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Effects of Extended Spaceflight on the Mandible and Incisor Teeth of Mice

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Effects of Extended Spaceflight on the Mandible and Incisor Teeth of Mice

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TABLE OF CONTENTS

ABSTRACT

The launch and flight of the Russian Bion-M1 satellite has enabled us to compare the effects of long duration (30 days) spaceflight on mandibles and teeth with those from a shorter (13 days) space shuttle (STS-135) mission. The primary goal of this study was to determine the effects of weightlessness on non-weight bearing bones as well as the development and mineralization of teeth. Rodent mandibular incisors are of particular interest because they show rapid and continuous eruption, which enables investigation of the cellular and molecular changes in teeth in response to microgravity even in short duration spaceflights. Microtomography (micro CT) was used to obtain data on 3D architecture of mandibular bone and teeth. Light microscopy combined with immunohistochemical analyses was helpful to understand signaling and regulatory responses of mandibular bone and teeth to spaceflight at the cellular level. Hemimandibles of a total of 41 adult C57Bl/6J mice were used in this study. Hemimandibles of 7 mice from a 13 day flight on space shuttle Atlantis (STS-135) and 6 mice from a 30 day flight on Bion-M1 were compared with 14 ground control mice housed in the same habitats used during flight and 14 ground control mice housed in standard vivarium cages. The results showed significant differences in enamel and dentin volume and density, and mandibular bone fraction, volume and density between flight and control groups after Bion-M1 and STS-135 flights. Additionally, significant differences between Bion-M1 flight and control groups were found in the point of initial mineralization of the mandibular incisors, and the overall length of the incisors in STS-135 flight mice vs. control mice. Immunohistochemical analyses showed that the expression of some proteins was either increased or decreased depending on their function. No significant alteration in tissue morphology was observed. Only limited information is available about the effects of spaceflight on the oral cavity as well as non-weight bearing bones. The

findings resulting from this study aid in the expansion of theoretical knowledge of space flight effects as well as the potential for clinical applications. These findings provide background data for devising effective countermeasures to bone loss.

INTRODUCTION

Human history in space started in the 1960s and has kept growing since then. While advancements in space technology made it possible to answer some of the questions about distant planets, understanding the effects of microgravity on biological processes still remains a very hot research topic for scientists. There are multiple ways to obtain microgravity-based data including actual spaceflight missions and ground-based studies (bed rest models, hindlimb suspension models, clinostats). Even though these ground-based studies are useful in obtaining data on the effects of weightlessness in the lack of an actual space environment, spaceflight missions have their own unique environment, which cannot be totally duplicated.

Rodents are the most commonly used animal models in space research due to their structural and functional similarities to humans, their small size, ease of maintenance, and less experimental expenses compared to sending larger animals or humans to space.

As a participant in a NASA project called "PROTEIN EXPRESSION IN SALIVARY GLANDS: EFFECTS OF EXTENDED SPACE FLIGHT", we were able to identify some of the cellular and molecular changes related to microgravity in salivary glands. Salivary proteins serve as biomarkers for a variety of physiologic functions. Environmental or hormonal stimulations regulating the cyclic AMP signaling pathway result in alterations in physiological responses. Our previous studies conducted on salivary glands of mice flown on STS-131, STS-135 space shuttle flights and the Bion-M1 satellite have shown altered protein expression in response to microgravity (1,2). In this study, we will utilize mouse samples from the Russian Bion-M1 satellite (30 days) and STS-135 US space shuttle (13 days) missions and compare the effects of

different flight durations.

Spaceflight has a variety of effects on human physiology including bone and muscle loss, cardiovascular deconditioning, altered immune function and disruption of taste. Bone loss is one of the well-known effects of prolonged spaceflight (3-7). Given the unique environment of spaceflight, bone loss seen in astronauts cannot be equated with skeletal changes due to aging in osteoporosis patients. Bone loss in astronauts is related to multiple risk factors including limited physical activity, adaptation to weightlessness, alterations in mineral metabolism and dietary intake (6). Skeletal changes can be a restrictive factor for sending humans to space because of increased risk of fractures after return to Earth. Conducting studies on the effects of spaceflight on human health is of great importance to develop effective countermeasures to bone loss. Several potential countermeasures have been suggested to date including exercise, creating artificial gravity by centrifuges, vibration and pharmaceuticals (8). Although it has not been tested in any long duration spaceflight, exercise combined with drug therapy has been considered to be an effective way of preventing microgravity induced bone loss.

The skeletal effects of spaceflight on weight-bearing bones such as the lower limbs and spine are well established; imbalance in bone formation and resorption results in bone loss in astronauts (6,9). Depending on the mechanical load imposed on the skeleton, bone mass changes can vary greatly from weight bearing bone to non-weight bearing bone. Previous studies have shown that weight bearing bones were more affected than non-weight bearing bones (10). Structural and molecular changes in bone can be shown by utilizing antibodies to proteins that serve as indicators of specific cellular function and composition. Sclerostin is a protein secreted by

osteocytes and other terminally differentiated cells embedded within a mineralized matrix, i.e., cementocytes and hypertrophic chondrocytes (11). It has a negative effect on bone formation by inhibiting proliferation and differentiation of osteoblasts and inducing osteoblast apoptosis. Sclerostin, a product of the SOST gene, shares sequence similarity with differential screening-selected gene aberrative in neuroblastoma (DAN) family of bone morphogenetic protein antagonists. However, it is believed that the effects of sclerostin on bone formation are due to inhibition of Wnt signaling pathways. Mechanical loading plays an important role in regulation of SOST/sclerostin expression by osteocytes. Several ground based microgravity studies have shown that sclerostin synthesis is elevated by mechanical unloading that results in bone loss (12,13). Analysis of SOST transgenic mice has shown decreased bone density (14).

Osteocalcin, also known as bone gamma carboxyglutamic acid (Gla protein) is an abundant non-collagenous protein in bone and a marker for osteoblasts (15). It may act as a regulator of bone mineralization. As osteocalcin is involved in the bone formation process, it has been used to measure the effectiveness of a given antiosteoporotic drug for bone formation (16). Osteocalcin does not only have a role in bone metabolism, but also presents an endocrine function resulting in increased insulin secretion by beta cells in the pancreas, target cell sensitivity, and testosterone production by Leydig cells of the testis (15).

Tartrate-resistant acid phosphatase (TRAP), also called type 5 acid phosphatase, is an iron containing enzyme that has been detected in bone and many non-skeletal tissues including skin, spleen, liver, lung, thymus and linings of the gastrointestinal tract (17). Several studies have demonstrated that TRAP has a critical role in normal bone development and mineralization

(18,19). It mainly serves as a histochemical marker of osteoclasts; however, it is also expressed by macrophages and dendritic cells (18). A variety of pathological conditions influence the expression of TRAP such as osteoporosis, hyperparathyroidism, Gaucher's disease and some cancers.

There are a variety of radiographic techniques to measure bone health including dual energy X-ray absorptiometry (DXA), quantitative computed tomography (QCT) and X-ray microtomography (micro CT). While DXA and QCT provide valuable information on skeletal integrity of astronauts, micro CT is widely used in animal studies. Micro CT provides a nondestructive way to measure mineral thickness and density of dental hard tissues as well as trabecular bone morphology. Previous studies on mandibles and maxillae with dental radiographs revealed that alterations in trabecular bone pattern are reflective of osteoporosis (20). Whalen et al. found that there is an association between oral bone loss and osteoporosis (21). Therefore, microgravity induced mandibular and alveolar bone loss assessment by 3D imaging using micro CT is expected to show correlations to the radiographic appearance in osteoporosis.

Rodent incisor teeth are excellent models to study the effects of microgravity on tooth development and mineralization. The continuous growth of rodent incisor teeth allows observation of the successive stages of amelogenesis along the entire length of the tooth. Inner enamel epithelial cells induce adjacent dental papilla cells to differentiate into odontoblasts. Secretion of predentin by odontoblasts is followed by enamel formation by ameloblasts. The process of amelogenesis consists of three main stages, which are presecretory, secretory, and

maturation stages. During the presecretory stage, inner enamel epithelial cells change their polarity, elongate and differentiate into ameloblasts. Presecretory ameloblasts prepare to secrete enamel matrix. In the secretory stage, ameloblasts develop a Tomes' process, secrete enamel proteins and partially mineralize the newly deposited matrix. Once the entire thickness of enamel has been formed, the organic matrix is replaced by a significant amount of mineral, which leads to mature enamel composed of more than 95% mineral (calcium hydroxyapatite crystals). This stage is called the maturation stage. Enamel formation progresses sequentially from posterior to anterior direction along the longitudinal axis of the tooth. The enamel covers only the labial surface of the rodent incisor tooth.

Ameloblast and odontoblast secretory products can be used to observe microgravity-induced changes in cellular and molecular activity. Amelogenin is the predominant enamel matrix protein, constituting 90% of developing enamel matrix (22). Amelogenin is encoded by AMELX and AMELY genes on X and Y-chromosomes, respectively. Defects in human AMELX gene are associated with amelogenesis imperfecta hypoplastic type I. Amelogenin plays an important role in regulation of hydroxyapatite crystal growth and the development of normal enamel thickness. The importance of amelogenin in enamel biomineralization has been demonstrated in *Amelx*$^{-/-}$ mice (23), which have hypoplastic or hypomineralized enamel.

Dentin sialophosphoprotein (DSPP), a member of the SIBLING (Small Integrin-binding ligand, N-linked Glycoprotein) family, is the major non-collagenous protein of dentin (24). The expression of DSPP is not restricted to dentin; it is also present in bone, cementum and transiently in presecretory ameloblasts. DSPP is the parent protein that is cleaved into two main

protein products: dentin phosphoprotein (DPP) and dentin sialoprotein (DSP). These two cleavage fragments differ in their biochemical structure and expression location. DPP is the COOH terminal cleavage product of DSPP that is found mostly at the mineralization front of dentin, whereas DSP is the NH_2 terminal cleavage product of DSPP mainly localized in predentin. Several studies have shown that mutations in DSPP cause dentinogenesis imperfecta and dentin dysplasia (25).

Cellular signaling from peptide hormone–receptor interactions or catecholamine binding to β-adrenergic receptors is mediated by cyclic AMP (26-28) by activating cyclic AMP-dependent protein kinase (PKA) and specific protein phosphorylation with its type II regulatory subunit (RII) serving as a marker. The PKA RII subunits are polyfunctional proteins that are involved in many cell functions including cell proliferation, cell differentiation and gene expression. Experimentally, PKA RII expression in animals and humans shows a measurable biphasic response: above baseline increases in acute stress, and sharp decreases in chronic stress (29,30). These findings are applicable to evaluating oral tissue responses to altered gravity. Studies of rats and mice flown in space have shown changes in the expression of PKA RII in salivary glands and heart muscles (2,31,32).The expression of PKA RII has been observed in rat dental follicles during tooth eruption (33). However, PKA RII expression has not previously been measured in dental tissues in response to microgravity

HYPOTHESIS

Non-weight bearing bones such as the mandible will show the effects of microgravity on bone formation and bone resorption. Moreover, the rates of formation and mineralization of enamel

and dentin are altered by exposure to microgravity. This hypothesis predicts that extended space flight will result in decreased bone mass, similar to that seen in weight-bearing bones, and increased tooth mineralization. The overall objective is to improve the understanding of mechanisms underlying space flight induced changes in biological systems so that effective countermeasures eventually may be developed.

SPECIFIC AIMS

1) To determine changes in morphology and mineralization of mandibular bones and teeth of mice flown in space by radiographic analyses

2) To assess mandibular bone remodeling by studying osteoblast and osteoclast numbers and function

3) To compare expression of selected bone, enamel and dentin related proteins between mandibles and teeth of flight mice to those of control mice. It is anticipated that after spaceflight the expression of proteins involved in enamel and dentin formation will be increased and the expression of bone related proteins will be decreased in flight mice.

