Effects of chronic hypergravity: from adaptive to deleterious responses in growing mouse skeleton

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Gnyubkin V, Guignandon A, Laroche N, Vanden-Bossche A, Normand M, Lafage-Proust MH, Vico L. Effects of chronic hypergravity: from adaptive to deleterious responses in growing mouse skeleton. J Appl Physiol 119: 908–917, 2015. First published July 30, 2015; doi:10.1152/japplphysiol.00364.2015.—One of the most important but least studied environmental factors playing a major role in bone physiology is gravity. While the knowledge of deleterious effects of microgravity on the skeleton is expanding, little is known about hypergravity and its osteogenic potential. Centrifugation was used to assess effects of 21-day continuous 2- or 3-g acceleration on femur and L2-vertebra of 7-wk-old male C57BL/6 mice. Under 3-g acceleration growth slowed down, and deleterious skeletal effects were found (P < 0.05 compared with control): cortical thinning, osteoclasts; sclerostin; DMP1; microcomputed tomography; immunohistochemistry; bone vascularization.

THE EVOLUTION OF LIFE ON EARTH has always been determined by variations of environmental factors. While some conditions have changed over time, gravity has remained constant. Alterations of gravity (microgravity or hypergravity) are unique and severe challenges for living beings. Spaceflights are an example of such a challenge, as weightlessness leads to dramatic physiological deconditioning from cellular (8) to organ level systems (65).

In relation to the skeleton, spaceflight-related site-specific decrease of bone mass has been observed in astronauts since the 1970s. Importantly, the weight-bearing sites of the skeleton are those most affected by microgravity (30, 62). For example, monthly decrease of bone mineral density (BMD) in the lumbar spine and hip could reach 1.5% of total bone mass (27, 29). Even though recently developed countermeasures, such as an advanced resistive exercise device on the International Space Station, provide better protection against the decrease of BMD than previous systems, still it is not always fully efficient (50, 52). In addition, bones are unable to rapidly restore their initial BMD after spaceflights (62). Mathematical modeling revealed that most crew members who have flown on long-duration missions (4–6 mo) would return their BMD to pre-flight level within 3 yr (51).

Spaceflights provide a unique opportunity for studying chronic microgravity effect; however, such experiments are rare and expensive. Hypergravity based on centrifugation is more accessible. It allows dose-dependent effects of gravity on living beings (5, 63) and adaptation mechanisms to altered gravity (3, 43) to be investigated. What can also be assessed is a capacity of hypergravity as a countermeasure against physiological deconditioning in space (7).

Short-time centrifugation (from minutes to hours) showed limited ability to prevent bone loss associated with unloading. Thus, in humans, daily centrifugation for 1 h at 2.5 g did not prevent bedrest-induced bone loss (53), even when combined with ergometric exercises (22). In rats, 1 h daily centrifugation at 1.5 or 2.6 g did not prevent tail-suspension-induced bone loss (68). However, site-specific increase of BMD and BMC in the cervical spine was found in military pilots after repetitive exposure to high head-feet directed acceleration (positive Gz loading) (37, 38).

In contrast to short-time hypergravity, rats centrifuged for 4 days at 2 g, showed increase of both bone formation activity and trabecular thickness in the tibia metaphysis (58). Exposure to 2.9 g for 28 days was similarly beneficial, and it led to a trabecular bone volume increase in both ovariectomized and sham-operated rats compared with controls (21). Also, sitespecific increase of bone mass was observed after 3 mo of chronic exposure to 2.5 g in dogs (41).

Centrifugation is still a rare experimental model, and available data assessing the impact of hypergravity on different organ systems are very limited, in particular for vulnerable growing organisms. Therefore, several laboratories decided to perform a comprehensive study designed to assess safety and effects of chronic 21-day continuous 3- and 2-g hypergravity on stress response (17), vestibular (3) and immune system (17), muscle functions, and cognitive abilities (3). In the first stage of the program, focused on young mice, our team aimed to analyze skeleton response to hypergravity and to find a g level which would be osteogenic. While the skeleton of young animals has been shown to benefit more from mechanical...
loading than adults (49, 56, 57), the potential impairment of the growth process (46) also had to be assessed.

