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Bone mineral density, bone mineral content, gingival crevicular fluid (matrix metalloproteinases, cathepsin K, osteocalcin), and salivary and serum osteocalcin levels in human mandible and alveolar bone under conditions of simulated microgravity

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Abstract: In astronauts and cosmonauts, exposure to microgravity has been associated with several physiological changes, including an osteoporosislike loss of bone mass. It has been reported that head-down tilt bed-rest studies mimic many of the observations seen in space flights. There has been no study of the effects of mandibular bone and alveolar bone loss in both sexes under conditions of simulated microgravity. This study was designed to investigate bone mineral density; bone mineral content; matrix metalloproteinase (MMP)-8, MMP-9, cathepsin K, and osteocalcin levels in gingival crevicular fluid (GCF); and salivary and serum osteocalcin levels in normal healthy men and women under conditions of simulated microgravity, namely, -6° head-down-tilt (HDT) bed rest. The subjects of this investigation were 10 male and 10 female volunteers who were exposed to 3 weeks of -6° HDT bed rest. Dual-energy X-ray absorptiometry was used to measure bone density and bone mineral content in alveolar bone from the mandibular canine to the third molar, as well as in the mandibular ramus, before, during, and after exposure to conditions of simulated microgravity. GCF (ie, MMP-8, MMP-9, cathepsin K, and osteocalcin) and salivary and serum osteocalcin levels were measured by enzyme-linked immunosorbent assays. Bone mineral density and bone mineral content were significantly lower under conditions of simulated microgravity in both sexes. The decreases were greater in women than in men, but the differences between sexes were not significant. Cathepsin, osteocalcin, MMP-8, and MMP-9 levels were significantly higher under conditions of simulated microgravity than under normal conditions; the increases were greater in women than in men, but the differences were not significant. Additional, more comprehensive, studies with larger sample sizes are now necessary for the investigation of simulated microgravity and microgravity. (J Oral Sci 52, 385-390, 2010)

Keywords: simulated microgravity; head-down-tilt; bone loss; MMP-8; MMP-9; bone density; bone mineral content; cathepsin K; osteocalcin.

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Introduction

Space flight results in many physiological adaptations, including, but not limited to, muscle atrophy, bone

demineralization, cardiovascular deconditioning, and orthostatic intolerance (1-5). The duration of microgravity (real or simulated) appears to correlate positively with the degree of adaptation (3-4). To lessen the adaptive effects of spaceflight, many countermeasures have been suggested and implemented. One that has been used extensively by the Russian and US space programs is physical exercise, and it has been shown that heavy exercise can lessen the effects of microgravity on muscle and cardiovascular deconditioning (1-5).

The effects of disuse on the weight-bearing bones of the skeleton have been well documented in both humans and rodents (6-7). Humans exposed to prolonged spaceflight (4 months) lose 1% to 2% of their bone density per month, and the loss is concentrated mostly in the cancellous compartments of the lower appendicular skeleton (8). Changes similar to those in humans have been documented in animals exposed to short-duration spaceflight (up to 16 days), including altered gene expression of bone matrix proteins, decrements in bone formation, and reduced mechanical strength (9-12). Hind-limb unloading is well established as a model to simulate spaceflight (13). A small number of studies of skeletally mature rats (5 months of age) have demonstrated distinct differences in the responses of these rats and those of growing animals (3 months of age). As compared with immature rats aged 3 months, unloading must be maintained 2 to 3 times longer in mature rats if it is to produce significant declines in bone calcium content, density, and formation rates, all of which are more adversely affected in skeletally mature animals (14-15).

