



## Evaluation of twelve vibration regimes applied to improve spine properties in ovariectomized rats



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### ABSTRACT

While whole-body vibration (WBV) has recently been introduced as a non-pharmacological therapy for osteoporosis, studies have shown that it has no significant effect on the lumbar spine in older women. However, the vibration protocols differed among studies, and the major factor influencing the outcomes is unclear. The intention of the present study was to evaluate the effect of WBV—vertical (v) or horizontal (h) and of different frequencies and application regimes (1× or 2×/d)—on lumbar spine properties in ovariectomized rats (Ovx). Three experiments were conducted. Thirteen-week old female Sprague–Dawley rats were Ovx or left intact (Non-Ovx). After eight weeks, all of the rats underwent metaphyseal osteotomy of the tibiae. Five days later, the rats were divided into six groups (n = 15): 1) intact, 2) Ovx, and 3–6) Ovx exposed to WBV. In Experiment 1, groups 3–6 underwent 35 Hz-v, 50 Hz-v, 70 Hz-v, and 90 Hz-v, respectively. In Experiment 2, groups 3–6 underwent 30 Hz-h, 50 Hz-h, 70 Hz-h, and 90 Hz-h, respectively. In Experiment 3, groups 3–6 underwent 35 Hz-v, 70 Hz-v, 35 Hz-h, and 70 Hz-h, respectively. Vibration exposure was 15 min 1×/d in Experiment 1 and 2 and 2×/d in Experiment 3 for up to 30 days. Vertebral bodies were used in micro-computed tomography, biomechanical, ashing, and gene expression analyses.

Vertical vibrations applied once a day favorably affected bone volume fraction (BV/TV) and  $\text{Ca}^{2+}/\text{PO}_4^{3-}$  and decreased Rankl gene expression. When applied twice a day, v-vibrations diminished mineral content. Horizontal vibrations (1×/d) reduced  $\text{Ca}^{2+}/\text{PO}_4^{3-}$  ratio and Opg mRNA level, whereas h-vibration (2×/d) normalized OC serum levels. Many of the other measured parameters did not reveal any significant differences between the vibrated groups and the untreated Ovx group. The effect of ovariectomy was confirmed by atrophied uterus, impaired biomechanical properties, and bone mineral density and BV/TV of the vertebral body.

The findings of the present study indicate that application frequency rate and direction of vibration might influence spine response differently. However, we were unable to find any clearly beneficial or harmful effect of vibration regimes on the osteopenic lumbar spine in rats.

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### 1. Introduction

The loss of ovarian hormones following menopause often leads to the development of postmenopausal osteoporosis, which is characterized by structural deterioration of bone tissue and increased risk of fracture (Ström et al., 2011).

Physical activity and exercise is highly recommended for the prevention of osteoporosis. Regular exercise not only increases bone

density, but also enhances coordination and strengthens the muscles, thereby reducing the risk of falling, which is the major risk factor for osteoporotic fractures (Lau et al., 2011). The influence of passive exposure to mechanical stimulation in the form of whole-body vibration (WBV) is increasingly gaining interest as a non-pharmacological method of protecting and improving the musculoskeletal system. It has the advantage of easy application with user-friendly, durable devices (Chan et al., 2013). The interaction of the human body with vibration is complex, and, despite a risk of serious injury, certain types of vibration possess healing effects and health benefits (Jordan et al., 2005). The International Organization for Standardization has defined human tolerance to vibration exposure as follows: at 30 Hz, 0.3 g,

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humans can be exposed safely for up to four hours per day; accelerating the vibration to more than 1.0 g leads to a drastic decrease in safe exposure duration (ISO, 1997).

Several clinical studies (randomized controlled trials) reported a significant improvement in hip bone mineral density (BMD) after brief WBV treatments (12–40 Hz) over 6–12 months in postmenopausal women (47–88 years old) with osteopenia or osteoporosis (Slatkowska et al., 2010). However, the response of the spine and tibia was less pronounced. The differential effect of WBV on different bone sites was explained by the variability in the transmission of vibratory signals from one anatomical site to another and differences in body positions (Slatkowska et al., 2010). The latest meta-analysis of scientific papers over 60 years concluded that WBV had no significant effect on the hip and lumbar spine BMD in older women, but it enhanced leg muscle strength significantly (Slatkowska et al., 2010). The vibration protocols differed among the studies; therefore, it was unclear whether the treatment frequency or the total number of vibration sessions was the major factor that influenced the outcomes (Lau et al., 2011).