As we found that 21-day centrifugation at 2 g stimulated bone formation, we repeated the experiment to assess effects on bone vascularization and osteocyte proteins expression. Angiogenesis and bone remodeling have proved to be closely linked when mechanical loading is altered (1). Thus treadmill running increases trabecular bone mass and stimulates bone formation and angiogenesis (66). Therefore, we hypothesized that chronic centrifugation would alter the bone vascular network, and we assessed vascularization parameters by using previously validated quantification technique (48).

Sclerostin and dentin matrix acidic phosphoprotein 1 (DMP1) were selected for immunohistochemistry analysis of osteocyte protein expression (4), as their expression is affected by mechanical loading (15, 19, 47) and because they control osteocyte protein expression (4), as their expression is affected previously validated quantification technique (48).

In this study, we repeated the experiment to assess effects of 21-day centrifugation at 2 g on bone vascularization and osteocyte proteins expression, as their expression is affected by mechanical loading (15, 19, 47) and because they control osteocyte protein expression (4), as their expression is affected previously validated quantification technique (48).

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MATERIALS AND METHODS

Animals

Seven-week-old C57BL/6J male mice (Charles River Laboratories, l’Arbesle, France) were used for all experiments. At the end of each experiment, the mice were euthanized with cervical dislocation; femurs and lumbar vertebrae were dissected out and processed as described below. Protocols and animal procedures followed the European Community standards on the care and use of laboratory animals (Ministère de l’Agriculture, France, Authorization No. 42-21-080) and were approved by the local animal care and use committee (Comité d’Éthique en Expérimentation Animale de la Loire - Université Jean Monnet, CEEAL-UJM).

Material Setup

Mice were housed by four in standard cages (36 x 20 x 14 cm) with bedding material, in a quiet room with constant temperature (22°C), 50% relative humidity, and a 12/12 h light-dark cycle. Food and water were provided ad libitum (Safe Diets A04, Augy, France). After a week of habituation, cages were transferred into the centrifuge’s gondolas preserving the same environmental conditions. The centrifuge (COMAT Aérospace, Florens, France) made it possible to maintain a permanent level of hypergravity for 21 days. A centrifuge with a radius of 1.4 m had four gondolas hanging on the periphery (S1 video) (23). Each gondola can accommodate up to three cages. All gondolas were equipped with a video surveillance system to control animals’ condition and food/water stocks. Animals were provided with enough food and water for the whole duration of the experiments, thus the centrifuge was stopped only twice (6 and 2 days before euthanasia) for 5 min each time to inject animals with tetracycline for dynamic histomorphometry (see below). We performed three experiments lasting 21 days: one at 3 g and two at 2 g. Control mice were also placed into gondolas to mimic experimental conditions, but were not exposed to centrifugation.

Experimental Groups

In the first 3- and 2-g experiments, 24 mice were randomly and equally divided in control and experimental groups (n = 12 per group). At the end of experiments, animals were processed for microcomputed tomography (μCT) and histomorphometry. For the second 2-g experiment, 30 mice were randomized in two equal groups (control and experimental, n = 15 per group). At the end of an experiment, femurs of five animals in each group were isolated and processed for immunohistochemistry (IHC) analysis. Others were analyzed for bone vascular parameters as described below. In all experiments, euthanasia and dissection were performed within 3 h after the end of centrifugation. Tissue collection was done within 15–20 min after euthanasia.

Investigation Tests

Microtomography. Formalin-fixed and ethanol-dehydrated femur and L2 vertebral body were scanned with a high-resolution μCT (Viva CT 40, Scanco Medical, Switzerland) and analyzed as described in David et al. (10). Data were acquired at 55 keV energy and 145 μA current for 10 μm cubic resolution. Right femur trabecular bone was scanned within the metaphysis below the growth plate, and the cortical bone was scanned in the diaphysis; L2 vertebra was scanned entirely (Fig. 1). Three-dimensional reconstructions were generated with the following parameters: Sigma: 1.2, Support: 2, Threshold: 160 (spongiosa) or 280 (cortex). In distal femur metaphysis, the secondary spongiosa was evaluated above the distal primary spongiosa and the cortical bone was evaluated at the level of diaphysis; in L2 vertebral body secondary spongiosa was evaluated between two primaries (Fig. 1). Three-dimensional structural parameters were obtained from 60 sections (0.6 mm) for both trabecular and cortical bone in femur, and from 180 sections (1.8 mm) for trabecular bone in L2 vertebral body. Regions of interest (ROI) were manually defined by an operator (Fig. 1). For cortical bone, we measured BMD (mg/cm³), cortical thickness (Cl.Th, mm), and cortical area (Cl.Ar, mm²). For trabecular bone, we measured trabecular thickness (Tb.Th, μm), trabecular number (Tb.N,
mm⁻³), trabecular separation (Tb.Sp, μm), bone volume (BV/TV, %), connectivity density (Conn.D, mm⁻³), structural model index (SMI), and degree of anisotropy (DA).