MATERIAL AND METHODS:

Animals and Tissue Collection:

The use of mice in these investigations was approved by the Institutional Animal Care and Use Committee of Moscow State University Institute of Mitoengineering (Protocol No–35, 1 November, 2012) and the Biomedical Ethics Commission of the Institute of Biomedical Problems (Moscow) (protocol No–319, 4 April, 2013), and by the Institutional and Animal Care and Use Committee at the National Aeronautics and Space Administration (NASA). The experimental procedures were conducted in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health. Hemimandibles containing molar and incisor teeth were obtained from 6 adult male C57Bl/6J mice flown for 30 days on the Russian Bion-M1 mission (15-16 weeks old at launch, 19-20 weeks old on landing) housed in cylindrical, acrylic "BOS" flight habitats (34). Hemimandibles from 7 adult female C57Bl/6J mice housed in Animal Enclosure Modules (AEMs) (30) were collected from the 13-day STS-135 mission (9 weeks old at launch, 11 weeks old on landing). The BOS flight habitats and the AEMs are enclosed rodent habitats, of two different designs, that provide ventilation, lighting, waste collection, food and water for the rodents. Seven ground control mice were housed in the BOS flight habitats and 7 in AEMs for the same length of time as the flight. For each flight 7 additional ground control mice were maintained under standard vivarium conditions.

Following rapid transport of mice to the laboratory, they were killed by cervical dislocation. The mandibles were removed, split into 2 hemimandibles and trimmed of excess soft tissue. After

dissection, the hemimandibles were fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, shipped to UConn Health, and stored in 1% paraformaldehyde at 4ºC.

Image Acqusition:

A two-fold experimental approach was taken: hemimandibles and teeth taken from flight and control groups were used for morphological and immunohistochemical experiments to determine changes in structure, density and mineralization of the mandibles and teeth due to microgravity exposure. Morphological studies showed specific functional responses of the tissues and cells to microgravity. The hemimandibles were scanned in 70% ethanol at a resolution of 12 μm voxels using a Scanco μCT 40 (Scanco Medical, AG, Bassersdorf, Switzerland) at the UConn Health MicroCT Facility. Serial images were acquired transverse to the longitudinal axis of the mandible at 55 kVp (145 μA), employing 1000 conebeam projections per revolution at an integration time of 300 ms within a 12.3 mm field of view. Three-dimensional images were reconstructed using standard convolution back-projection algorithms with Shepp and Logan filtering, and rendered at isometric 12 μm voxels. The morphometric variables to be analyzed included bone volume (mm^3), mineral density (mg/cm^3), and enamel and incisor (enamel+dentin) volume (mm^3). Three locations along the anteroposterior length of the incisor were selected for morphometric and densitometric analysis: at the level of the (1) first molar (center of the mesial root), (2) second molar and (3) third molar. At each of these locations, the bone volume fraction was defined as bone volume measured within the osseous margins, excluding the molar and incisor teeth. To quantify the degree of mineralization of incisors, tissue density of the enamel and dentin was measured volumetrically through 10 serial sections (120 μm span) after calibrating X-ray attenuation to a hydroxyapatite phantom (mg HA/cm^3). In

addition, 3D reconstructed images provided data about the point of first visible mineralization of the incisors with respect to the molars. In order to have a consistent reference point a line between the first and second molars, which was perpendicular to a line through the bottom of the occlusal sulci of the molar teeth, and another perpendicular line at the first mineralization point of the incisor were drawn. The distance between these two perpendicular lines was measured to determine how far from the first line the mineralization begins and compared between flight and control groups (Fig. 1A).

Two-dimensional images were captured by a Faxitron X-ray machine (Field Emission, McMinnville, Oregon) at 20 kVp, 8 s. These images were used to measure the entire length of the incisor to determine if there were differences between flight and control mice with regard to eruption rate or incisor wear. The length of the incisor was measured from the incisal tip to the bone covering the apical end, as shown by the red line in Figure 1B.

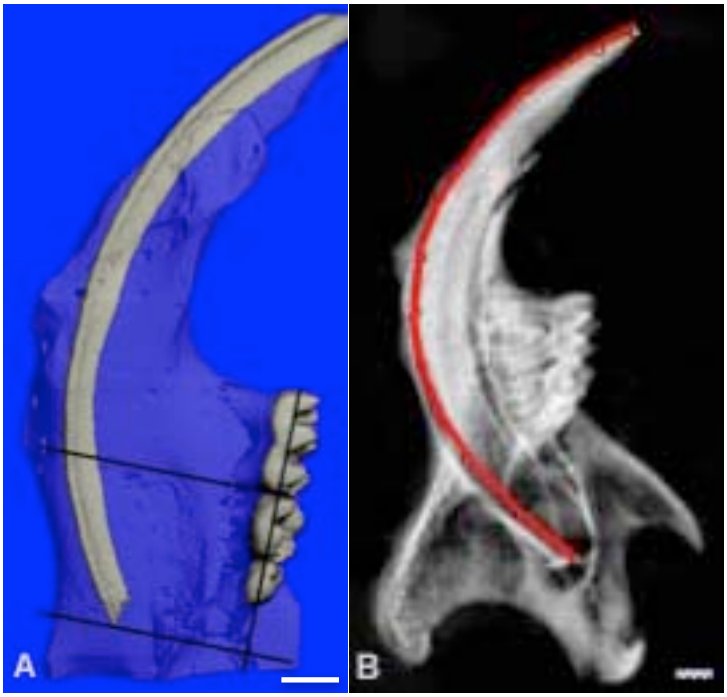

Fig 1. A-3D reconstruction image, B-Faxitron image. Scale bar = 1mm

Immunohistochemical Procedures:

Selected proteins were visualized using immunohistochemistry to determine the effects of extended travel in space on their expression and localization (Table 1). These included signaling molecules (sclerostin) and matrix proteins (osteocalcin) in bone; and matrix proteins (amelogenin, DSP) and signaling molecules (PKA RII) in teeth. Rabbit antibodies to amelogenin and osteocalcin were purchased from Santa Cruz Biotechnology (Dallas, TX), goat antibody to sclerostin was purchased from R&D Systems (Minneapolis, MN), rabbit antibody to DSP was obtained from Dr. Larry Fisher (NIDCR, NIH, Bethesda, MD) (35) and rabbit antibody to PKA RII was provided by Dr. Maija Mednieks (UConn Health)(36,37).

Tissue	Cells/ Matrix	Function	Antibody/Protein (dilution)
Bone	Osteoblasts	Bone formation	Osteocalcin (1:500) PKA RII (1:500)
	Osteocytes	Bone maintenance Mechanosensation	Sclerostin (1:50)
	Osteoclasts	Bone resorption	TRAP
Enamel	Ameloblasts	Enamel formation	Amelogenin (1:400) PKA RII (1:500)
Dentin	Odontoblasts	Dentin formation	DSP (1:2000)

Table 1. Selected Antibodies/Proteins for immunohistochemical study.

For immunohistochemical studies, the hemimandibles were demineralized in 14% EDTA containing 1% paraformaldehyde, pH 7.1, for 6 weeks. Following extensive washing in phosphate buffered saline (PBS), the tissues were dehydrated in an ascending series of ethanol, cleared in xylene, and embedded in paraffin (Paraplast PLUS, Fisher Scientific). A Leica RM2235 microtome (D-69226; Leica, Inc., Nussloch, Germany) was used to obtain 5-μm sagittal sections. Paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated in a graded ethanol series and antigen retrieval was performed in sodium citrate buffer solution (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) overnight using a water bath set to 60ºC. Then the sections were treated with 0.3 % hydrogen peroxide (H_2O_2) in 85% methanol solution for 30 minutes to block endogenous peroxidase. Afterwards, they were processed with PBS containing 1% bovine serum albumin (BSA) and 5% normal serum for 30 minutes at room temperature to block non-specific binding. Primary antibodies at various dilutions in PBS containing 1% BSA and 5% normal serum were applied overnight at 4ºC in a humidified chamber. Control sections were incubated without the primary antibody. After rinsing with PBS, the sections were incubated with Vectastain biotinylated secondary antibody diluted in PBS containing 1% BSA for 30 minutes at room temperature. After PBS washes, the sections were treated with the ABC reagent (Vectastain Elite kit, Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature and immunopositive loci were detected by incubation with Vector NovaRED. Finally, the sections were counterstained with hematoxylin solution, dehydrated in graded ethanol solutions, cleared in xylene, and coverslips were mounted with DPX (Electron Microscopy Sciences, Hatfield, PA).

Osteoclast numbers and function were assessed by TRAP staining, using the Leukocyte Acid

Phosphatase (TRAP) Kit from Sigma-Aldrich (St. Louis, MO).

Immunohistochemical procedures were conducted in two groups with 20 sections in each group. Immunohistochemical data were analyzed semi-quantitatively based on reaction intensity, using a scale of 0 (no reaction) to +5 (intensely reactive). Scoring was done by a blinded observer using 4X and 25X objectives on a Leitz Orthoplan microscope. Sections were photographed using an Olympus DP72 microscope with a digital camera (Olympus America Inc., Center Valley, PA).

STATISTICAL ANALYSIS

Quantitative data from micro CT and faxitron images, and the immunohistochemical reactivity, were analyzed using ANOVA and post-hoc t-tests. $P<0.05$ was considered statistically significant.

RESULTS

Forty-one mice were included in our study. One mouse was excluded due to poor quality of its tissue sections. Therefore, a total of 40 mice were analyzed. Radiographic analyses of the mouse mandibles were done by using Micro CT and Faxitron imaging. In addition, the expression of a variety of proteins involved in tooth and bone formation and mineralization in mouse mandibles and incisor teeth was analyzed by immunohistochemistry. Slides were stained, and reaction intensity was evaluated in two batches. Hence, two intensity values were provided for each flight and control group in the tables.

Immunohistochemical Analysis:

Immunohistochemistry showed that strong to very strong staining for DSP occurred in the predentin and dentinal tubules (Fig. 2A). The surface layer of the predentin exhibited the strongest reactivity. DSP reactivity was significantly reduced in STS-135 flight mice vs. both vivarium mice and ground control mice in the predentin layer (Table 2). Intertubular dentin also was reactive, but to a lesser extent than the dentinal tubules. Odontoblasts and incisor pulp strongly reacted with DSP (Fig. 2B). Enamel matrix was also positive for DSP. This staining was weak in the secretory stage, but became stronger as degradation of the matrix occurred in the maturation stage. Cementum and some cementocytes of the molars showed reactivity for DSP. Most cementocytes were unreactive, but those closest to root dentin exhibited strong to intense reactivity, which was significantly increased in STS-135 flight mice vs. ground control mice (Table 3). The reactivity in cementum, as well as that in bone, was generally weak. Osteoblasts were also positive for DSP staining. A significant decrease was noted in DSP reactivity of osteoblasts in STS-135 ground control mice vs. vivarium mice (Table 3). There were no significant differences in DSP reactivity among any of the Bion mice groups.