Studies of cosmonauts/astronauts have included so few women subjects that conclusions specific to females are not possible. However, because age-related bone loss occurs at a higher rate in women than in men, there is a clear need to define the effects of spaceflight on the female skeleton (6,9). Microgravity also causes skeletal redistribution of mineral, as was shown in another long-term human study in which skeletal responses to microgravity were not uniform. For example, certain regions of the body, such as the skull, that experience elevated loads during spaceflight (because of cephalic fluid shifts) displayed either increased or unchanged calcium deposition. In contrast, unloaded areas, such as the tibia, exhibited loss of calcium (16). However, when the net effect of microgravity on the entire skeleton was examined, mineral density was decreased, with 1 study estimating the average loss at 0.5% per month (based on values calculated from urinary and serum levels of calcium from astronauts on Skylab missions) (17). Head-down tilt bed-rest studies appear to mimic many of the conditions seen in spaceflight. Both the bed-rest and in-flight observations indicate that loss of bone mass is continuous and progressive in weightlessness and in microgravity (5).

Several biomarkers that may be diagnostic of bone loss have been studied. Serum osteocalcin is regarded as a valid marker of bone turnover when resorption and formation are coupled, and as a specific marker of bone formation when formation and resorption are uncoupled (18). Gingival crevicular fluid (GCF) is an exudate that can be harvested from the sulcus or a periodontal pocket, and has been studied as a promising marker of bone loss (19,20). In periodontal disease (bone loss), a number of proteases degrade collagen and extracellular matrix. The matrix metalloproteinases (MMPs) are the principal group of enzymes that degrade extracellular matrix. The onset of collagen destruction in periodontitis is caused by the action of collagenases, which are a subgroup of MMPs. In a healthy oral environment, the periodontal ligament apparatus is protected from MMP-mediated proteolytic attack by tissue inhibitors of metalloproteinases (TIMPs). In chronic periodontitis, TIMP levels are low and thus inadequate to inhibit elevated levels of MMPs, activated neutrophil pro-collagenase, and progelatinase. Furthermore, mobilization and activation of inflammatory cells such as lymphocytes and neutrophils, alternation of immuno-modulators, and secretion of inflammatory proteases occur (21,22). Cathepsin K is predominantly expressed in osteoclasts and is a potent extracellular matrix-degrading enzyme that plays a critical role in osteoclast-mediated bone resorption (23). There has been no study of the effects of simulated microgravity on loss of mandibular bone and alveolar bone in men and women. This study was designed to assess bone mineral density, bone mineral content, GCF (ie, cathepsin K, MMP-8, MMP-9, and osteocalcin), and salivary and serum osteocalcin in normal healthy men and women under conditions of simulated microgravity, namely, -6° head-down tilt (HDT) bed rest.

Materials and Methods

We enrolled 20 volunteers: 10 men (age 18-24 years, mean weight 76.5 ± 3.2 kg, mean height 172.9 ± 3.4 cm) and 10 women (age 19-26 years, mean weight 72.6 ± 6.5 kg, mean height 169.6 ± 4.3 cm). The volunteers underwent 3 weeks of -6° HDT bed-rest exposure. They had not participated in systemic endurance training for 10 days before the study. Each subject was given a detailed explanation of the experimental protocol and provided written and verbal consent. The average energy and calcium expended by the subjects during the simulation was 2,300 kcal/ day (range 2,080-3,010 kcal/day) and 1,200 mg/day, respectively. Each subject completed a questionnaire on

their medical and dental history to determine the status of systemic diseases, smoking, and history of alcohol and drug use. They also underwent a clinical examination for systemic diseases, chronic diseases, and oral and dental diseases. Patients were excluded if they had a systemic or chronic disease, an oral or dental disease, if they were smokers, or if they had a history of alcohol or drug abuse. We used dual-energy X-ray absorptiometry – the "gold standard" for the measurement of bone mineral density (BMD) – to measure bone loss under conditions of simulated microgravity. BMD measurements were performed before, during, and after the simulation of microgravity, as described in a previous study (5). BMD was measured in alveolar bone from the canine to the third molar, and in the mandibular ramus, of both sides of the mandible. Samples of GCF, saliva, and serum were taken before, during, and after the simulation of microgravity. One to 4 sites per patient were randomly selected for GCF collection. GCF was collected by inserting periopaper strips in a gingival pocket for 45 seconds. Strips contaminated by blood or saliva were discarded. After collecting GCF, the levels of MMP-8 and -9 were measured by enzyme-linked immunosorbent (ELISA) assays (Human Quantikine MMP-8, -9 ELISA kit). Osteocalcin measurements in serum, saliva, and GCF were made using an electrochemiluminescence technique described previously (21). The concentration of cathepsin K was determined by an ELISA kit (Quantikine Human cathepsin K immunoassay, Biomedica, Vienna, Austria, Catalogue no. BI-20432). All statistical analyses were performed using the SPSS software package (version 11.0). The paired *t*- test was used to compare bone density, bone mineral content, GCF (MMP-8, MMP-9, cathepsin K, and osteocalcin), and salivary and serum osteocalcin levels under normal conditions and conditions of simulated microgravity.