**Histomorphometry.** Centrifugation was stopped twice for 5 min, 6 and 2 days before death, and mice were injected intraperitoneally with 200 μl of a 0.37% tetracycline solution. Distal femur metaphysis and L2 vertebral body were processed undecalcified for histomorphometry as described (9). Bone cellular parameters were measured semiautomatically with digitizing tablet (Summasketch, Summagraphics, as described (9). Bone cellular parameters were measured semiautomatically with digitizing tablet (Summasketch, Summagraphics, and a software designed in our laboratory (9). In brief, we evaluated longitudinal growth rate (LGR, μm/day) by measuring the distance in micrometers per day between two fluorochrome labelings (parallel to the growth plate) in the primary spongiosa. In the secondary spongiosa we measured double labeled surfaces (dLS/BS, %) and mineral apposition rate (MAR, μm/day), from which the bone formation rate (BFR/BS, μm²/μm²/day) was calculated. Osteoid surfaces (OS/BS, %) were detectable in femurs only. Osteoclasts surfaces (Oc.S/BS, %) were assessed through tartrate-resistant acid phosphatase labeling.

**Bone vessel quantification.** Vascular assessment was processed as previously described by Roche et al. (48). In brief, after intracardiac perfusion of prewarmed contrasting solution [50% BaSO₄ (Micropaque Guerbet, Paris, France), 1.5% gelatin in PBS] with a peristaltic pump (MasterFlex, L/S, Cole-Parker Instrument, Germany), femoral bones were embedded in medium for frozen sections (Neg50, Thermo Scientific, Ref. No. 6502) by snap freezing in liquid nitrogen. Nine-micrometer-thick sections of whole bone were cut with 27 micrometer-thick sections of whole bone were cut with 27 μm increments with a cryotome (Microm HM 525, Thermo Scientific). The secondary spongiosa of the distal femur was selected as ROI (4 mm²) and manually analyzed with a 100-point eyepiece grid. The measured bone section (no primary antibody applied during IHC). The measured bone surface; MAR, mineral apposition rate; LGR, longitudinal growth rate.

Quantitative assessment of immunohistochemical data by measuring relative fluorescence has been previously proposed as a method for clinical pathology (14). We also used fluorescent IHC to perform semiautomated quantitative analysis of protein expression levels in near-lacunar space. Our technique made it possible to conduct an osteocyte-by-osteocyte analysis. Black and white pictures of the entire cortical area were obtained with an AxioObserver Z1 microscope (Zeiss, Darmstadt, Germany), at a magnification of 400 (oil immersed objective), and automatically merged by using the Mosaic plugin of Axiovision 4.7 software (Zeiss). Immunolabeling and imaging were done with the same reagents and imaging parameters for all samples to minimize any possible variations. Mosaic images were directly analyzed with ImageJ software (http://imagej.nih.gov/ij/) without any brightness or contrast correction. A cortical bone ROI was manually defined. Bone marrow, peristeum, blood vessels, and cutting/staining artifacts were excluded from ROI. A mask of DAPI positive cells within the ROI was merged with sclerostin or DMP1 staining to restrict the areas of measurements to areas of DAPI staining, providing fluorescence intensity data for individual osteocytes. As a lacunar size exceeds the area of DAPI staining, we applied two successive dilations of the DAPI mask to fit with lacunar area. The dilated DAPI does not entirely match the area of the osteocyte lacuna. Therefore the pixels of surrounding bone matrix having zero gray level might be included in the ROI. Hence, parameters based on calculations of mean gray level were not appropriate for the analysis of fluorescence. Considering this, we used Raw Integrated Density (RawIntDens) parameter of ImageJ, which represents a sum of all gray level pixels within the ROI. Collagen and osteocytes autofluorescence were defined with the Plot Profile function of ImageJ from a negative control bone section (no primary antibody applied during IHC). The measured gray level of autofluorescence was removed with the Subtract function.