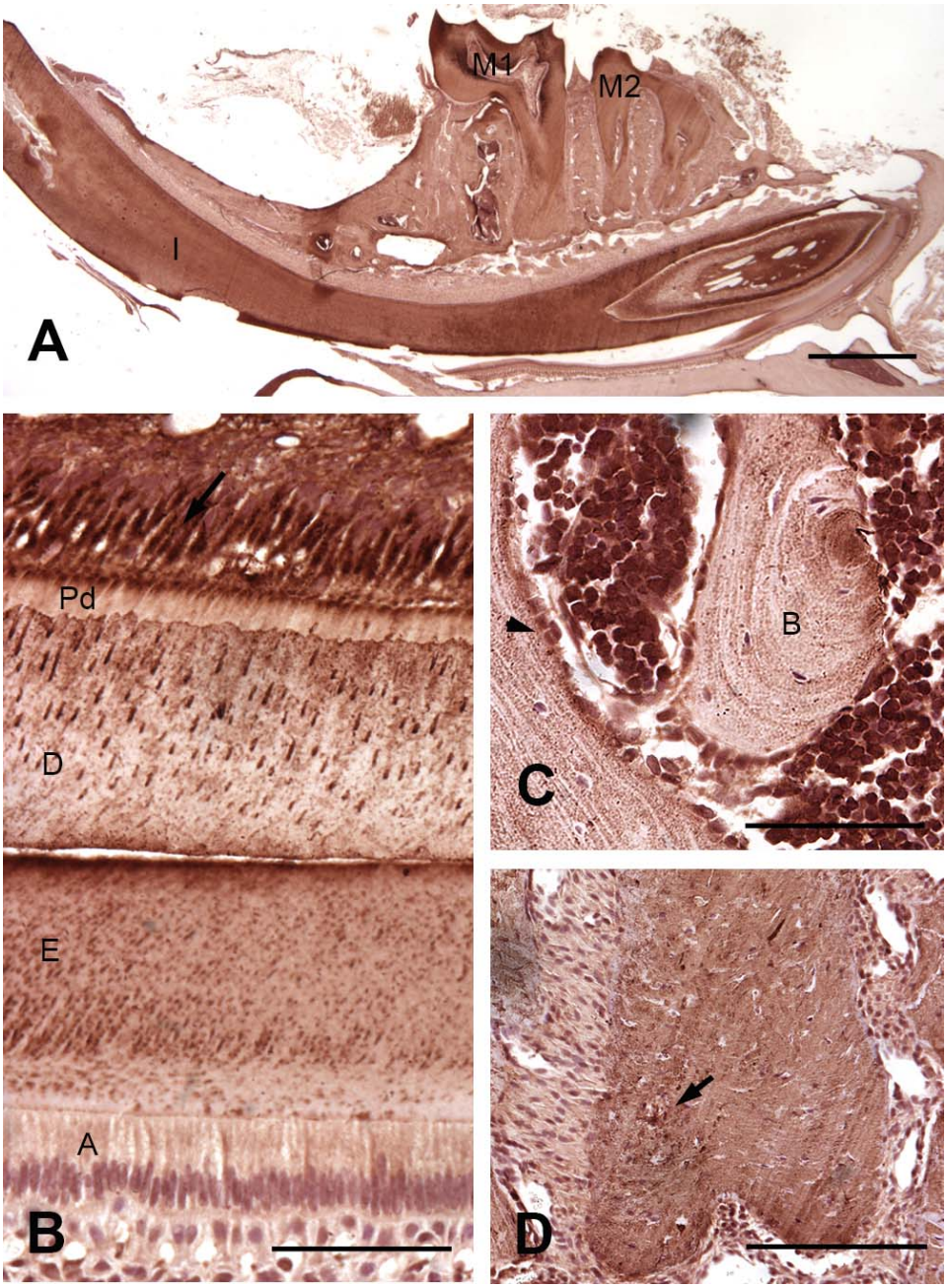

Fig. 2. Light micrographs of immunoreactions for DSP protein. A- DSP staining was observed within predentin, dentin, enamel matrix and bone. B- Odontoblasts (arrow), incisor pulp and predentin reacted strongly for DSP. C- Osteoblasts were also positive for DSP staining (arrow head). D- Cementum and some cementocytes of the molars showed reactivity for DSP (arrow). M1- First molar, M2- Second molar, I- Incisor, Pd- Predentin, D-Dentin, E- Enamel, A- Ameloblasts, B- Bone. Scale bars (A) 1000 μm, (B, C, D) 100 μm.

	Dentin Mean ± SEM (n)	Predentin Mean ± SEM (n)	Bone Mean ± SEM (n)	Cementum Mean ± SEM (n)
Bion-M1 Flight	3.66 ± 0.33 (3)	3.33 ± 0.66 (3)	1.33 ± 0.33 (3)	1.33 ± 0.16 (3)
	3 ± 0.57 (3)	2.5 ± 1.5 (2)	1.33 ± 0.33 (3)	1.16 ± 0.16 (3)
Bion-M1 Vivarium	3.66 ± 0.33 (3)	1.33 ± 0.33 (3)	1 ± 0 (3)	1 ± 0 (3)
	2 ± 0 (4)	1.75 ± 0.25 (4)	0.62 ± 0.12 (4)	0.87 ± 0.12 (4)
Bion-M1 Ground Control	3.25 ± 0.47 (4)	2.5 ± 0.64 (4)	1.12 ± 0.12 (4)	1 ± 0 (4)
	2.66 ± 0.33 (3)	2.16 ± 0.44 (3)	1.5 ± 0.28 (3)	1.33 ± 0.16 (3)
STS-135 Flight	4.33 ± 0.33 (3)	3.33 ± 0.33 (3) * ∞	1.5 ± 0.28 (3)	1.5 ± 0.76 (3)
	2.83 ± 0.72 (3)	1.16 ± 0.16 (3)	0.5 ± 0.28 (3)	1 ± 0.28 (3)
STS-135 Vivarium	3.75 ± 0.47 (4)	4.25 ± 0.25 (4) *	1.37 ± 0.23 (4)	1.12 ± 0.12 (4)
	2.66 ± 0.33 (3)	2.66 ± 0.33 (3)	0.66 ± 0.16 (3)	0.75 ± 0.25 (2)
STS-135 Ground Control	4.66 ± 0.33 (3)	5 ± 0 (3) ∞	1.16 ± 0.16 (3)	1 ± 0 (3)
	3.25 ± 0.25 (4)	1.37 ± 0.23 (4)	1.12 ± 0.37 (4)	1.12 ± 0.31 (4)

Table 2. DSP reactivity in Bion-M1 and 135-STS flight mice. $p<0.05$ (* flight vs. vivarium, ∞ flight vs. ground control)

	Odontoblasts Mean ± SEM (n)	Osteoblasts Mean ± SEM (n)	Cementocytes Mean ± SEM (n)
Bion-M1 Flight	4.33 ± 0.66 (3)	1 ± 0.57 (3)	4.66 ± 0.33 (3)
	2 ± 0 (3)	0.5 ± 0 (3)	4.33 ± 0.33 (3)
Bion-M1 Vivarium	4 ± 0 (3)	1 ± 0.28 (3)	4.33 ± 0.66 (3)
	2.5 ± 0.28 (4)	0.62 ± 0.12 (4)	4.75 ± 0.25 (4)
Bion-M1 Ground Control	4.25 ± 0.25 (4)	1.75 ± 0.25 (4)	4.5 ± 0.28 (4)
	2 ± 0.5 (3)	0.83 ± 0.44 (3)	4.33 ± 0.66 (3)
STS-135 Flight	4 ± 0 (3)	2.33 ± 0.33 (3)	4 ± 0.57 (3)
	2.33 ± 0.33 (3)	0.5 ± 0.28 (3)	5 ± 0 (3) ∞
STS-135 Vivarium	4.25 ± 0.47 (4)	3 ± 0 (4)	4.66 ± 0.33 (3)
	1.83 ± 0.16 (3)	1 ± 0.28 (3) §	4 ± 0 (2)
STS-135 Ground Control	3.66 ± 0.66 (3)	1.66 ± 0.16 (3)	4.66 ± 0.33 (3)
	2.62 ± 0.37 (4)	0.87 ± 0.23 (4) §	4.25 ± 0.25 (4) ∞

Table 3. DSP reactivity in Bion-M1 and 135-STS flight mice. $p<0.05$ (§ ground control vs. vivarium, ∞ flight vs. ground control)

Amelogenin staining was observed within secretory ameloblasts and maturation ameloblasts (Fig. 3A). Amelogenin staining was stronger in ruffle ended maturation ameloblasts than in smooth ended maturation ameloblasts (Fig. 3B). The supranuclear cytoplasm of secretory ameloblasts, along with numerous cytoplasmic granules, possibly lysosomes (38), showed reactivity (Fig. 3C). Cytoplasmic staining was weaker in maturation ameloblasts than in secretory ameloblasts, but maturation ameloblasts also contained reactive granules, again possibly lysosomes. Early secretory enamel matrix generally showed weak reactivity but the recently deposited matrix located around the Tomes' processes of the ameloblasts was strongly reactive. A strong reaction was seen throughout the matrix in the maturation stage before most of the matrix was degraded.

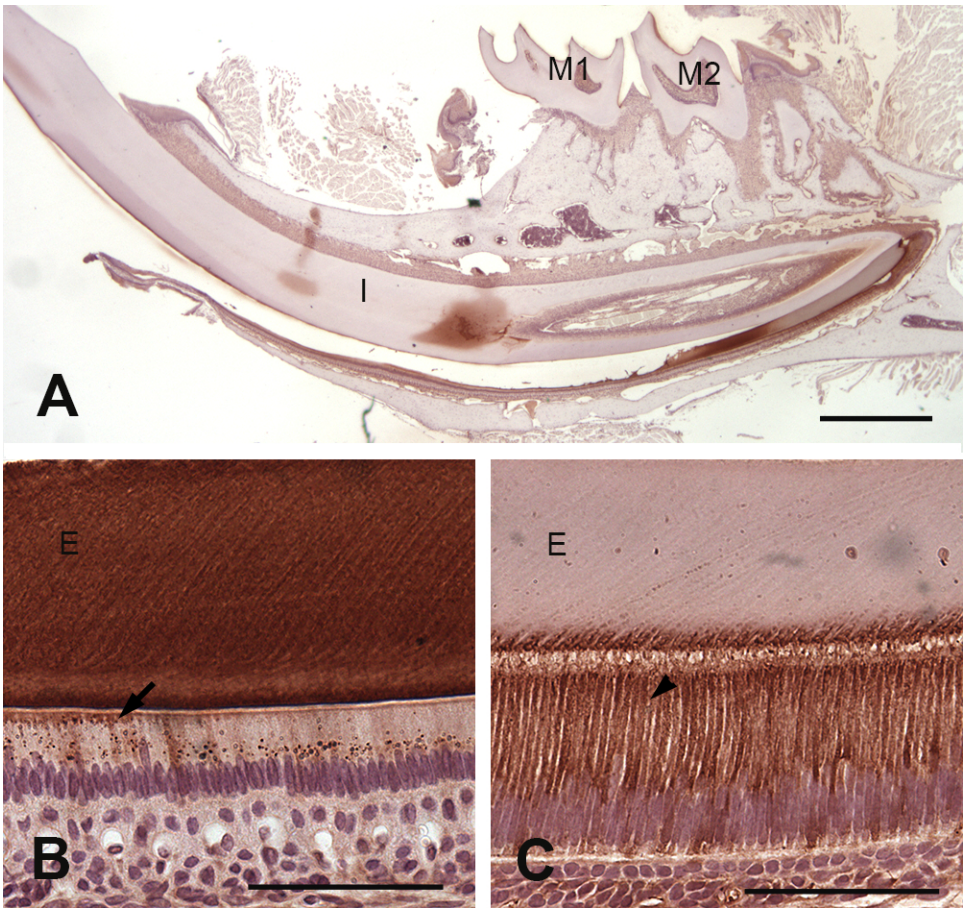

Fig. 3. Light micrographs of immunoreactions for amelogenin protein. A- Amelogenin staining was observed within secretory ameloblasts, maturation ameloblasts and enamel matrix. B- Amelogenin staining was stronger in ruffle ended maturation ameloblasts than in smooth ended maturation ameloblasts (arrow). C- The supranuclear cytoplasm of secretory ameloblasts, along with numerous cytoplasmic granules, possibly lysosomes, showed reactivity (arrow head). M1- First molar, M2- Second molar, I- Incisor, E- Enamel. Scale bars (A) 1000 μ m, (B, C) 100 μ m.

There was a significant decrease in amelogenin reactivity in STS-135 flight mice maturation ameloblasts compared to those of the vivarium group (Table 4). There were no significant differences in amelogenin reactivity among any of the Bion mice groups.

