resorbing activity.**

A slight modification of the method of Raisz (13) was employed. After the measurement of the length and wet weight, the diaphyses of the femora were incubated in $^{45}$Ca (37 Kbp/ml)-containing medium (BGJB-HW2, ref.15) for 2 hr. Then they were washed with PBS (-), transferred to chase medium, and incubated for 72 hr to measure the release of $^{45}$Ca into the medium from bone. Biphasic releases of radioactivity from bone into the medium were observed (Fig. 1). Within 24 hr after the start of the chase period, the first sharp decrease in $^{45}$Ca in the bone was observed, and attributed to physico-chemical Ca turnover. The second slow linear decrease then followed, indicating the real bone-resorbing activity. Therefore, to determine the bone-resorbing activity, we taransferred labeled bones to the chase medium and incubated them for 24 hr to remove physico-chemically contaminating $^{45}$Ca. An aliquot of the medium was withdrawn for the measurement of $^{45}$Ca released into the medium from bone during the first 24 hr. The medium was changed to fresh chase medium and incubated further for 48 hr with or without human parathyroid hormone (1-34, 0.4μM). At the end of the culture period, the radioactivity released into the medium during this period was determined by liquid scintillation counting. The bone was immersed in 1 N HCl overnight at room temperature to determine the remaining $^{45}$Ca and the calcium and phosphorus contents by Ca-test Wako and P-test Wako, respectively. Bone-resorbing activity was expressed as the percentage release of $^{45}$Ca:

\[
% \text{ release of } ^{45}\text{Ca} = \left( \frac{^{45}\text{Ca released into the medium during 24 to 72 hr of chase culture}}{^{45}\text{Ca released into the medium during 24 to 72 hr of chase culture} + ^{43}\text{Ca remaining in the femur}} \right) \times 100
\]

**Determination of bone-forming activity**

The second slow linear decrease in $^{45}$Ca in the bone shows the real bone-resorbing activity. Therefore, putative bone-forming activity would be calculated from the second linear decrease by extending the line to vertical bar. The formula of the second slow linear line is expressed as

\[
\log Y - \log A = (\log B - \log A) \times \left( \frac{X - 24}{72 - 24} \right)
\]

where A and B are the % of $^{45}$Ca remaining in the bone at 24 and 72 hr, respectively of the chase culture. Then, $X = 0$ was inserted into this formula, resulting in $Y = A \times (A/B)^{1/2}$, where $Y$ is the % of $^{45}$Ca utilized for the real bone formation when the bones were incubated for 2 hr with isotope. Therefore, bone-forming activity is expressed by the following formula:

bone-forming activity (cpm) = $A \times (A/B)^{1/2}$

$\times$ total radioactivity incorporated into bone during a 2-hr incubation with $^{45}$Ca ($^{45}$Ca released into the medium during first 24 hrs’ incubation) + ($^{45}$Ca released into the medium during 24 to 72 hr of incubation) + ($^{43}$Ca remaining in the femur).

**Determination of alkaline phosphatase activity of the tibiae**

Minced tibiae were homogenized in 9 vol of 50 mM Tris (pH 7.5) containing 0.1 % Triton X-100. The homogenate was centrifuged at 5,000
rpm for 10 min at 4°C. The supernatant was 
adquately diluted and used as the enzyme 
solution. The activity of alkaline phosphatase 
was measured as described previously (8). The 
assay system consisted of 50 mM sodium 
carbonate (pH 10.5), 5mM MgCl₂, and 2 mM p-
nitrophenylphosphate in a total of 2 ml, including 
0.1 ml of the enzyme solution. After incubation 
at 37°C for 10 min, the reaction was stopped by 
the addition of 2 N NaOH, and the released p-
nitrophenol was determined spectrophotometrically 
at 405 nm.

**Gene expression of fibroblast growth factor 
receptors (FGFRs) and hepatocyte growth 
factor (HGF) in the hind limbs**

All embryos were perfused with 4% 
paraformaldehyde buffered with 0.15 M 
phosphate, pH 7.3, from the left ventricle as soon 
as possible. Embryos were post-fixed in the same 
fixative for 2 hrs at 4°C. For decalcification, hind 
limbs were treated with 5% L-ascorbate in 0.1M 
sucrose for 3 days at 4°C.

After fixation, tissues were dehydrated with a 
ethanol series and embedded in paraffin 
(Paraplast+). Five micrometer thick serial 
sections were mounted on glass slides coated with 
3-aminopropyltriethoxysilane (Sigma). The 
sections were deparaffinized through conventional

![Image](image_url)

**Fig. 2** A low-power view of sagittal sections of humerus 
in C7 (A), GC7 (B), and F7 (C) groups.

Decalcified sections were prepared from the samples 
treated with cyanuric chloride as described in Materials 
and Methods.