**Table 1. Animals’ body weight after centrifugation**

<table>
<thead>
<tr>
<th>Group/Treatment</th>
<th>Control</th>
<th>3 g</th>
<th>Control</th>
<th>2 g Exp 1</th>
<th>Control</th>
<th>2 g Exp 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>24 ± 2</td>
<td>21 ± 1*</td>
<td>25 ± 2</td>
<td>24 ± 2</td>
<td>24 ± 1</td>
<td>23 ± 1</td>
</tr>
</tbody>
</table>

Data are shown as means ± SD. Nonparametric Mann-Whitney test, 3 or 2 g compared with control; *p ≤ 0.05.

**Table 2. μCT and histomorphometry analysis of femur diaphysis and distal metaphysis and L2 vertebral body in control and 3 g centrifugation groups**

<table>
<thead>
<tr>
<th>Group/Treatment</th>
<th>Control</th>
<th>3 g</th>
<th>Control</th>
<th>3 g Vertebral Body</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical bone</td>
<td>1,186 ± 12</td>
<td>1,190 ± 11</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Trabecular bone</td>
<td>13.8 ± 4</td>
<td>12.5 ± 4</td>
<td>23.5 ± 3</td>
<td>21.8 ± 2</td>
</tr>
<tr>
<td>BV/TV, %</td>
<td>38 ± 5</td>
<td>34 ± 7</td>
<td>40 ± 4</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>Tb.Th, μm</td>
<td>36.6 ± 0.6</td>
<td>36 ± 0.5</td>
<td>5.9 ± 0.4</td>
<td>57.0 ± 3</td>
</tr>
<tr>
<td>Tb.N, mm⁻¹</td>
<td>244 ± 50</td>
<td>251 ± 40</td>
<td>130 ± 13</td>
<td>136 ± 9</td>
</tr>
<tr>
<td>Conn.D, mm⁻³</td>
<td>112 ± 27</td>
<td>114 ± 25</td>
<td>257 ± 21</td>
<td>267 ± 26</td>
</tr>
<tr>
<td>SMI</td>
<td>2.4 ± 0.4</td>
<td>2.6 ± 0.3</td>
<td>1.1 ± 0.3</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>DA</td>
<td>1.62 ± 0.08</td>
<td>1.56 ± 0.13</td>
<td>1.84 ± 0.11</td>
<td>1.78 ± 0.07</td>
</tr>
<tr>
<td>OS/BS, %</td>
<td>17.9 ± 3.8</td>
<td>11.7 ± 4.6*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>dLS/BS, %</td>
<td>27.5 ± 6</td>
<td>22.1 ± 4.9*</td>
<td>11 ± 2</td>
<td>8.6 ± 2.4*</td>
</tr>
<tr>
<td>MAR, μm/day</td>
<td>1.2 ± 0.1</td>
<td>1 ± 0.06*</td>
<td>0.9 ± 0.05</td>
<td>0.7 ± 0.06*</td>
</tr>
<tr>
<td>LGR, μm/day</td>
<td>11.1 ± 1.7</td>
<td>8.7 ± 1*</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are shown as means ± SD. Nonparametric Mann-Whitney test, 3 g compared to control; *p ≤ 0.05. μCT, microcomputed tomography; BMD, bone mineral density; ND, not done (parameters were too small for reliable measurements); BV/TV, bone volume over total volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; Conn.D, connectivity density; SMI, structural model index; DA, degree of anisotropy; OS/BS, Osteoid surfaces over bone surface; dLS/BS, double labeled surfaces over bone surface; MAR, mineral apposition rate; LGR, longitudinal growth rate.
of ImageJ before evaluating fluorescence of proteins. To calculate intra- and interobserver coefficients of variation, we repeated measurements three times with the same operator and three times with a different operator on five histological sections. Each time every operator had to independently define an ROI and perform related measurements. Variations between measurements were always less than 2% and not statistically significant. To ensure representativity of osteocyte population, we analyzed two to three entire cortical transversal sections per bone sample (~450 osteocytes per section) from five animals per group; average number of analyzed osteocytes per group was 5,000.