The vibration treatments applied in clinical and experimental trials differ in frequency, magnitude, force direction (vertical or horizontal), and duration, and they are performed using different WBV devices (Fritton et al., 2000). Numerous animal studies have investigated the effects of WBV on long bones and muscles (Komrakova et al., 2013; Cullen et al., 2001; Tezval et al., 2011).

While evaluating the spine is of high clinical relevance in the treatment of osteoporosis, studies of the spine are rare and controversial (Christiansen and Silva, 2006; Wehrle et al., 2014; Sehmisch et al., 2009a; Holguin et al., 2011). In one study, 45 Hz vibrations of different magnitudes (0.1, 0.3, and 1.0 g) increased the bone volume fraction in the tibiae of male mice, whereas other skeletal sites, including the lumbar spine, appeared to be unresponsive (Christiansen and Silva, 2006). In another study, 35- and 45 Hz vibrations were found to have no effect on the trabecular bone of the lumbar spine (Wehrle et al., 2014). In contrast, Sehmisch et al. (2009a) reported a significant improvement in the spine properties of ovariectomized (Ovx) rats after 90 Hz, vertical vibration treatments. Brief daily exposure (15 min per day) to the 90 Hz vertical vibrations was found to mitigate the degradation of the intervertebral disk caused by the hindlimb unloading of the rat (Holguin et al., 2011).

In the present study, 12 vibration regimes, differing in frequency (30–90 Hz), type (vertical or horizontal), and application frequency (1× or 2×/day) were applied to ovariectomy-induced osteopenic rats to evaluate their effects on lumbar vertebrae. The Oxv rat has been widely used as a model of postmenopausal osteoporosis and osteoporosis-related fractures (Komrakova et al., 2013; Kalu, 1991). In the present study, all of the rats underwent bilateral osteotomy of the tibia. The experimental conditions were similar to the clinical situation in which patients with osteoporotic fracture might be treated with WBV to improve their mobility, bone healing, and muscle strength. The bone healing and muscle tissue results have been published in part (Komrakova et al., 2013) and are discussed in the present paper.

## 2. Materials and methods

### 2.1. General procedures

The animal experiments and data analyses were performed at the University Medical Center Goettingen, Germany. All animal procedures were approved by the government in accordance with German animal-protection laws (Az: 011/07, Braunschweig) prior to performing the study.

Three experiments were conducted using 270 female Sprague–Dawley rats; the 12-week-old rats were obtained from Harlan Winkelmann (Borchen, Germany). The rats received a standard pellet diet (ssniff Special Diet, Soest, Germany) and water without restrictions during the entire experimental period. Individual body weight (BW) and average food consumption were recorded weekly. After a one-

week acclimatization period, the rats were assigned randomly to the severe osteopenic groups, and they underwent bilateral ovariectomy under intraperitoneal ketamine and xylazine anesthesia (115 and 8 mg/kg BW, respectively). The rats designated as healthy controls were left intact (Non-Ovx). Thereafter, the rats were housed in groups of four or five in standard cages under standard conditions (12 h dark–light regime,  $22 \pm 1$  °C) for up to eight weeks to induce bone loss in the Oxv rats (Kalu, 1991). Thereafter, all of the rats underwent a bilateral osteotomy and osteosynthesis of the tibia (data on tibia published in part by Komrakova et al. (2013)). Five days after the osteotomy, the Oxv rats designated for WBV began exposure to the different vibration treatments according to the experimental protocols. There were six groups of 15 rats in each experiment.

In Experiment 1, the Oxv rats were exposed to vertical WBVs (v) of different frequencies for 15 min per day for 30 days. The groups were as follows: 1) Non-Ovx, 2) Oxv, 3) 35 Hz-v 1×/d, 4) 50 Hz-v 1×/d, 5) 70 Hz-v 1×/d, and 6) 90 Hz-v 1×/d.