Results

Bone mineral density and bone mineral content were significantly lower under conditions of simulated microgravity than under normal conditions in both sexes (Table 1, $P < 0.01$). The decreases of bone mineral density and bone mineral content were greater in women than in men, but the differences between sexes were not significant. Osteocalcin levels in serum, saliva, and GCF were significantly higher under conditions of simulated microgravity than under normal conditions ($P < 0.01$). The increases were greater in women than in men, but the differences between sexes were not significant. Levels of MMP-8, MMP-9, and cathepsin K were significantly higher under conditions of simulated microgravity than under normal conditions $P < 0.01$). The increases were greater in women than in men, but the differences between sexes were not significant.

Discussion

Both bone mineral density and bone mineral content were significantly lower under conditions of simulated microgravity in both sexes, as has been reported in previous studies (5-7,11-20). In the present study, the decreases in bone mineral density and bone mineral content were greater in women than in men, perhaps due to the activity of hormones, such as estrogens (21). Because of the limited number of studies, it is difficult to compare BMD loss in cosmonauts and healthy humans exposed to the bed-rest conditions used for simulating some aspects of microgravity. In an experiment by Leblanc and colleagues, BMD data from dual-energy X-ray absorptiometry were obtained from 6 individuals after 17 weeks of bed rest. The results showed substantial BMD loss in the lower extremities and lumbar vertebrae (22). BMD loss in the legs was estimated to be 0.4% per month. In a study of 11 volunteers exposed to 12 weeks of bed rest, researchers observed a BMD decrease of 0.95% per month in the greater trochanter (23). The monthly differences measured in space are similar to those noted in bed-rest studies, although the healthy volunteers in the bed-rest studies did not benefit from countermeasures such as drugs or physical activity programs.

The MMPs are host proteinases that degrade and remodel tissue (24-26). MMP-8 and -9 are the most prevalent MMPs in both diseased periodontal tissue with bone loss and GCF (27-35). In the present study, levels of MMP-8 and -9 were significantly higher under conditions of simulated microgravity than under normal conditions, possibly because simulated microgravity increased the virulence of bacteria (36). The MMPs are a family of proteolytic enzymes involved in the degradation of the extracellular matrix of various tissues, including bone (18-19). More than 20 different mammalian MMPs have been identified. These have been divided into 4 subgroups: the collagenases (MMP-1, -8, -13, and -18), the gelatinases (MMP-2 and -9), the stromelysins (MMP-3 and -10), and the membrane-type metalloproteinases (MMP-14 and -17). Osteoblasts produce various MMPs, such as MMP-1, -2, -13 (collagenase 3), and -14 (MT1-MMP), and osteoclasts selectively produce MMP-8 and MMP-8. We previously reported that the induction of MMPs (such as MMP-2, -3, and -13) in osteoblasts is essential for bone resorption (19).