	Secretory Ameloblasts Mean ± SEM (n)	Maturation Ameloblasts Mean ± SEM (n)	Enamel Matrix Mean ± SEM (n)
Bion-M1 Flight	3.5 ± 0.5 (2)	3 ± 0.57 (3)	3 ± 0 (3)
	3.66 ± 0.33 (3)	3.33 ± 0.33 (3)	3.33 ± 0.33 (3)
Bion-M1 Vivarium	3.33 ± 0.33 (3)	3.33 ± 0.33 (3)	3.33 ± 0.33 (3)
	4 ± 0 (3)	3.5 ± 0.28 (4)	3 ± 0.4 (4)
Bion-M1 Ground Control	2.75 ± 0.47 (4)	3 ± 0.25 (4)	2.5 ± 0.28 (4)
	3.5 ± 0.5 (2)	3.5 ± 0.5 (2)	2.5 ± 0.5 (2)
STS-135 Flight	2.33 ± 0.43 (3)	2.33 ± 0.33 (3)	3 ± 0.57 (3)
	3 ± 0.57 (3)	2 ± 0 (3) *	3 ± 0.57 (3)
STS-135 Vivarium	2.25 ± 0.25 (4)	2.5 ± 0.28 (4)	3.25 ± 0.25 (4)
	3.66 ± 0.33 (3)	3.33 ± 0.33 (3) *	3 ± 0 (3)
STS-135 Ground Control	3 ± 0.57 (3)	3.33 ± 0.33 (3)	3 ± 0 (3)
	2.75 ± 0.25 (4)	3 ± 0 (4)	3.5 ± 0.25 (4)

Table 4. Amelogenin reactivity in Bion-M1 and 135-STS flight mice. $p<0.05$ (* flight vs. vivarium)

Osteocalcin reactivity was strong to intense in osteoblasts and odontoblasts (Fig. 4B,C). Although bone and intertubular dentin were only weakly reactive, the dentinal tubules showed a positive reaction for osteocalcin (Fig. 4A). Occasionally strong staining was observed in cementocytes in cellular cementum of the molars near the root dentin, however, those closer to the apex were unstained. A significant increase in osteocalcin reactivity of odontoblasts was found in Bion flight mice vs. ground control mice and a significant decrease in ground control

mice vs. vivarium mice. There were no significant differences in osteocalcin reactivity of osteoblasts among any groups in both flights (Table 5).

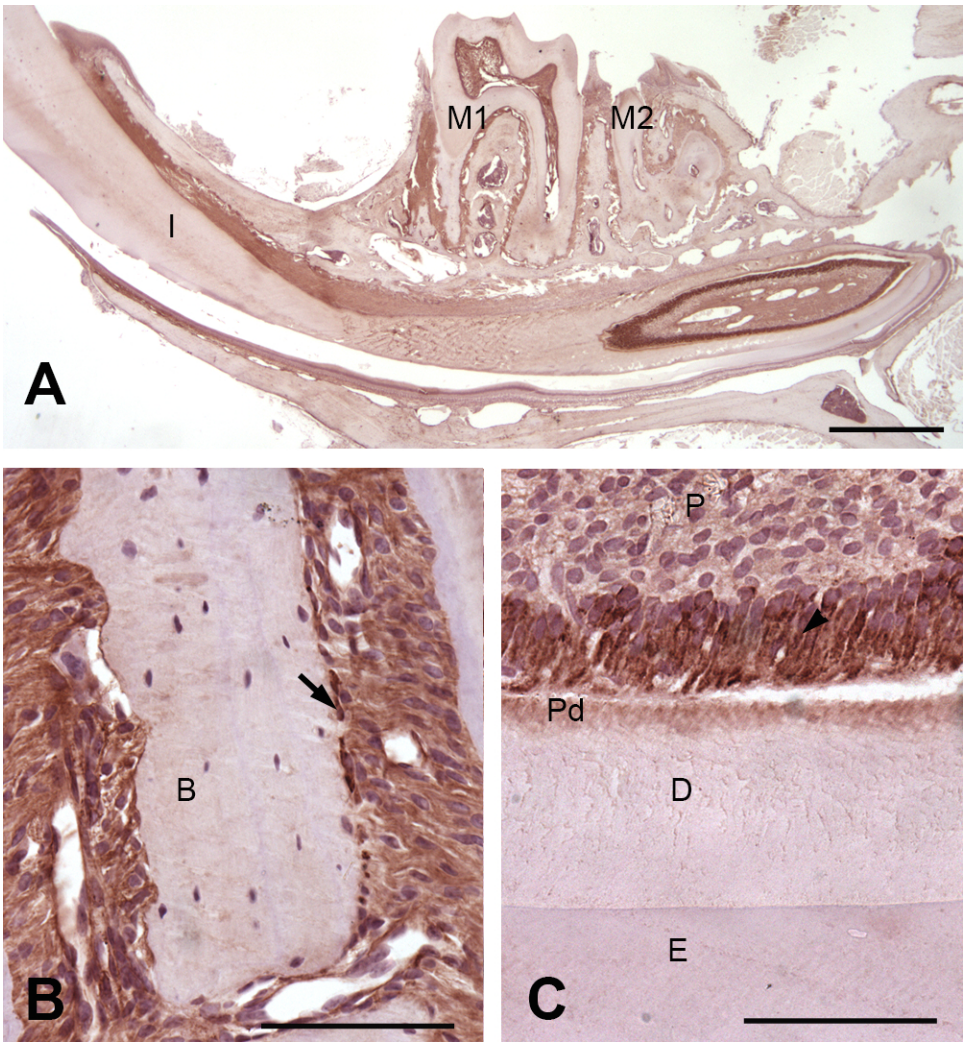

Fig. 4. Light micrographs of immunoreactions for osteocalcin protein. A- Osteocalcin staining was observed within bone, dentin and predentin. B- Osteocalcin reactivity was strong to intense in osteoblasts (arrow). C- Osteocalcin reactivity was strong to intense in odontoblasts. (arrow head). M1- First molar, M2- Second molar, I- Incisor, Pd- Predentin, D-Dentin, E- Enamel B- Bone, P- Pulp. Scale bars (A) 1000 µm, (B, C) 100 µm.

	Odontoblasts Mean ± SEM (n)	Osteoblasts Mean ± SEM (n)
Bion-M1 Flight	3.66 ± 0.33 (3) ∞	5 ± 0 (3)
	2.16 ± 0.44 (3)	2.66 ± 0.88 (3)
Bion-M1 Vivarium	3.75 ± 0.25 (4) §	4.75 ± 0.25 (4)
	2 ± 0 (3)	2 ± 0.57 (3)
Bion-M1 Ground Control	2.16 ± 0.44 (3) ∞ §	4 ± 0.57 (3)
	2 ± 0.4 (4)	2.25 ± 0.47 (4)
STS-135 Flight	3.25 ± 0.47 (4)	4 ± 0.7 (4)
	2.5 ± 0.5 (2)	3 ± 0 (2)
STS-135 Vivarium	3.33 ± 0.66 (3)	5 ± 0 (3)
	1.5 ± 0.28 (4)	3.25 ± 0.25 (4)
STS-135 Ground Control	3 ± 0.4 (4)	5 ± 0 (4)
	2.33 ± 0.33 (3)	3.33 ± 0.33 (3)

Table 5. Osteocalcin reactivity in Bion-M1 and 135-STS flight mice. $p<0.05$ (∞ flight vs. ground control, § ground control vs. vivarium)

Staining for sclerostin was intense in osteocytes and cementocytes, and moderate to strong in osteoblasts (Fig. 5). Osteoid was reactive in all sections, however the proportion of reactive vs. nonreactive osteoblasts, osteocytes and cementocytes varied among samples. There were no differences in sclerostin reactivity between the Bion and STS-135 flight groups and their respective control groups (Table 6).

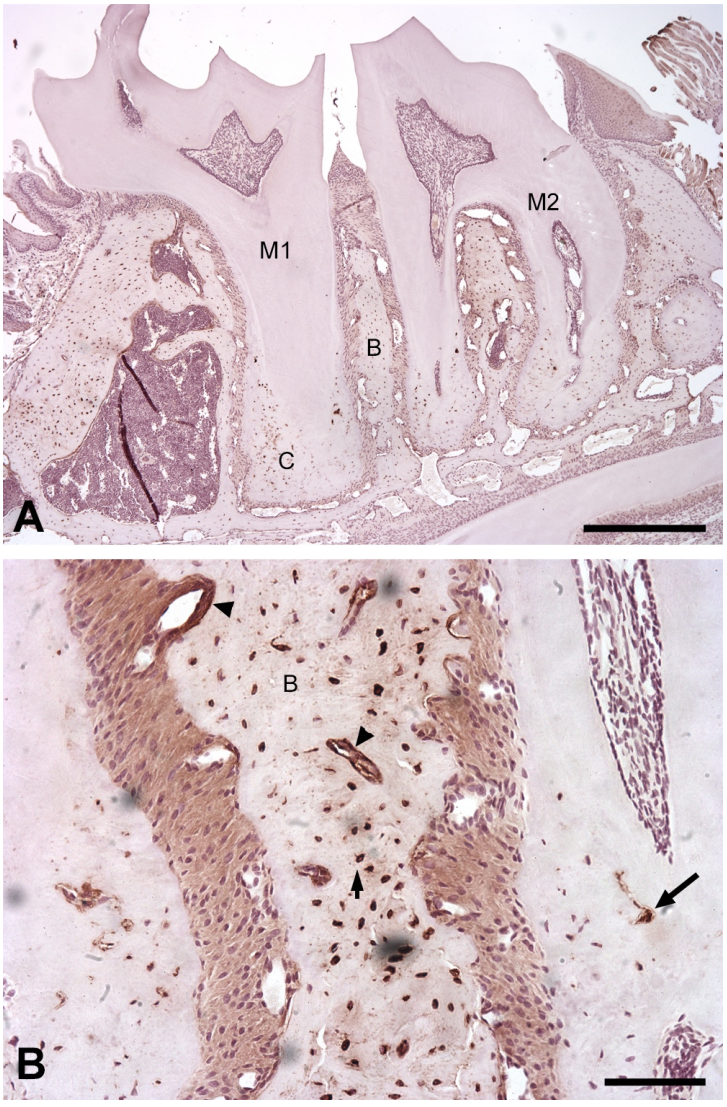

Fig. 5. Light micrographs of immunoreactions for sclerostin protein. A- Sclerostin staining was observed within bone and cementum. B- Staining for sclerostin was intense in osteocytes (short arrow) and cementocytes (long arrow), and moderate to strong in osteoblasts (arrow head). M1- First molar, M2- Second molar, B- Bone, C- Cementum. Scale bars (A) 500 μm, (B) 100 μm.

	Osteoblasts Mean ± SEM (n)	Osteocytes Mean ± SEM (n)	Cementocytes Mean ± SEM (n)
Bion-M1 Flight	2.66 ± 0.33 (3)	4.66 ± 0.33 (3)	5 ± 0 (3)
	3.33 ± 0.33 (3)	5 ± 0 (3)	5 ± 0 (3)
Bion-M1 Vivarium	2 ± 0 (3)	4.66 ± 0.33 (3)	4.33 ± 0.33 (3)
	3.5 ± 0.28 (4)	5 ± 0 (4)	5 ± 0 (4)
Bion-M1 Ground Control	1.87 ± 0.12 (4)	4.75 ± 0.25 (4)	4.5 ± 0.28 (4)
	2.66 ± 0.66 (3)	5 ± 0 (3)	4.66 ± 0.33 (3)
STS-135 Flight	2.66 ± 0.33 (3)	5 ± 0 (3)	4.66 ± 0.33 (3)
	3.66 ± 0.33 (3)	5 ± 0 (3)	3 ± 0 (1)
STS-135 Vivarium	3.75 ± 0.25 (4)	5 ± 0 (4)	4.75 ± 0.25 (4)
	4 ± 0 (2)	5 ± 0 (2)	5 ± 0 (2)
STS-135 Ground Control	3.33 ± 0.33 (3)	5 ± 0 (3)	4.66 ± 0.33 (3)
	3.75 ± 0.25 (4)	5 ± 0 (4)	5 ± 0 (4)

Table 6. Sclerostin reactivity in Bion-M1 and 135-STS flight mice.