In Experiment 2, the Oxv rats were treated with horizontal WBV (h) of different frequencies for 15 min per day for 30 days. The groups were as follows: 1) Non-Ovx, 2) Oxv, 3) 30 Hz-h 1×/d, 4) 50 Hz-h 1×/d, 5) 70 Hz-h 1×/d, and 6) 90 Hz-h 1×/d.

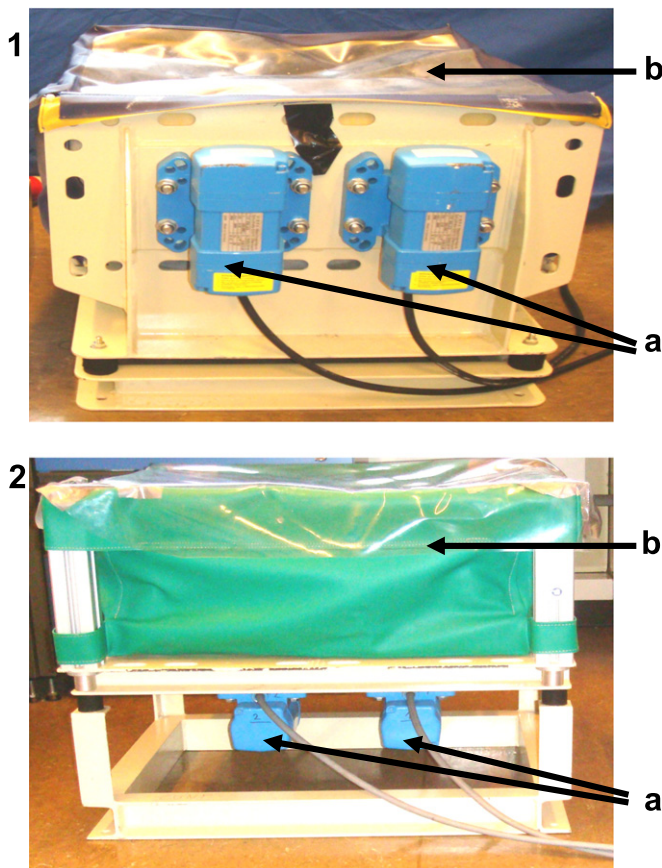
The vibration frequencies that caused significant changes in the lumbar spine properties were identified in Experiments 1 and 2 (35 and 70 Hz) and applied further in Experiment 3 with the purpose of intensifying their effects by increasing treatment frequency to 2×/day. In Experiment 3, the Oxv rats were subjected to vertical or horizontal WBV of 35 or 70 Hz for 15 min, twice a day for 30 days. The rats were divided into the following groups: 1) Non-Ovx, 2) Oxv, 3) 35 Hz-v 2×/d, 4) 70 Hz-v 2×/d, 5) 35 Hz-h 2×/d, and 6) 70 Hz-h 2×/d.

The WBV treatments were conducted using two vibration devices (Vibra Maschinenfabrik Schultheis, Offenbach, Germany) that generated vertical or horizontal vibrations (Komrakova et al., 2013; Stuermer et al., 2010). During the WBV sessions, seven or eight rats at a time were maintained in a plastic cage (50 × 50 × 25 cm) attached to the vibration platform (Fig. 1). Non-vibrated rats from the Non-Ovx and Oxv groups were maintained in the same room during the WBV sessions. Vibration parameters were measured on the bottom of the cage with a handheld vibration monitoring system (SWM 3000; REO Elektronik, Berlin, Germany). Transmission of the vibration from the cage bottom to the lumbar spine region was measured in four additional rats. The sensor of the SWM device was attached to the lumbar region of the shaved back of the rats with adhesive tape, and the measurements were taken within a few seconds, when the rats were not moving.