The findings of this study suggest that elevated MMP-8 levels exert protective and anti-inflammatory effects that prevent bone loss under conditions of simulated

Table 1 Bone density, bone mineral content, gingival crevicular fluid (matrix metalloproteinases -8 and-9, cathepsin K, osteocalcin), and salivary and serum osteocalcin levels under normal conditions and those of simulated microgravity

Variables	Sex	Normal	Simulated microgravity
Bone density of mandibular bone (g/cm^2)	M	1.138 ± 0.67	1.002 ± 0.54
		(0.998-1.787)	(0.845-1.543)*
	F	1.113 ± 0.68	0.967 ± 0.63
		(1.024-1.875)	(0.678-1.576)**
Bone mineral content of mandibular bone (g/cm^2)	M	2789 ± 1121	2662 ± 1176
		(1587-3985)	(1478-3457)*
	F	2713 ± 1082	2611 ± 1212
		(1682-3998)	(1402-3678)**
Bone mineral content of alveolar bone (g/cm^2)	M	2734 ± 1127	2569 ± 1289
		(1567-3884)	(1236-3398)*
	F	2713 ± 1082	2611 ± 1212
		(1682-3998)	(1402-3678)**
Bone density of alveolar bone (g/cm^2)	M	1.243 ± 0.783	0.845 ± 0.654
		(0.876-1.989)	(0.564-1.123)*
	F	1.189 ± 0.564	0.823 ± 0.435
		(0.765-1.658)	(0.589-1.235)**
GCF MMP-8 (pg/µl)	M	9.90 ± 4.57	25.39 ± 6.82
		(5.45-13.45)	(17.89-31.03)*
	F	9.87 ± 6.34	29.67 ± 8.56
		(5.86-13.96)	(15.76-37.69)**
GCF MMP-9 (pg/µl)	M	12.34 ± 10.23	24.12 ± 8.67
		(10.21-23.41)	(21.47-32.48)*
	F	10.98 ± 11.67	26.67 ± 9.41
		(6.48-21.06)	(15.78-28.98)**
GCF cathepsin K (pmol/l)	M	4.23 ± 1.45	11.65 ± 4.05*
		(3.22-5.78)	(7.89-15.03)
	F	5.03 ± 2.21	12.45 ± 4.42
		(4.01-7.32)	(8.02-16.93)**
Salivary osteocalcin (ng/ml)	M	2.3 ± 2.1	6.9 ± 1.3
		(1.6-4.3)	(4.4-7.9)*
	F	2.9 ± 2.2	7.3 ± 1.9
		(1.9-5.3.)	(5.2-8.6)**
Serum osteocalcin (ng/ml)	M	4.1 ± 1.9	9.3 ± 2.4
		(3.2-6.3)	(7.3-10.5)*
	F	5.2 ± 2.5	10.5 ± 3.9
		(4.4-7.8)	(7.1-12.6)**
GCF osteocalcin (ng/ml)	M	3.4 ± 2.4	7.2 ± 2.9
		(1.6-4.3)	(5.4-11.9)*
	F	3.6 ± 2.6	7.9 ± 2.1
		(2.0-6.2)	(5.6-10.1)**

*$P < 0.01$ as compared with normal conditions, **$P < 0.05$ as compared with normal conditions

microgravity. Our data further indicate that deficiencies in MMP-8 and -9 might influence leukocyte accumulation in the gingiva by regulating increased cell migration or, alternatively, by hampering the resolution of inflammation after a bacterial challenge. Osteocalcin levels in serum, saliva, and GCF were significantly higher under conditions of simulated microgravity than under normal conditions. Although the increases were greater in women than in men, the differences between sexes were not significant. These results are not surprising, as levels of bone markers are typically much higher in women than in men (23). Osteocalcin levels are determinants of bone formation, (24) and it has been shown in vitro that osteocalcin has a role in bone resorption. Cathepsin K levels were significantly higher under conditions of simulated microgravity than under normal conditions, although the differences between men and women were insignificant. There was also an increase in the concentration of cathepsin K from normal conditions to those of simulated microgravity. The similar levels under conditions of simulated microgravity in our study further confirm that, although cathepsin K is secreted by macrophages and fibroblasts, it is predominantly

expressed in osteoclasts (24).

Although there were a number of limitations in this study, including the fact that dietary factors, demographic data, and other important factors were not included in the analysis, our data provide more evidence of the mechanisms underlying bone loss due to microgravity. Moreover, the findings suggest that bone loss is greater in women than in men. Additional comprehensive studies with larger sample sizes are required for the investigation of simulated microgravity and microgravity.

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