Unexpectedly, we observed that overall reactivity for PKA RII was weak to moderate (Fig. 6A). PKA RII staining was observed in maturation ameloblasts and odontoblasts (Fig. 6B-C). Thus

we were not able to provide any quantitative data on PKA RII reactivity due to this overall weak staining.

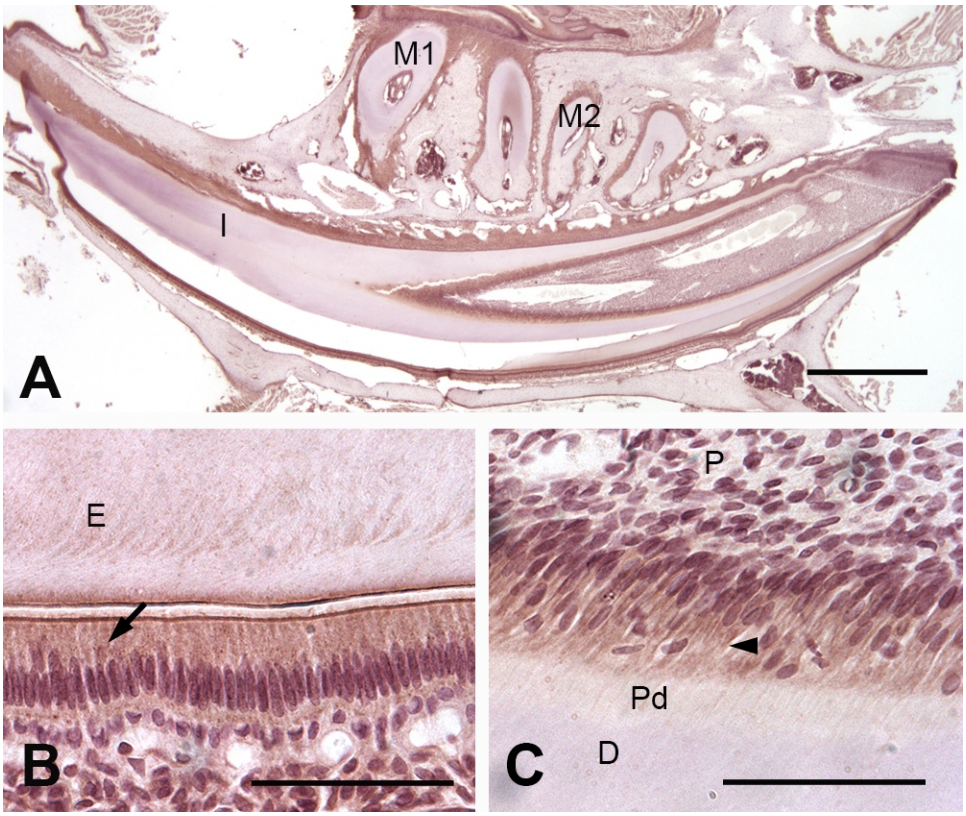

Fig. 6. Light micrographs of immunoreactions for PKA RII antibody. A- PKA RII staining was observed within periodontal ligament, ameloblasts and odontoblasts . B- PKA RII reactivity was weak to moderate in maturation ameloblasts (arrow). C- PKA RII reactivity was weak to moderate in odontoblasts. (arrow head). M1- First molar, M2- Second molar, I- Incisor, Pd- Predentin, D-Dentin, E- Enamel B- Bone, P-Pulp. Scale bars (A) 1000 μ m, (B, C) 100 μ m.

We attempted to assess osteoclast numbers and function by TRAP staining (Fig. 7A-B), however we were not able to quantify the staining, due to variability in the amount of alveolar bone visible in individual sections. More bone sections need to be analyzed in order to get meaningful results.

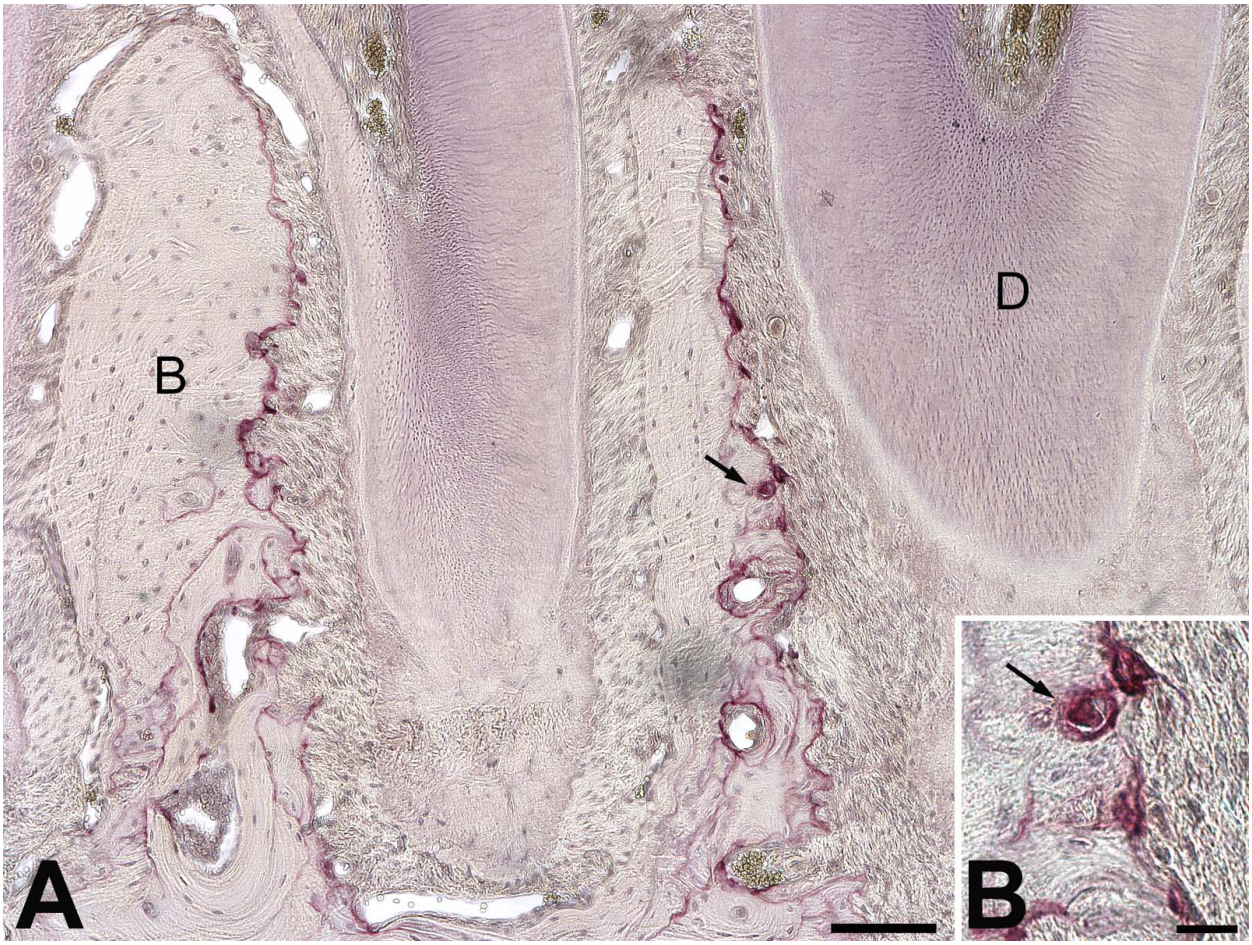

Fig. 7. Light micrographs of TRAP staining. A- TRAP staining was observed in osteoclasts. B- Higher magnification of TRAP staining in osteoclasts (arrow). D-Dentin, B- Bone. Scale bars (A) 100 µm, (B) 20 µm.

MicroCT Imaging Analysis:

The results obtained from the micro CT analyses showed a significant increase between Bion flight mice and vivarium mice and in ground control mice vs. vivarium mice for incisor volume (enamel + dentin volume) and in ground control mice vs. vivarium mice for enamel volume. A trend toward an increase in incisor volume was seen in flight mice vs. ground control mice (Table 7) (Fig. 8)

	VF (IV/TV) (%)		Apparent Density (mg HA/ cm^3)		Tissue Density (mg HA/ cm^3)		Incisor Volume (mm^3)		Enamel Volume (mm^3)	
	mean	p	mean	p	mean	p	mean	p	mean	p
Flight	95.7%	*	1350	*	1460	*	1.798	*	0.266	*
Vivarium	92.3%		1218		1375		1.429		0.148	
Flight	95.7%		1350		1460		1.798	∞	0.266	
Ground Control	94.6%		1330		1457		1.639		0.236	
Vivarium	92.3%	*	1218	*	1375	*	1.429	*	0.148	*
Ground Control	94.6%		1330		1457		1.639		0.236	

Table 7. Means and p values from t-tests comparing mean incisor volumes and dentin density determined by microCT analyses of two hemimandibles for each condition from Bion-M1 flight. * $p<0.05$; ∞ $0.1>p>0.05$ (trend).

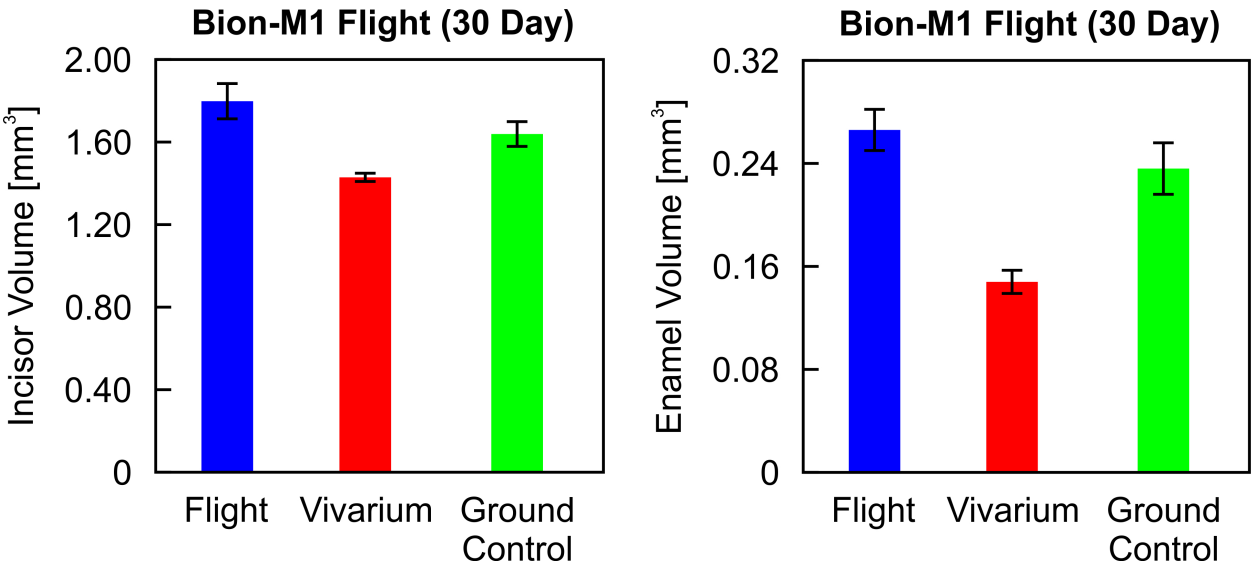

Fig. 8. Incisor volume and enamel volume in Bion-M1 flight (30 day) mice.

A significant decrease was seen in the mandibular bone volume fraction (BVF) and bone volume in Bion flight mice vs. vivarium mice and in ground control mice vs. vivarium mice (Table 8) (Fig. 9).