After 30 days of vibration treatments, the rats were anesthetized with CO<sub>2</sub> and decapitated. Blood samples were collected for electrochemiluminescence immunoassay analysis of osteocalcin (Oc) and colorimetric assay of alkaline phosphatase (Alp). Serum analysis was conducted at the Department of Clinical Chemistry, University of Goettingen, using an automated chemistry analyzer (Roche/Hitachi Modular, Mannheim, Germany) and commercially available kits (Roche Diagnostics) according to the manufacturer's instructions. The lumbar vertebral bodies (L1–L5) were dissected free of soft tissues and stored at –20 °C until further analysis. The sixth vertebral body (L6) was immersed in liquid nitrogen and stored at –80 °C for quantitative real-time polymerase chain reaction (qRT-PCR) analysis of gene expression.

### 2.2. Biomechanical analysis

A Zwick/Roell type 145660 Z200/TND mechanical testing device (Ulm, Germany) was used to analyze the fourth lumbar vertebral body (L4), applying a previously developed test (Sehmisch et al., 2009b). Briefly, the vertebral body was placed with the cranial end plate on an angled base. The angle of the base corresponded to the shape of the vertebral body. The surface of the stamp was designed according to the size and angle of the caudal vertebral body end plate. The spinal process was fixed with clips to prevent lateral slipping. A



**Fig. 1.** Vibration devices generated (1) horizontal vibrations and 2) vertical vibrations. (a) two motors; (b) cage for rats.

compression load was applied at the caudal end plate along the craniocaudal axis of the vertebral body. After fixing L4 with a preload of 1 N, the measurements were performed at a feed motion rate of 5 mm/min and recorded with testXpert software (Zwick/Roell). The test was automatically terminated when the maximum compressive strength was achieved and the decline of the loading curve was more than 10 N. Stiffness, a slope of the linear rise of the curve (N/mm); yield load, end point of elastic deformation (N); and maximal compressive strength (Fmax, N) were calculated using Excel (MS Office 2003).

### 2.3. Micro-computed tomography (micro-CT)

The third vertebral body (L3) was scanned using a micro-CT device (eXplore Locus SP Scanner; GE Healthcare, London, Ontario, Canada). The scan protocol was as follows: 72 kVp, 90  $\mu$ A, 1600 ms exposure time, 360° rotation, 0.029 mm pixel size, and 900 views. Three-dimensional (3D) reconstruction was performed in the MicroView program (v2.1.2, GE Healthcare). After the data was reconstructed, the analysis was conducted using a program (3DOsteoAnalyze, v1.000.4) developed at the Department of Radiology, University of Goettingen. The L3 body was used in micro-CT analysis. Bone volume fraction (BV/TV, %) and bone density were estimated by defining the bone tissue (including cortical and cancellous bones) and total tissue on the density histogram. Bone density was assessed in G-value and converted into the BMD ( $\text{g}/\text{cm}^3$ ) using a linear regression equation formulated by measuring five hydroxyapatite standards of different mineral densities. The mean cortical width (Ct.Wi) was determined with the aid of at least five vectors drawn on the dorsal aspect of the vertebral body, cut sagittally (Fig. 2A).

To conduct a 3D analysis of the trabecular structure, a rectangle ( $3.6 \times 1.1 \times 1.1$  mm) was placed into the vertebral body so that only trabecular bone was included within it. Trabecular number (Tb.N), number

of trabecular nodes (N.Nd), mean trabecular separation (Tb.Sp), mean trabecular junctions at one node (Tb.N/Nd), and trabecular thickness (Tb.Th) (Bouxsein et al., 2010) were assessed within the rectangle (Fig. 2B).

### 2.4. Ashing

The second vertebral body (L2) was ashed in a muffle oven at 750 °C for 30 min. The L2 was weighed before and after ashing to the nearest 0.000001 g, and mineral content was determined by the ash weight. Organic content was calculated as the difference between the wet tissue weight and the ash weight. Organic content and mineral content were expressed relative to the wet weight of each vertebra (%).

Calcium content was assessed by atomic absorption spectrometer (4100; PerkinElmer, Burladingen, Germany) according to The European Committee for Standardization (CEN, French: Comité Européen de Normalisation) (CEN, 2002). Orthophosphate content was determined using the colorimetric method (DM4 spectral photometer; Zeiss, Germany) according to CEN (2004).