**Statistical Analysis**

Data analysis was performed with the STATISTICA software (version 8.2, StataCorp, College Station, TX). Data were assessed with the nonparametric Mann-Whitney U-test.

**Results**

**Centrifugation at 3 g**

After 21 days at 3 g, mice displayed 12% (P ≤ 0.001) loss of body weight with a 22% (P ≤ 0.05) decrease of LGR compared with controls (Table 1 and 2). While Ct.Th and Ct.Ar were reduced by 5 and 11%, respectively (P ≤ 0.05), trabecular compartments of femur and L2 vertebral body did not change (Table 2, Fig. 2). BFR/BS was reduced (−34%, P ≤ 0.05) and Oc.S/BS increased ( +41%, P ≤ 0.05) in femur. In L2 vertebral body BFR/BS decreased by 36% (P ≤ 0.05) and Oc.S/BS increased by 20% (P ≤ 0.05) (Table 2 and Fig. 2).

**Body weight.** In both 2-g experiments, at the end of the experiment there was no change in body weight compared with controls (Table 1).

**µCT and histomorphometry.** In femur cortex, exposure to 2 g did not change mass-structural parameters (Table 3). However, trabecular bone in both distal femur and vertebral body was affected. In the femur there was an increase of BV/TV (+24%, P ≤ 0.05), Tb.N (+18%, P ≤ 0.05), and Conn.D (+36%, P ≤ 0.05) and decrease of Tb.Sp (−22%, P ≤ 0.05) and SMI (−17%, P ≤ 0.05) compared with controls (Fig. 3). Tb.Th and DA were not affected (Table 3). In vertebral body

![Fig. 2. Microarchitecture and histomorphometry parameters in control and 3-g centrifugation groups. Femoral distal metaphysis and L2 vertebral body: cortical thickness (Ct.Th) (A), cortical area (Ct.Ar) (B), osteoclasts surface over bone surface (Oc.S/BS) (C), and bone formation rate (BFR/BS) (D). Boxes represent 50% of values (25 to 75%), whiskers represent minimum and maximum values, and middle point represents median. Nonparametric Mann-Whitney test, *P ≤ 0.05, ***P ≤ 0.001.](#)
Table 3. μCT and histomorphometry analysis of femur diaphysis and distal metaphysis and L2 vertebral body in control and 2 g centrifugation groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Femur</th>
<th>L2 Vertebral Body</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>2 g</td>
</tr>
<tr>
<td>Cortical bone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD, mg/cm³</td>
<td>1.212 ± 13</td>
<td>1.206 ± 14</td>
</tr>
<tr>
<td>Ct.Th, μm</td>
<td>184 ± 16</td>
<td>178 ± 12</td>
</tr>
<tr>
<td>Ct.Ar, mm²</td>
<td>0.95 ± 0.1</td>
<td>0.91 ± 0.1</td>
</tr>
<tr>
<td>Trabecular bone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb.Sp, μm</td>
<td>206 ± 60</td>
<td>161 ± 21°</td>
</tr>
<tr>
<td>SMI</td>
<td>2.1 ± 0.5</td>
<td>1.8 ± 0.3°</td>
</tr>
<tr>
<td>DA</td>
<td>1.56 ± 0.12</td>
<td>1.51 ± 0.04</td>
</tr>
<tr>
<td>OS/BS, %</td>
<td>16.5 ± 3.1</td>
<td>22 ± 3**</td>
</tr>
<tr>
<td>dLS/BS, %</td>
<td>13.8 ± 4.9</td>
<td>18 ± 4.4°</td>
</tr>
<tr>
<td>MAR, μm/day</td>
<td>1.2 ± 0.1</td>
<td>1 ± 0.18°</td>
</tr>
<tr>
<td>LGR, μm/day</td>
<td>11.2 ± 1.9</td>
<td>10.2 ± 2.1</td>
</tr>
</tbody>
</table>