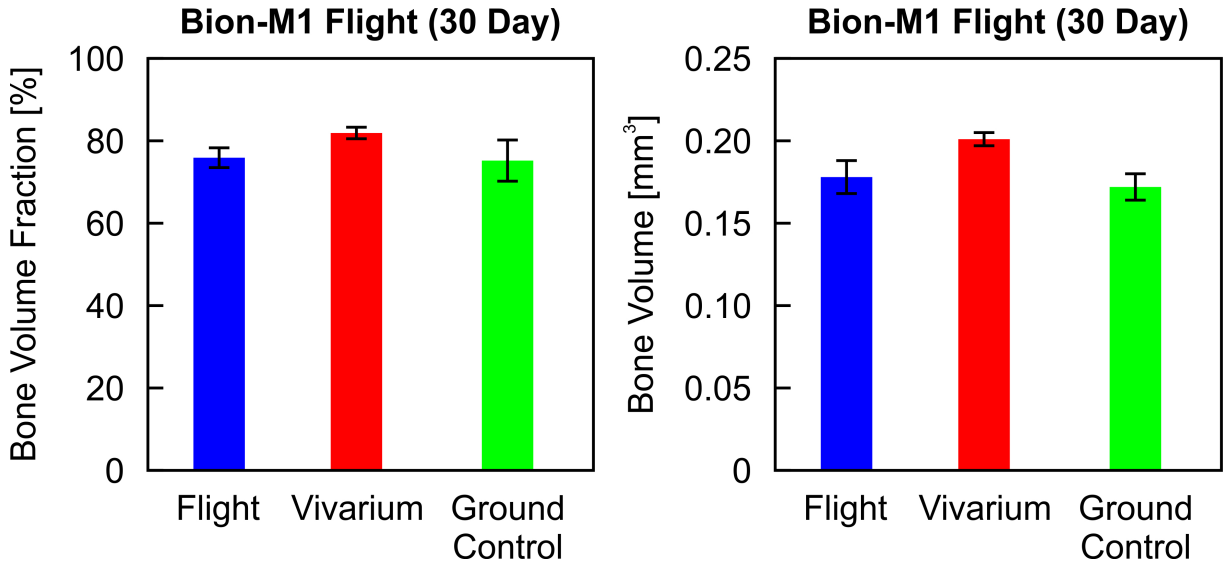

Fig. 9. Bone volume fraction and bone volume in Bion-M1 flight (30 day) mice.

	BVF (BV/TV) (%)		Apparent Density (mg HA/ cm^3)		Tissue Density (mg HA/ cm^3)		Total Volume (mm^3)		Bone Volume (mm^3)	
	mean	p	mean	p	mean	p	mean	p	mean	p
Flight	75.9%	*	966	*	1215		0.234		0.178	*
Vivarium	81.9%		1030		1224		0.246		0.201	
Flight	75.9%		966		1215		0.234		0.178	
Ground Control	75.2%		960		1216		0.229		0.172	
Vivarium	81.9%	*	1030	*	1224		0.246	∞	0.201	*
Ground Control	75.2%		960		1216		0.229		0.172	

Table 8. Means and p values from t-tests comparing mean bone volume and density determined by microCT analyses of two hemimandibles for each condition from Bion –M1 flight.
* $p<0.05$; ∞ $0.1>p>0.05$ (trend).

There was a trend toward an increase in incisor volume between flight and vivarium control group in the STS-135 mice (Table 9) (Fig. 10).

	VF (IV/TV) (%)		Apparent Density (mg HA/ cm^3)		Tissue Density (mg HA/ cm^3)		Incisor Volume (mm^3)		Enamel Volume (mm^3)	
	mean	p	mean	p	mean	p	mean	p	mean	p
Flight	88.6%		1166		1360		1.144	∞	0.116	
Vivarium	86.9%		1129		1338		1.037		0.086	
Flight	88.6%		1166		1360		1.144		0.116	
Ground Control	87.3%		1146		1352		1.105		0.107	
Vivarium	86.9%		1129		1338		1.037		0.086	
Ground Control	87.3%		1146		1352		1.105		0.107	

Table 9. Means and p values from t-tests comparing mean incisor volumes and dentin density determined by microCT analyses of two hemimandibles for each condition from STS-135 flight. ∞ 0.1>p>0.05 (trend).

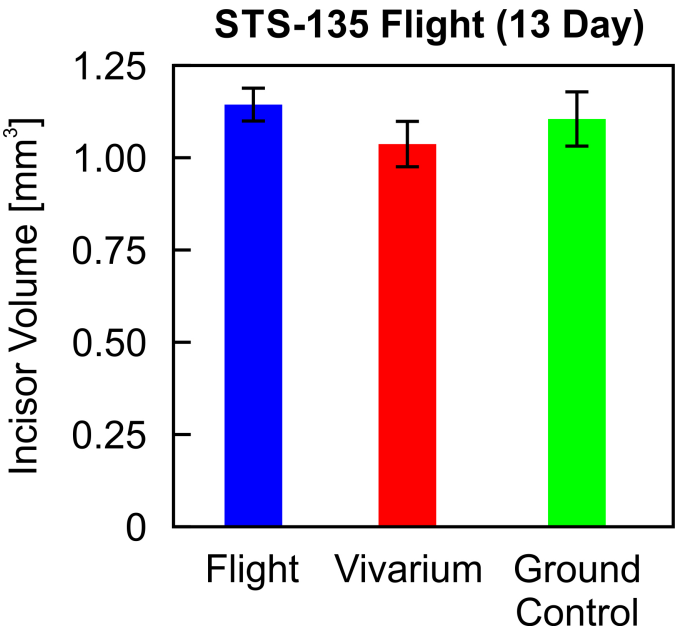

Fig. 10. Incisor volume in STS-135 flight (13 day) mice.

A significant increase was seen in bone volume in STS-135 flight mice vs. vivarium mice and ground control vs. vivarium mice. There was a significant increase in ground control mice vs. vivarium mice and a trend toward an increase in flight mice vs. vivarium for BVF (Table 10) (Fig. 11).

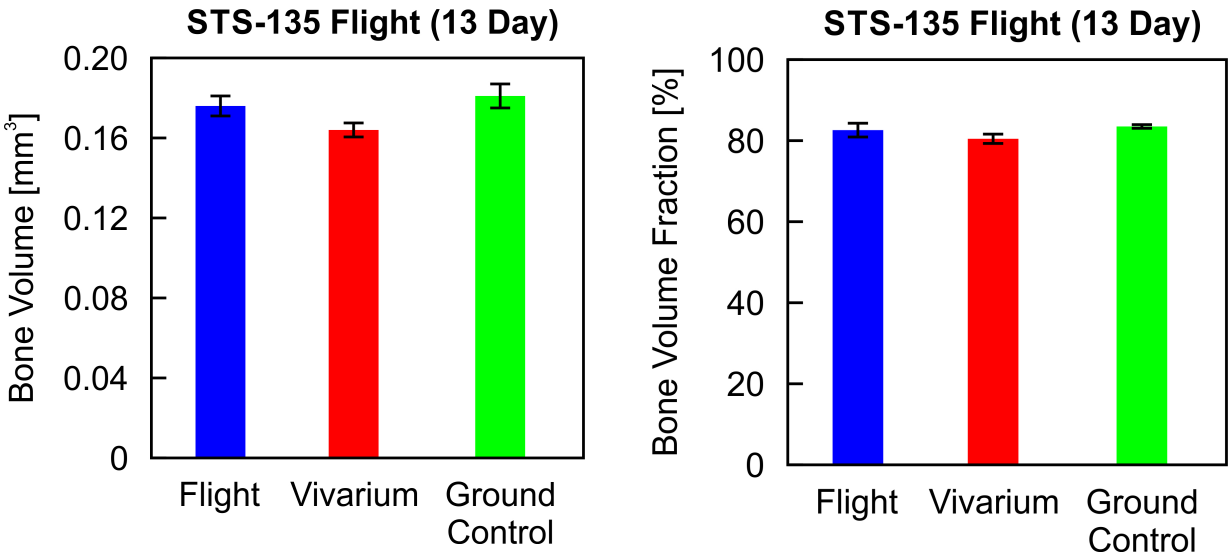

Fig. 11. Bone volume and bone volume fraction in STS-135 flight (13 day).

	BVF (BV/TV) (%)		Apparent Density (mg HA/ cm^3)		Tissue Density (mg HA/ cm^3)		Total Volume (mm^3)		Bone Volume (mm^3)	
	mean	p	mean	p	mean	p	mean	p	mean	p
Flight	82.6%	∞	1004	∞	1173		0.213		0.176	*
Vivarium	79.6%		972		1162		0.206		0.164	
Flight	82.6%		1004		1173		0.213		0.176	
Ground Control	83.5%		1023		1188		0.217		0.181	
Vivarium	79.6%	*	972	*	1162	*	0.206	∞	0.164	*
Ground Control	83.5%		1023		1188		0.217		0.181	

Table 10. Means and p values from t-tests comparing mean bone volume and density determined by microCT analyses of two hemimandibles for each condition from STS-135 flight. * $p<0.05$; ∞ $0.1>p>0.05$ (trend).

Analysis of 3D reconstruction images demonstrated that the incisor mineralization of Bion-M1 flight and ground control groups began significantly more posteriorly compared to that of the vivarium mice (Fig. 12).

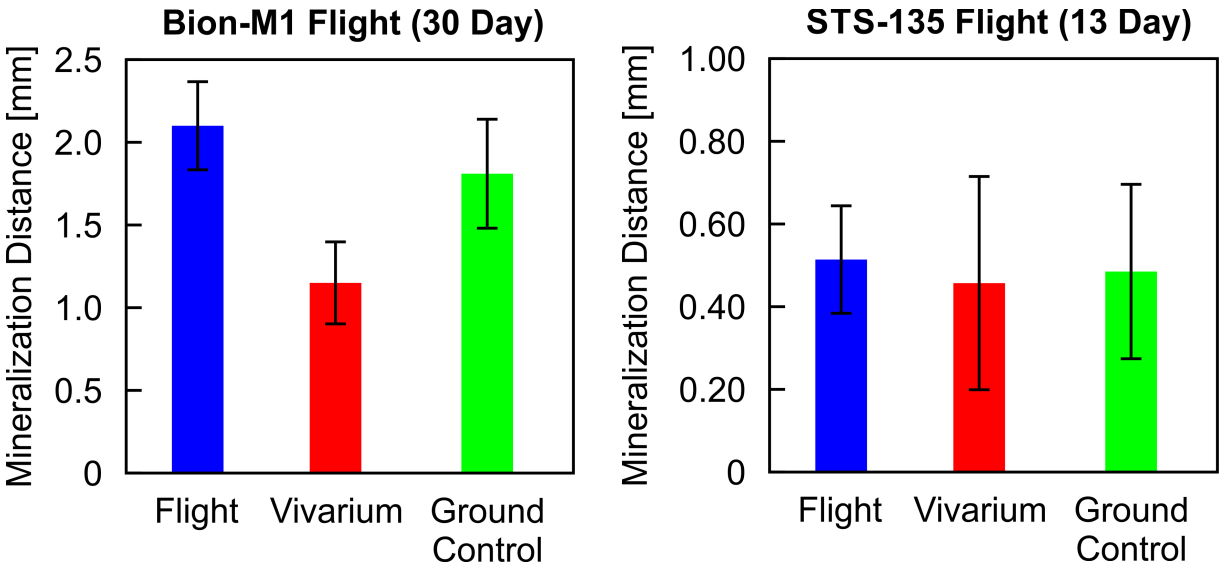

Fig. 12. Beginning of incisor mineralization distance posterior to reference line between 1st and 2nd molars in Bion-M1 and STS-135 mice on 3D reconstructed images.

It was noted that in the flight animals mineralization began posterior to the third molar, in the ground control animals, mineralization began at the distal (posterior) edge of the second molar, while in the vivarium control animals, mineralization began more anteriorly (Fig. 13). Cross-sections of the incisor at the level of the first, second and third molar teeth reveal thicker enamel (E) and dentin (D) in the incisors of the flight animals compared to the control animals. Similar results were obtained with incisors from ground control animals vs. vivarium controls.