### 2.5. Expression of bone genes

Total RNA was extracted (RNeasy™ Mini Kit; Qiagen, Hilden, Germany) from the L6, which was homogenized using a micro-dismembrator (Sartorius, Goettingen, Germany). RNA quantity was assessed by a photometer (Biometra, Goettingen, Germany). RNA samples (100 ng) were then reverse-transcribed using SuperScript™ RNase H-reverse transcriptase (Promega, Mannheim, Germany). Ready-to-use primer pairs (QuantiTect® Primer Assays) and SYBR Green dye (Qiagen) were used in qRT-PCR to study the expression of rat genes, tartrate-resistant acid phosphatase (Trap), collagen type 1 alpha 1 (Coll1 $\alpha$ 1), Alp, Oc, insulin-like growth factor-1 (Igf-1), receptor activator of nuclear factor kB ligand (Rankl), and osteoprotegerin (Opg). qRT-PCR was performed using an iCycler (CFX96; Bio-Rad Laboratories, Munich, Germany). Relative gene expression was calculated using the  $2^{-\Delta\Delta\text{CT}}$  method (Livak and Schmittgen, 2001) for each gene of interest relative to the value observed in the intact group ( $n = 10$ ), where the rats were non-Ovx, untreated, and maintained under the same conditions and of the same age and comparable BW as the treatment groups. The reference gene was  $\beta$ -2 microglobulin.

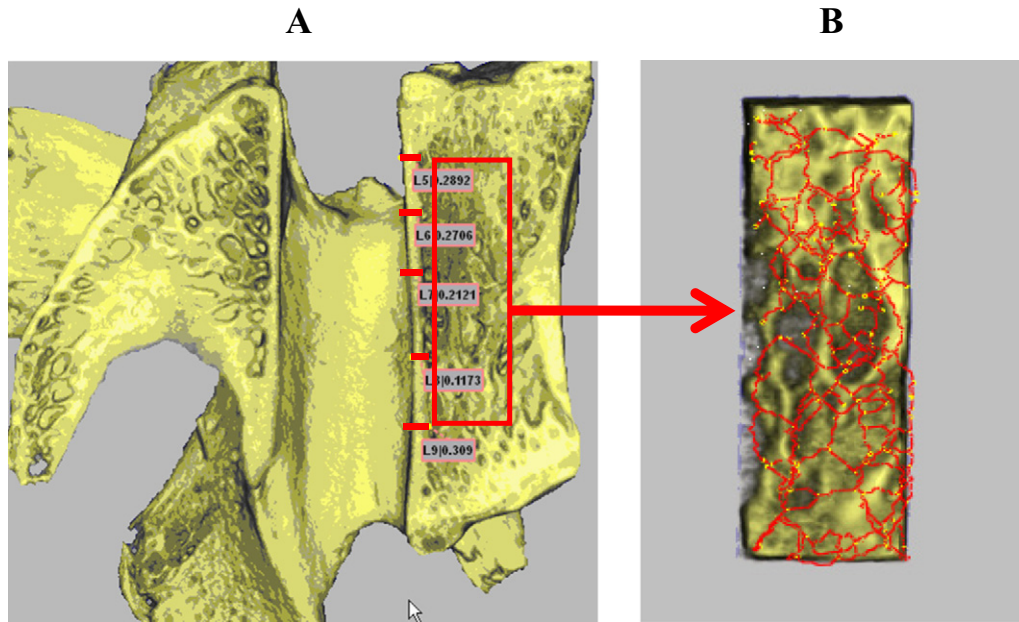
### 2.6. Statistical analyses

Statistical analyses were conducted using the SAS program (Version 9.3; SAS Institute, Cary, NC). ANOVA (F-test,  $P < 0.05$ ) was applied to reveal the impact of the treatments on the respective variables. The weight of the rats was taken as a covariate to examine its effect on the variables, and it was found that the effect of BW on bone parameters was not significant ( $P > 0.05$ , F-test). Differences between individual means were estimated using Tukey's test ( $P < 0.05$ ). The relationships among organic mass, mineral mass, and biomechanical parameters (stiffness, yield load, Fmax) were assessed by correlation analysis (Person's coefficient) using GraphPad Prism (Version 4.0; San Diego, CA). Relative gene expression was analyzed using the non-parametric Kruskal–Wallis test and Dunn's multiple comparison test in GraphPad Prism. Data are shown as mean and standard deviation (SD).