Data are shown as means ± SD. Nonparametric Mann-Whitney test, 2 g compared to control, *P < 0.05, ***P < 0.001. ND, not done (parameters were too small for reliable measurements); Ct.Th, cortical thickness; Ct.Ar, cortical area.

we found similar positive alterations: increase of BV/TV (+13%, P ≤ 0.05) and Tb.N (+4%, P ≤ 0.05) and decrease of Tb.Sp (−9%, P ≤ 0.05) and SMI (−33%, P ≤ 0.05). However, contrary to femur, Tb.Th increased by 9% (P ≤ 0.05) and Conn.D was not affected (Table 3).

Bone cellular activities were altered in both femur and vertebral body regions. In femur 2 g led to decrease of Oc.S/BS (−36%, P ≤ 0.05) and increase of OS/BS (+33%, P ≤ 0.001) and dLS/BS (+31%, P ≤ 0.05). At the same time, we found a decrease of MAR (−15%, P ≤ 0.05), and BFR/BS did not change (Fig. 3). There were also no changes in LGR (Table 3). In L2 vertebral body, we observed increase of dLS/BS (+48%, P ≤ 0.001) and BFR/BS (+45%, P ≤ 0.05), and a decrease of Oc.S/BS (−16%, P ≤ 0.05). MAR remained unchanged (Table 3).

Bone vascularization. Exposure to 2 g led to an increase of both number of vessels (+22%, P ≤ 0.05) and their volume (+44%, P ≤ 0.01) in distal femur secondary spongiosa compared with controls (Table 4).

Circulating sclerostin immunoassay. When analyzing circulating sclerostin with the ELISA kit, no difference was found between control group (205 ± 32 pg/ml, n = 7) and 2-g group (225 ± 24.8 pg/ml, n = 7).

Quantitative analysis of immunohistochemical data. No significant differences between groups were detected in osteocyte density, percentage of sclerostin, or DMP1 positive cells (Table 5). However, the fluorescence intensity (RawltDens; see MATERIALS AND METHODS) of individual osteocytes showed differences between 2 g and controls. In the 2-g group, osteocyte sclerostin fluorescence (Fig. 4) was 35% less than in control group (medians: 365 vs. 560, respectively; P < 0.001). Osteocyte DMP1 fluorescence (Fig. 4) was 60% higher than in controls (medians: 3,610 vs, 2,258, respectively; P < 0.001) (Table 5).

DISCUSSION

It has been assumed that chronic hypergravity might induce physiological responses, which would be opposite to microgravity effects (5, 17, 63). We found this assumption to be true for 2 g. While microgravity led to an increase of bone resorption and decrease of trabecular BV/TV in rodents (59–61), 2-g centrifugation resulted in lower osteoclastic activity and augmented BV/TV in both femur and vertebra. Ikawa et al. (21) reported a bone gain in rats centrifuged at 2.9 g. Bojados and Jamon (3) found in male C57Bl/6j mice that hypergravity acted as endurance training on muscle force until 3 g, and then became deleterious only at 4 g. Thus it could be expected that adaptation to a 3-g environment would also lead to improvement of bone tissue. However, in our study μCT evaluation after exposure to 3 g demonstrated a decrease of Ct.Ar and Ct.Th in young mice. In the Ikawa et al. study (21), beside the fact that animals were adult female 20-wk-old rats, the centrifuge had a 50-cm radius (1.4 m in our study) and much smaller cages, which raises concerns that animals may have experienced increased stress due to close confinement; also, hypergravity was interrupted daily for 15 min. This makes direct comparison difficult.

Even though μCT demonstrated that femoral and L2 vertebral body trabecular compartments remained the same, histology revealed inhibition of bone formation and stimulation of bone resorption, suggesting that longer exposure to 3 g might result in trabecular bone loss. Similarly to other authors (17, 67), we observed body weight loss, which could be a result of low food intake (67) and high stress as indicated by serum corticosterone assay (17). It can be assumed that potential osteogenic effects of high mechanical loading were counterbalanced by stressful 3 g environment.