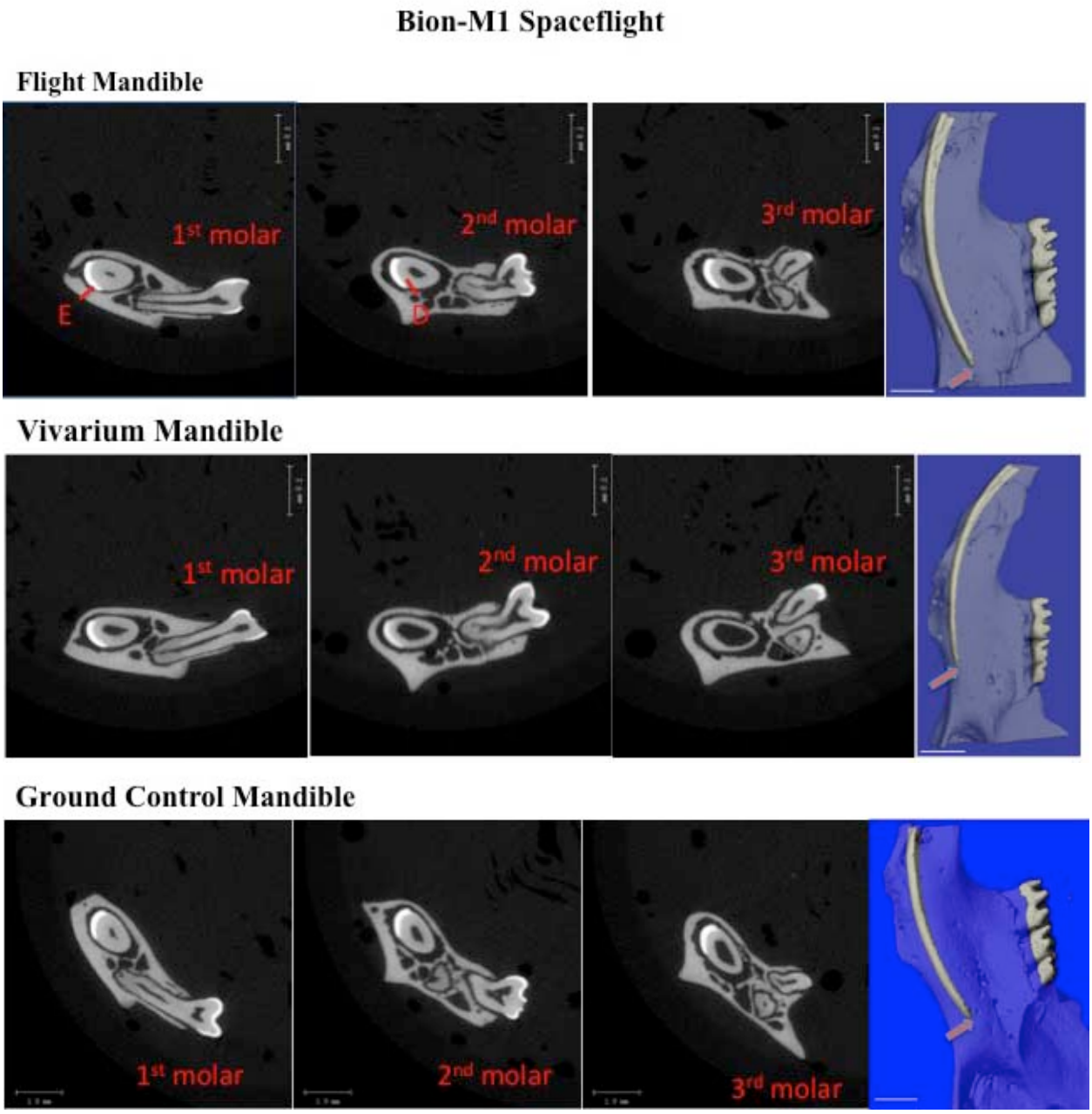

Fig. 13. MicroCT imaging of hemimandibles from flight, vivarium ground control, and habitat ground control mice from the 30-day Bion-M1 mission. Left panels: Cross-sections of the incisor tooth at the level of the first, second and third molar teeth, E: Enamel D: Dentin. Right panels: Lateral views of semi-transparent 3D reconstructions to show the beginning of incisor enamel and dentin mineralization (arrow). Scale bar =1 mm.

On the other hand, there was no significant difference in the initiation of incisor mineralization between flight and control groups in the STS-135 mice (Fig. 14). Cross-sections of the incisor at

the level of the first, second and third molar teeth reveal thicker enamel (E) and dentin (D), in the incisor of the flight animals compared to vivarium control animals.

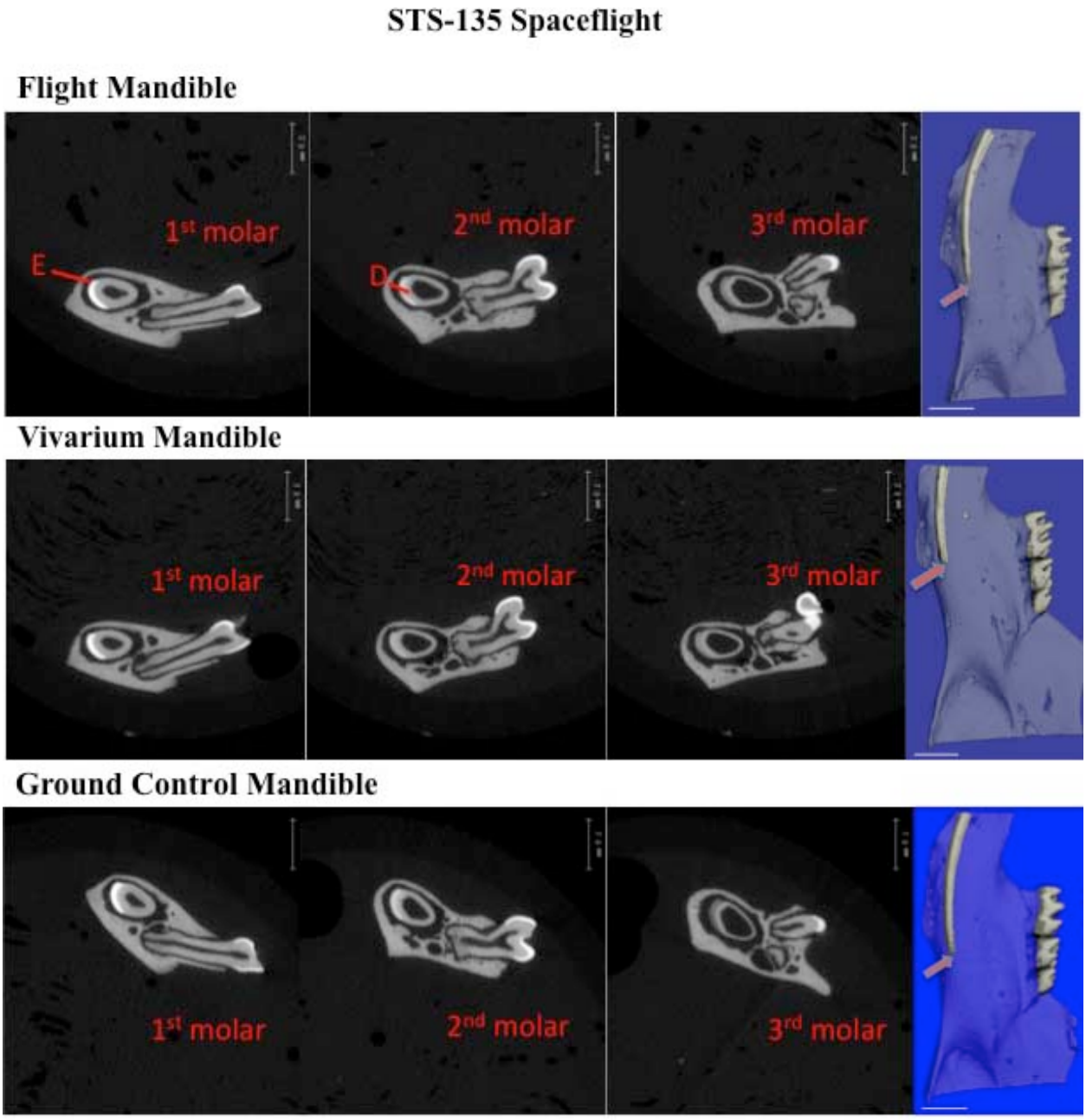

Fig. 14. MicroCT Imaging of hemimandibles from flight, vivarium ground control, and habitat ground control mice from the 13-day STS-135 mission. Left panels: Cross-sections of the incisor at the level of the first, second and third molar teeth. E: Enamel D: Dentin. Right panels: Lateral views of semi-transparent 3D reconstructions to show the beginning of incisor enamel and dentin mineralization (arrow).

Faxitron Imaging Analysis:

Faxitron images of the hemimandibles showed a significant increase in the length of incisors in the STS-135 flight group compared to vivarium and ground control groups. However, no significant difference was noted in incisor length between Bion-M1 flight and control groups.

	Bion-M1 Flight Mean ±SEM	Bion-M1 Vivarium Mean± SEM	Bion-M1 Ground Control Mean± SEM	STS-135 Flight Mean ±SEM	STS-135 Vivarium Mean ±SEM	STS-135 Ground Control Mean ±SEM
Incisor Length (mm)	14.9 ± 0.15	15.13 ± 0.04	14.73 ± 0.21	14.58 ± 0.07 * ∞	14.06 ± 0.1 *	14.26 ± 0.08 ∞

Table 11. Incisor lengths of Bion-M1 and STS-135 mice on Faxitron images. $p<0.05$ (* flight vs. vivarium, ∞ flight vs. ground control)

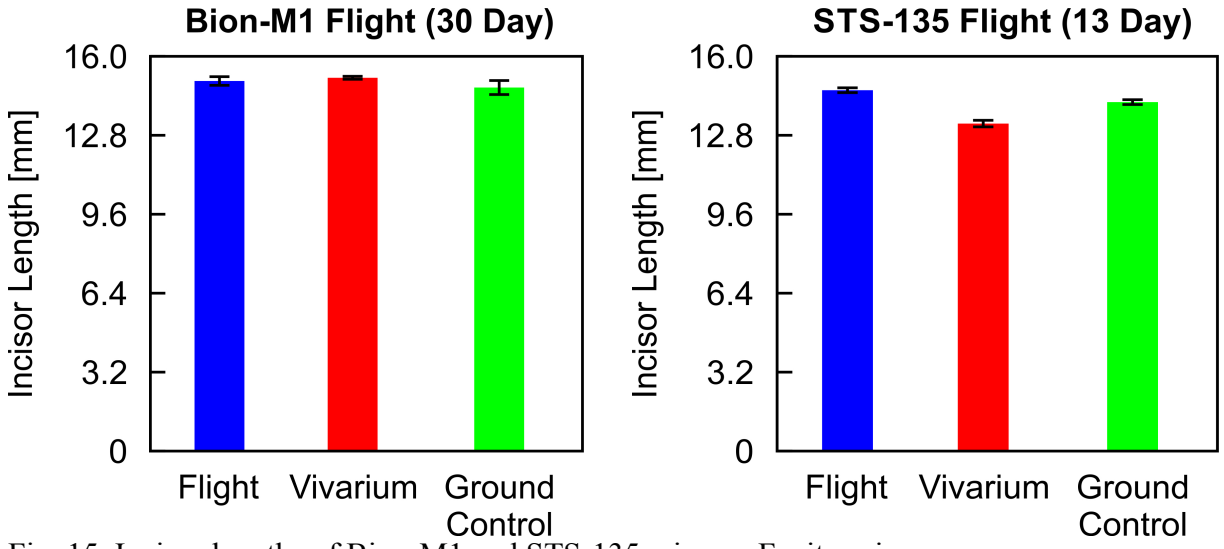

Fig. 15. Incisor lengths of Bion-M1 and STS-135 mice on Faxitron images.