![Image](image_url)

**Fig. 3** Decalcified sections prepared from humerus in 
C10 (A), GC10 (B) and F10 (C) groups after treatment 
with cyanuric chloride as described in Materials and 
Methods. Small amounts of osteoid tissues (arrows), 
stained deeply with eosin, are seen in the cortical bones.

xylene and ethanol steps. The sections were next 
treated with glycine and with acetic anhydride 
for acetylation to reduce background in 
autoradiography. In situ hybridization and 
washing were performed on serial sections with 
35S-labeled antisense riboprobes, according to 
the method of Noji et al (11). For autoradiography, the 
slides were immersed in emulsion (Kodak NTB-
2, diluted 1:2 with water), air-dried, and exposed 
for 2 weeks at 4°C. Then, the slides were 
developed with D-19 (Kodak) and finally stained 
with hematoxylin and eosin.

**Results**

**Light and electron microscopical examination 
of humerus and tibia**

Fig. 2 shows low-power views of sagittal 
sections of humerus in C7, GC7, and F7 groups. 
The humerus in each group was composed of 
well-developed epiphysis, metaphysis, and 
diaphysis. In decalcified sections prepared from 
cyanuric chloride-treated humerus, small 
amounts of osteoid tissues were found at the bone 
collar adjacent to the periosteum of the diaphysis 
in each group of both 7- and 10-day-old embryos. 
The amount and distribution of osteoid tissues 
were almost the same in each group (Fig. 3). 
Alcian blue staining revealed no morphological 
difference in the cartilaginous matrix at the
epiphysis in each group (Fig. 4).

In undecalcified sections prepared from tibiae in each group, the diaphysis was composed of a well-calcified bone collar stained with von Kossa’s (Fig. 5). Calcification of the bone collar had progressed more in 10-day-old embryos than in 7-day-old ones. Small foci of calcification was observed in the cartilaginous matrix in the hypertrophic layer adjacent to the bone collar (Fig. 5). The enzyme histochemistry indicated that the bone surface was covered with numerous ALP-positive osteoblasts (Fig. 5). Numerous TRAP-positive osteoclasts were found on the eroded bone and cartilage surfaces (Fig. 6). The distribution of both ALP-positive and TRAP-positive cells were almost the same among F, GC, and C groups (Figs. 5, 6).

Electron microscopic observation revealed no significant difference in ultrastructures of osteoblasts, osteocytes, and osteoclasts among the groups (data not shown).

**Length, wet weight, calcium and phosphorus contents, and bone-resorbing and -forming activity of the femoral diaphysis**

Bone-resorbing activity estimated as % release of $^{45}\text{Ca}$ was not different among F, GC, and C groups (Fig. 7), although the activity was a lower in 10-day incubation groups and hatched groups than in the 7-day groups. Parathyroid hormone (0.4 mM) stimulated the release of $^{45}\text{Ca}$ from bone into the medium to the same extent in F, GC, and C groups (Fig. 7). Bone-forming activity also showed no difference between control (GC, C) and space chicks (F), as seen in Fig. 8. Microgravity did not affect the length, wet weight, or calcium and phosphorus contents at any stage of embryo (Fig. 9).
Fig. 7 Effect of microgravity on the bone-resorbing activity (% release of $^{45}$Ca) of the femora.

The release of $^{45}$Ca into the medium from bone was determined and expressed as % release. Results are expressed as means ± SEM.

**Akaline phosphatase activity of tibia**

To assess the effect of microgravity on bone development, we measured the alkaline phosphatase activity of F, GC, and C groups.

Fig. 8 Effect of microgravity on the bone-forming activity (cpm) of the femora.

The curve (- - ) shows the values obtained from the chick embryos grown under normal conditions in a separate experiment. Results are expressed as means ± SEM.

Tibiae were dissected and separated into 3 portions (resting cartilage, growth plate, and cortical bone). Of the three portions of tibiae examined, the cortical region showed the highest

Fig. 9 Effect of microgravity on the length, wet weight, and calcium and phosphorus contents of the femora.

The length (A) and wet weight (B) were measured immediately after return to the ground from the space flight. Calcium (C) and phosphorus (D) content were determined after the culture of the femora used for the assessment of the bone-resorbing activity. The curve (- - ) shows the values obtained from the chick embryos grown under normal conditions in a separate experiment. Results are expressed as means ± SEM.
Fig. 10  Effect of microgravity on alkaline phosphatase activity of tibiae isolated from embryos pre-incubated for 7 (C7, GC7, and F7) and 10 (C10, GC10, and F7) days on the ground. Alkaline phosphatase activity of resting cartilage, growth cartilage, and cortical bone of tibia was measured when the eggs were returned to the ground from the space flight. Results are expressed as means ± SEM.