Although infrared video surveillance was not quantitative, it seemed that the animals’ motion activity was lower compared with control. Thus it is plausible that less movement could reduce the dynamic aspect of mechanical loading while the static component was greater. It is well accepted that the same mechanical stimulus is beneficial when applied intermittently and deleterious for cortical bone when applied statically (28). At the same time, during static loading muscle strength might benefit from isometric contractions (32). This might explain why at 3 g an increase in muscle force was occurring along with bone growth reduction.

In contrast to 3 g, centrifugation at 2 g did not lead to any significant alteration of body weight. The same was previously observed after 21 (17) or 30 (39) days of 2-g centrifugation with the same facility, mice, and conditions as in present study. In addition, no differences in plasma corticosterone level (17) or adrenal gland weights (39) were found between control and 2-g experimental groups.

Longitudinal bone growth can be impaired by high static or dynamic mechanical loading, as it was demonstrated in the rat ulna (46). In contrast to 3 g, centrifugation at 2 g did not lead to any decrease of longitudinal growth rate, which further indicates that 2 g is a safe model.

It is known that microgravity (27) primarily affects trabecular compartment. Interestingly, centrifugation at 2 g also altered trabecular bone in the first place, and the weight-bearing bones (femur) were more affected than nonweight-bearing bones (vertebra) in relation to BV/TV and Conn.D parameters. These findings are important as the biomechanical properties of bones are highly dependent on trabecular organization (16, 25).

In addition, we observed that 2 g modified trabecular microarchitecture in femur and vertebra in different ways. In-
crease of Tb.Th and Tb.N in vertebra without alteration of Conn.D can be a consequence of adaptation to greater gravitational forces applied along a typical loading vector, which animals were exposed to (6, 13, 20, 42). In contrast to alterations in vertebra, we found no increase of Tb.Th in femoral metaphysis; however, we detected an increase of Conn.D. In relation to biomechanical properties, increase of Conn.D is more beneficial than trabeculae thickening (18). Combined
vascularization in the femoral distal metaphysis.

Table 4. Effects of 21 days 2 g centrifugation on vascularization in the femoral distal metaphysis

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>2 g Centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ves.N/Mar.Ar, mm²</td>
<td>61.6 ± 11.3</td>
<td>74.9 ± 6.8*</td>
</tr>
<tr>
<td>Ves.V/Mar.V, %</td>
<td>12.7 ± 3.2</td>
<td>18.3 ± 2.3**</td>
</tr>
</tbody>
</table>

Data are shown as means ± SD. Nonparametric Mann-Whitney test, 2 g compared to control, *P ≤ 0.05, **P ≤ 0.01. Ves.N/Mar.Ar, vessel number per marrow area; Ves.V/Mar.V, vessel volume per marrow volume.

with higher Th.N, it results in more uniform mechanical stress distribution (57a). Such adaptation suggests that during locomotion at 2 g, femoral bones experienced specific strain distribution and mechanical forces that were unusually directed. This can be linked to the fact that exposure to 2 g modifies gait parameters, as it was demonstrated in younger animals (2). At the same time, we detected no effects of 2-g continuous hypergravity on the cortical bone, which demonstrates that cortical and trabecular bones respond differently to continuous gravitational challenge.

As we registered changes in trabecular compartments of femur and L2 vertebral body, we analyzed cellular activities there. Exposure to 2 g resulted in increased osteoid and mineralized surfaces, presumably due to increase of osteoblast recruitment and/or lifespan. At the same time, MAR decreased in femur and did not change in vertebra. Similar changes were detected in rats centrifuged at 2.9 g for 4 wk (lower MAR, BFR/BS, and osteoblast surface); however, authors provided little explanations regarding the phenomena of trabecular bone gain along with lower bone formation (21). Our findings suggest that hypergravity might alter individual osteoblast function and mineralization in a transient manner, or that reduction of osteoclastic parameters has played a major role in trabecular bone gain. Future in vitro investigations of osteoblastic cells from centrifuged animals may shed light on the underlying mechanisms.

Centrifugation at 2 g resulted in notable reduction of osteoclasts’ parameters both in weight-bearing and nonweight-bearing bones. It is particularly important for microgravity-related bone loss, as resistive physical exercises cannot reduce high bone resorption level, which has been constantly observed in crewmembers (54).