DISCUSSION

The aim of this study was to investigate the effects of microgravity on the mandible and incisor teeth of mice. In this study, two approaches – immunohistochemical and radiographic – were used to determine the effects of weightlessness. A variety of proteins that are specific cell type markers in bone and teeth or are indicators of specific cell functions were used for this purpose; amelogenin for enamel formation (39), DSP for dentin formation (35), osteocalcin for bone formation (40), sclerostin for bone maintenance (41), TRAP for bone resorption (18), and PKA RII as an intracellular regulatory protein and marker of stress (31). The expression patterns of these proteins were compared between flight and control groups. Analyses of these data suggested that protein expression was altered during spaceflight. Light microscopic analyses showed overall tissue morphology and cellular function (e.g., tartrate-resistant acid phosphatase [TRAP] staining of osteoclasts). No significant alteration in tissue morphology was observed.

This study is one of the few studies to examine the effects of spaceflight on the mandible. To date, spaceflight research on skeletal tissues focused on weight bearing bones of the lower body. Previous studies reported that spaceflight induces bone loss in weight bearing bones (42,43). However, recent studies on the mandible and calvaria revealed that non-weight bearing bones were also affected by spaceflight (44,45). In this study, a significant increase was seen in bone volume in STS-135 flight mice vs. vivarium mice and ground control vs. vivarium mice. There was a significant increase in ground control mice vs. vivarium mice and a trend toward an increase in flight mice vs. vivarium for BVF. The difference in response of mandibular bone to microgravity could be explained by the head-ward shift of interstitial fluid, which causes increased arterial pressure to the head and greater bone blood flow to the skull and mandible

(44). After a longer spaceflight mission as in the Bion-M1 mission, a significant decrease in the mandibular bone volume fraction (BVF) and bone volume was observed in flight mice vs. vivarium mice and in ground control mice vs. vivarium mice. Although the findings in the Bion flight mice are consistent with spaceflight induced bone loss in weight bearing bones, the reason for differences between the two flights are unknown. Additionally, it is not clear why the ground control (habitat) mice showed similar changes as the flight mice in both missions. These changes may be related to the decreased masticatory forces developed by Bion flight and ground control mice fed a soft diet whereas STS-135 flight and control mice were fed hard diet. Previous studies on the effects of masticatory muscle function on mandibular bone morphology showed that reduced mechanical loads cause decreased mandibular growth (46,47).

We expected to confirm the quantitative measurements of micro CT results by histological examination. However, the results from microCT were not always consistent with those of immunohistochemistry. For example, microCT analysis on mandibular bone showed a significant decrease in the mandibular bone volume fraction (BVF) and bone volume in Bion flight mice vs. vivarium mice and in ground control mice vs. vivarium mice; a significant increase in bone volume in STS-135 flight mice vs. vivarium mice and ground control vs. vivarium mice, a significant increase in ground control mice vs. vivarium mice and a trend toward an increase in flight mice vs. vivarium for BVF. However, immunohistochemical analysis of bone formation and maintenance proteins indicated no significant difference among any groups in both flights. These contradictory results could be attributed to the differences in quantification of tissue samples. MicroCT is a volume averaging method, which discloses properties of hard tissues averaged over a stack of slices. In contrast to microCT, microscopy

methods do not average over a volume; light microscopy of the sections addresses individual spaceflight induced changes in bone. Immunohistochemical analyses revealed that there was a range of staining intensities for most antibodies, and we used the highest values for the analyses. Additional analyses to determine the number or proportion of cells or structures exhibiting different staining intensities could provide further insight into the effects of microgravity on protein expression.

Due to the small number of astronauts it is challenging to detect skeletal health trends after spaceflight (6). Rodents are commonly used animals because of their small size, which enables them to be housed in larger numbers, and at lower expense per animal. Animal models have been proven to be a suitable model to understand the underlying mechanisms of bone loss (48-50). Accumulated data from animal experiments revealed that rodents also suffer from microgravity-related bone loss (51). Previous studies on the effects of microgravity on bone and teeth mineralization showed contradictory results. While some studies reported increased Ca and P content of incisor dentin (52), others reported unchanged results (10,53-55). In the non-weight bearing bones, ribs and regions of mandible covered by muscle attachment, the rates of bone formation and mineralization and their content was unchanged (10). Weightlessness simulated by tail suspension of rats caused impaired mineralization of dentin (56). A possible explanation for some of these different findings is the differences in flight duration. The results from this study, comparing mandibles and teeth from a 30-day flight to those from a 13-day flight, have shed light on this issue. The microCT analysis revealed an increased incisor volume in flight mice vs. control mice in Bion-M1 and STS-135 flights. This finding supports our hypotheses that microgravity will cause increased enamel and dentin volume in the flight groups. The increase in

osteocalcin reactivity of odontoblasts between Bion flight mice vs. ground control mice correlate with the microCT analysis. However, no significant difference was noted in DSP reactivity of odontoblasts among any of the Bion and STS-135 mice groups. Immunohistochemical analyses of amelogenin reactivity in STS-135 flight mice maturation ameloblasts vs. those of the vivarium group revealed contradictory results to the microCT analysis. In addition, there were no significant differences in amelogenin reactivity among any of the Bion mice groups.

Three dimensional reconstructed images showed that in the Bion flight animals mineralization began posterior to the third molar, in the ground control animals, mineralization began at the distal (posterior) edge of the second molar, while in the vivarium control animals, mineralization began more anteriorly. However, no significant difference was noted in incisor length between Bion-M1 flight and control groups in Faxitron images. The earlier initiation of mineralization in Bion flight and ground control animals suggests that there is an effect of flight habitat environment, and a likely candidate is the difference in diet, paste for the habitat vs. chow for vivarium. The earlier mineralization may indicate decreased incisor eruption rate. Previous studies reported that soft diet decreases the eruption rate of rat incisors (57), and dentin thickness and enamel and dentin mineralization were increased in mice in which eruption rates were decreased (58). However, there is a discrepancy in the literature regarding the effects of the consistency of the diet on eruption rates and lengths of incisor teeth in rodents (59). While some studies reported that when rats were fed harder diet, decreased incisor length due to wear caused faster eruption rate (60), other studies found no correlation between the diet and eruption rate (61).

On the other hand, there was no significant difference in the initiation of incisor mineralization between flight and control groups in the STS-135 mice. Faxitron images of the STS-135 flight mice showed a significant increase in the length of incisors compared to vivarium and ground control mice. Whether these differences result from a different incisor eruption rate, different mineralization rate, or a difference in attrition rate, is unknown, and further investigation is needed for better understanding. Rodent incisor teeth with tetracycline injection can be used in studies that seek to determine the relationship between the incisor eruption rate and mineralization rate; increased tetracycline incorporation reflects higher mineralization rate. Previous studies revealed that alterations in tooth length could affect eruption rate (62). There is an inverse relationship between the eruption rate and the length of impeded (shortened) mandibular incisors in young rats (63).

A technical disadvantage of this study was that the unique nature of space flight samples makes this experiment unrepeatable. In order to minimize this technical problem, initial handling, preservation and shipping stages were carried out with procedures that previously were proven to work under spaceflight conditions. After landing, the mice were transported to the laboratory as rapidly as possible. For the Bion-M1 mice, transport took approximately 13 hours; for STS-135, 2 hours. Hemimandibles were fixed in 4% paraformaldehyde, shipped to UConn Health, and stored in 1% paraformaldehyde. Another disadvantage was the small sample size. Tissues from 13 mice that were flight animals, 14 mice that were vivarium animals and 14 mice that were ground control animals were used in this study. In order to get meaningful statistical analyses, samples from individual animals were analyzed separately rather than being pooled.

Interpretation of the results of this study was also complicated due to the sex and age differences of animals in each flight. Bion-M1 mice were male and 15-16 weeks old at launch, 19-20 weeks old on landing; whereas STS-135 mice were female and 9 weeks old at launch, 11 weeks old on landing. Bone and tooth growth continue throughout entire lifespan of rodents and slow down after puberty. Male mice generally have a larger bone size and grow faster than female mice. Both male and female mice reach skeletal maturation at 5-6 months of age (64). Therefore, the skeletally immature STS-135 mice may have suffered less from microgravity related bone loss than the older Bion-M1 mice.

Different food consumption in each flight and control group was another significant issue. The spaceflight diet plays an important role in supporting rodent growth and maintenance in the space environment. This unique diet should deliver adequate nutrition as well as avoid inducing unintended physiological effects (65). In this study, Bion-M1 flight and ground control animals were fed a paste diet whereas vivarium control animals were provided with standard pelleted chow. STS-135 flight and ground control animals were fed food bars that were attached to the walls of cages. Vivarium control animals were provided with standard pelleted chow. Given the fact that there were no significant differences between the average daily food consumptions in flight mice and their respective vivarium and ground controls both in Bion and STS-135 flights, the nutritional status of the mice was similar (2,34). Each diet has its own nutrient composition, which may eventually affect rodent bone and teeth maintenance. For example, paste diet has a softer nature than other diets due to its high water (60%) content. Paste diet does not present a promising usage for future long-term flights due to its potential for dental issues and limited shelf life (65).

Changes in bone and teeth may serve as physiological responses to spaceflight conditions. The question whether these changes are homeostatic or harmful can be answered by understanding the readaptation ability of biological systems to Earth conditions. Post-flight follow-up studies will help to determine if these microgravity-induced changes return to preflight status after returning to Earth, and if so, how long it takes to readapt. If the changes are irreversible or result in pathology, it is important to understand affected metabolic pathways in order to develop effective pharmacologic agents and other countermeasures.

CONCLUSIONS

Conducting spaceflight research has gained much more importance in recent years, as NASA plans to start a new era of space exploration in which humans will travel beyond low-earth orbit to deep space and planets such as Mars. Before moving further in the space research era, the effects of space environment on biological systems should be comprehensively understood. Only limited information is available about the effects of spaceflight on the oral cavity as well as non-weight bearing bones. Access to space as a research tool advances fundamental knowledge of the way in which weightlessness interacts with biological processes.

The results of this study yielded the following findings:

Radiological Findings:

- The micro CT analyses showed a significant increase between Bion flight mice and vivarium mice and in ground control mice vs. vivarium mice for incisor volume (enamel + dentin volume) and in ground control mice vs. vivarium mice for enamel volume. A trend toward an increase in incisor volume was seen in flight mice vs. ground control mice.
- A significant decrease was seen in the mandibular bone volume fraction (BVF) and bone volume in Bion flight mice vs. vivarium mice and in ground control mice vs. vivarium mice.
- There was a trend toward an increase in incisor volume between flight and vivarium control group in the STS-135 mice.
- A significant increase was seen in bone volume in STS-135 flight mice vs. vivarium mice and ground control vs. vivarium mice.

- There was a significant increase in ground control mice vs. vivarium mice and a trend toward an increase in flight mice vs. vivarium for BVF.
- Analysis of 3D reconstruction images demonstrated that the incisor mineralization of Bion-M1 flight and ground control groups began significantly more posteriorly compared to that of the vivarium mice.
- There was no significant difference in the initiation of incisor mineralization between flight and control groups in the STS-135 mice.
- Faxitron images of the hemimandibles showed a significant increase in the length of incisors in the STS-135 flight group compared to vivarium and ground control groups.
- No significant difference was noted in incisor length between Bion-M1 flight and control groups.

Immunohistochemical Findings:

- There was a significant decrease in amelogenin expression in STS-135 flight mice maturation ameloblasts compared to those of the vivarium group.
- There were no significant differences in amelogenin expression among any of the Bion mice groups.
- Osteocalcin expression of odontoblasts was significantly increased in Bion flight mice vs. ground control mice.
- No significant difference was noted in DSP expression of odontoblasts among any of the Bion and STS-135 mice groups.
- Immunohistochemical analyses of sclerostin expression by osteocytes and osteocalcin expression by osteoblasts indicated no significant difference among any groups in both flights.

The resulting findings of this study aid in the expansion of theoretical knowledge of spaceflight effects on the mandible and teeth as well as the potential for clinical applications. These findings provide background data for devising effective countermeasures to bone loss.

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