Fig. 11  Effect of microgravity on alkaline phosphatase activity of tibiae isolated from hatched groups (HC7, HGC7, HF7, HC10, HGC10 and HF10). Alkaline phosphatase activity of resting cartilage, growth cartilage, and cortical bone of tibia were measured at the day of hatching. Results are expressed as means ± SEM.
activity of alkaline phosphatase (ALP). ALP activity of tibiae did not change in any of the portions during the space flight (Fig. 10).

Microgravity did not affect ALP activity in bone of hatched chickens either (Fig. 11).

Fig. 12 Autoradiographs of longitudinal sections of 3rd digits of the 3 groups following in situ hybridization with HGF antisense probe.

Bright-field views (a) show cellular periosteum (CP), trabeculae (Tr), tendon of the extensor digitorum muscle (Te), and perichondrium (PC). Dark-field views (b) show that hybridization signals are principally located in CP, Tr, and Te. Labelling of other tissues is at the background level only. 1, F group; 2, C group; 3, GC group
Gene expressions in musculoskeletal systems of hind limbs (digits and tarsometatarsuses)

The length of hind limbs of the F group was slightly shorter than that of other groups as judged by external observation. However, each organ and tissue had developed normally when viewed microscopically. The expression patterns of FGFR1-4 and HGF in the musculoskeletal systems of the hind limbs were observed by the in situ hybridization method. Since intense signals for actin transcripts were observed in all tissues examined, we concluded that mRNAs in the samples had been well preserved. A summary of the findings is given in Table 1, and typical results are shown in Fig.12. Transcripts of FGFR1, FGFR4 and HGF were detected in the cellular periosteum, in osteoblasts overlaid on trabeculae, in the perichondrium, and in the immature tendon and dorsal interosseus and extensor digitorum muscles. Signals for FGFR2 and FGFR3 transcripts were weak in the cellular periosteum and a trabeculae, and were not observed at all in the perichondrium or in muscle. No significant signals of FGFR and HGF were detected in osteocytes and chondrocytes. These results indicate that the FGFR and HGF genes

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Intense gene expressions were observed in the area of endochondral and intramembranous ossification. Intense signals of actin indicate that mRNAs were well preserved. Specimens were from the 7-day incubation groups.
play some important roles in active endochondral and intramembranous ossification. However, no significant differences in the gene expression patterns were found among the three groups.

**Discussion**

Prolonged space flight brings about dramatic bone loss in humans and in experimental animals mainly by the inhibition of bone formation and partly by the stimulation of bone resorption. Space flight also induces muscle atrophy (6). Head-down bed rest causes myofibrillar disorganization and results in a decrease in the size of skeletal muscle fibers (4). Human model of unilateral lower limb unloading, which simulates microgravity, reduces the mass and strength of muscles (1). On the contrary, in cell culture, hypergravity increases DNA synthesis and inhibits the alkaline phosphatase activity of osteoblastic MC3T3-E1 cells (10) and stimulates the incorporation of $^{35}$S-sulfate into proteoglycan of growth plate and articular chondrocytes (7). These results indicate that the gravitational alteration induces a great change in skeletal tissues including bone, cartilage, and muscle. In order to confirm the effect of microgravity on bone tissues obtained previously and to study the effect of microgravity on the growth and development of embryonic bone, we loaded 0-, 7-, and 10-day-old fertilized chicken eggs onto the space shuttle Endeavor and investigated both morphologically and biochemically the embryonic bone metabolism after a 7 days' space flight.

Light microscopical examination of longitudinal sections of humerus from 7- and 10-day-old embryos showed that microgravity caused no remarkable differences in cortical bone and cartilaginous tissues. The formation of osteoid and the amount of calcification were not changed by the space flight. Electron microscopical examinations indicated that ultrafine structures of osteoblasts, osteocytes and osteoclasts in the tibiae isolated from the embryos flown in space (F) were not different from those of control (C and GC) embryos grown on earth. The distribution of tartrate-resistant acid phosphatase (TRAP)-positive cells was not different among the three groups.