After 21 days of hypergravity, we observed an increase of bone marrow vascularization (as assessed by vessel number and volume), although the exact underlying mechanism is not known. A link between load-induced bone gain and vascularization has been previously reported (1, 11, 40, 66). Beside the thoraco-cephalic fluid shift in microgravity (36), we can also assume that hypergravity induces blood pooling in extremities, which might contribute to increased vessel volume. Further, higher capillary density and enhanced microcirculation were reported after gravitational therapy with a short-arm centrifuge at 1.5–3 GZ, in humans with fractures or osteomyelitis (26).

Mechanical stimulation has been shown to alter osteocyte protein expression. In rodent ulna, axial cycling loading (47) results in a decrease of sclerostin expression, while in the orthodontic appliance model (15) DMP1 expression is increased. In our study, we observed for the first time similar changes for sclerostin (~35%) and DMP1 (~60%) expression in osteocytes of the femur diaphysis after an exposure to chronic hypergravity. However, in contrast to axial loading or orthodontic appliance models (15, 47) we did not detect any significant difference in percentage of positive/negative osteocytes (Table 5). This could be explained by the fact that these experimental models vary significantly from ours. It must also be taken into account that while during normal locomotion compressive mechanical strains are about 200–350 με (44, 10a), the models of cycling axial loading provide strains up to 2,200 με (47). Therefore, even assuming that chronic 2-g centrifugation provides twice higher strains during locomotion, centrifugation is still a milder challenge for bones, which results in modest but statistically significant alterations of osteocyte protein expressions. It should be noted that decrease of sclerostin level and low resorption can be linked together as it was shown that sclerostin affects osteocytes and promotes osteoclastogenesis and osteoclasts activation (64). Moreover, we did not find any changes in serum sclerostin after 2-g centrifugation. These findings imply either that changes in serum sclerostin levels do not necessarily reflect alterations of sclerostin production in osteocytes, as it has been shown in ovarietomized mice (24), or that decreased protein expression in femur diaphysis represents a local and not a generalized effect of 2-g exposure.

As there was no alteration of cortical bone after 21 days, we hypothesized that continuous 2-g hypergravity affects trabecular bone in the first place, and longer exposure to hypergravity is needed to stimulate cortical bone formation.

This study has the following limitations: 1) there is no data regarding possible increase of bone strength after 2-g centrifugation, as no mechanical testing was done; 2) data obtained do not represent dynamics of adaptation processes to hypergravity; 3) we did not study recovery processes after centrifugation; 4) because of stressful conditions of 3-g centrifugation, it is difficult to distinguish between the stress effects and impact of mechanical loading on bones; and 5) no quantitative analysis of food/water intake or animal activity during exper-

Table 5. Effects of 21 days 2 g centrifugation on osteocyte quantitative parameters in femur cortical bone compared to controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>2 g Centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sclerostin</td>
<td>0.95 ± 0.06</td>
<td>0.98 ± 0.05</td>
</tr>
<tr>
<td>Positive osteocytes, %</td>
<td>61.8 ± 5.2</td>
<td>87.1 ± 3.4</td>
</tr>
<tr>
<td>RawIntDens per group</td>
<td>560</td>
<td>2258</td>
</tr>
</tbody>
</table>

Data are shown as means ± SD for osteocytes’ density (DAPI staining) and percentage of positive cells (immunohistochemistry); as medians for level of fluorescence (RawIntDens, arbitrary unit). Nonparametric Mann-Whitney test, 2 g compared to control, ***P ≤ 0.002.

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ments was presented, as the video surveillance system was not designed for it.

In conclusion, our main findings are that effects of centrifugation are bone compartment-specific and depend on the gravity level, and that centrifugation at 2 g appeared to be a safe model for young animals in relation to bone and body growth. Centrifugation at 2 g brought down osteoclastic resorption and local sclerostin expression. It stimulated osteoid deposition and angiogenesis, and increased DMP1 level. Thus centrifuge is a promising platform for investigating bone mechanosensitivity and gravity-related alterations. In our study we argue that further research investigating the bone metabolism dynamics is needed to define optimal level and duration of hypergravity, and that future experiments can be performed in combination with bone loss animal models to assess protective or/and regenerative effects of hypergravity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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