In harmony with the morphological studies, many biochemical data showed that the bones isolated from the flight group were almost the same as those from C and GC groups. Bone-resorbing and -forming activities of the femora from 7-day- and 10-day-old embryos were not changed by space flight. As it takes a relatively long time (3 days) to determine the bone-resorbing and -forming activities, the possibility remains that the bones adapted rapidly to the gravity on earth; and thus bone-resorbing and -forming activities in the flight group became the same as those of the control (C and GC) groups. But this is not the case because the calcium and phosphorus contents of flight groups after culture of their femora were the same as those of the bones cultured from the GC and C groups. The determination of mineral content was made after culture rather than immediately after receipt of the eggs from the shuttle because of the limited number of embryos available. The post culture values are valid, however, for the following reasons: As no differences were observed in bone-resorbing and -forming activities between flight and control groups, the rates of decrease in calcium and phosphorus contents of the bone from the flight group must have been the same as those rates for the control groups during culture. Therefore, any group difference in calcium and phosphorus contents of the femora after culture would reflect that present before culture. Thus, the findings of no difference in calcium and phosphorus contents of the bone from the different groups indicates that space flight had no effect on the mineralization of the femora, supporting our conclusion that bone-resorbing and -forming activities were not changed by microgravity. In addition, PTH stimulated the bone resorption to the same extent in F, GC, and C groups, suggesting that microgravity did not change the responsiveness to hormones.

It is well known that ALP activity is an important enzyme for bone formation. Therefore, we determined the ALP activity of 3 different regions (resting cartilage, growth cartilage, and cortical bone) of tibiae isolated from embryos immediately after their return to the ground from
the space flight. The enzyme activity was different in each region, being lowest in resting cartilage and highest in cortical bone in F7 and F10 groups, and highest in growth cartilage in HF-7 and HF-10 groups. However, no discernible effect of space flight was observed on the ALP activity in each region, indicating also that microgravity has no effect on bone formation.

FGF and HGF are well known as regulators of the differentiation and development of bone (5, 19). FGF receptors are abundant in cartilage and bone (12). Therefore, next we investigated how the microgravity affects the gene expression of various kind of FGF receptors (FGF-1,2,3 and 4) and HGF by in situ hybridization on sections of tarsometatarsus of F7, GC7 and C7 groups. Hybridization signals for FGF-receptor transcripts were observed in the perichondrium, and in osteoblasts in regions in which active ossification was taking place, although expression sites were slightly different for each FGFR. However, no significant differences in their expression patterns or intensity were observed between F, GC, and C groups. In addition, morphological observation also showed that the tissues and organs in hind limbs had normally differentiated and grown in F and C groups. These results reflect the probability that the gene expressions of FGFRs and HGF had occurred normally.

All these results indicates that the microgravity has no effect on the morphology and the metabolism of bone of chicken embryo pre-incubated 7 days and more on earth. However, bone densitometric analysis of vertebral bone showed that space flight caused a slight, but significant, decrease in bone density in the F7 group (submitted elsewhere). Image analysis of humerus and cervical vertebrae by computed radiograph and bioimage analyzer also showed that immature calcification occurred in the bone from the F7 group, although the extent was very small (submitted elsewhere). No difference was observed between F10 and control (GC10 and C10) groups. These results indicate that microgravity has very small effect on the calcification of the embryonic bone at an early developmental stage. The discrepant results obtained by our morphological and biochemical experiments and physical experiments just mentioned can not be clearly explained at present. One possibility is that different regions of the same organs or different tissues may respond differently to microgravity. Another possibility may be differential sensitivity of the method used for analysis. Usually image analysis is highly sensitive for the determination of bone changes. Therefore, it is able to differentiate the small changes which can not be recognized by the ordinal biochemical and morphological techniques.

Therefore, we conclude that microgravity has essentially no effect on bone metabolism of the chick embryo although highly sensitive methods for analysis of physical properties do detect a very small inhibitory effect on bone growth and development, but only in the early developmental stage. This conclusion is largely different from that obtained for adult animals and humans who have flown in space. The bone loss of adult animals and humans was remarkable after space flight. Adult animals and humans are always receiving many kinds of mechanical stress such as exercise and gravity on earth. Such mechanical stresses may induce normal bone growth and development. Under the condition of microgravity the bones are free from the mechanical stresses, leading to the bone loss. On the contrary, the chick embryo is always flown in the egg in its early developmental stages, suggesting that chick embryos receive little mechanical stresses even on earth. Therefore, the microgravity obtained by space flight may have little effect on bone metabolism of the chick embryo. All the tissues of embryonic stages are actively developing and bone formation exceeds bone resorption in chick embryos, suggesting that chick embryo are in the modeling process. In contrast, bone tissues are actively being remodeled in mature animals and adult humans. Therefore, the possibility exists that the remodeling process may be more susceptible to microgravity than the modeling process. However, unresponsiveness to microgravity may be specific for chick embryonic bone, because cultured fetal mouse long bone (metatarsus) showed significant inhibition of mineralization
and significant increase of bone resorption under microgravity (17). Microgravity generated by 14 days’ space flight had no effect on the mineralization and physical properties of cortical bone of the mature (110 days old) rat (16). As young growing rats are well known to be sensitive to microgravity on space flight, the age must be an important factor in mammals. It is necessary in the future to investigate the effect of microgravity on skeletal tissues of chickens at various times after hatching.

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