## 3. Results

### 3.1. Transmission of different vibrations

The measurements of the vibration parameters in the cage produced the following results (shown for vertical and horizontal vibrations): the mean amplitude was 0.47 mm (range, 0.46–0.48 mm) and acceleration was 1, 2, 3, and 5 g at frequencies of 30, 50, 70, and 90 Hz, respectively. The measurements taken on the backs of the rats revealed 100%



**Fig. 2.** Micro-CT analysis of L3; (A) cortical width determined with the aid of five vectors drawn on the dorsal aspect of L3 cut sagittally; rectangle placed in the middle of the vertebral body for analysis of trabecular nets; (B) rectangle with trabecular nets.

transmission of the vibration frequencies (30–90 Hz), whereas the displacement was transmitted at 17% and 19% (0.08 mm and 0.09 mm) for vertical and horizontal vibrations, respectively. Transmitted acceleration rate ranged from 0.17 to 0.37 g and 0.15 to 0.48 g for vertical and horizontal vibrations, respectively, of 30–90 Hz.

3.2. Body weight, food intake, and uterus weight

At the beginning of the experiments, the BW of the rats did not differ ( $P > 0.05$ ) among the groups (Tables 1, 2, and 3). In all three

experiments, ovariectomy caused a significant increase in BW and food intake (data not shown); the vibration treatments did not change these parameters. The weight of the uterus was significantly lower in all Ovx rats than in the Non-Ovx rats, and vibration had no effect (Tables 1, 2, and 3).

3.3. Serum analyses

In Experiments 1 and 2, when the vibration treatments were applied once a day, they had no effect on Alp or Oc serum levels. Alp levels were

**Table 1**  
Vertical WBV applied once a day for 15 min for 30 days in Ovx rats. Body weight (BW), uterus weight (UW), and results of serum, ashing, biomechanical, and micro-CT analyses (Exp. 1).

Parameters	Non-Ovx		Ovx		35 Hz-v-1 ×/d		50 Hz-v-1 ×/d		70 Hz-v-1 ×/d		90 Hz-v-1 ×/d	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Initial BW (g)	233	17	239	13	233	16	236	8	252	13	237	8
Final BW (g)	293	18	326 <sup>a</sup>	19	313 <sup>a</sup>	26	314 <sup>a</sup>	15	331 <sup>a</sup>	21	309 <sup>a</sup>	16
UW (mg)	604	192	115 <sup>a</sup>	16	105 <sup>a</sup>	20	104 <sup>a</sup>	15	101 <sup>a</sup>	17	100 <sup>a</sup>	11
<i>Serum</i>												
Alp (U/L)	64.1	11.2	83.1 <sup>a</sup>	15.0	86.1 <sup>a</sup>	12.2	79.0 <sup>a</sup>	22.1	83.5 <sup>a</sup>	18.7	83.2 <sup>a</sup>	16.7
Oc (µg/L)	20.1	3.8	23.0	3.6	20.6	4.1	20.2	3.7	20.4	4.2	19.9	3.3
<i>Ashing</i>												
Organic content (%)	61.9	3.1	65.5 <sup>a</sup>	2.3	67.6 <sup>a</sup>	4.1	66.5 <sup>a</sup>	2.4	65.8 <sup>a</sup>	2.0	66.5 <sup>a</sup>	2.0
Mineral content (%)	38.1	3.1	34.5 <sup>a</sup>	2.3	32.4 <sup>a</sup>	4.1	33.5 <sup>a</sup>	2.4	34.2 <sup>a</sup>	2.0	33.5 <sup>a</sup>	2.0
Ca <sup>2+</sup> /PO <sub>4</sub> <sup>3-</sup>	1.58	0.11	1.59	0.08	1.58 <sup>c</sup>	0.05	1.65	0.10	1.73 <sup>a,b</sup>	0.05	1.69 <sup>a,b</sup>	0.05
<i>Biomechanics</i>												
Stiffness (N/mm)	147	29	108 <sup>a</sup>	19	117 <sup>a</sup>	20	115 <sup>a</sup>	28	112 <sup>a</sup>	24	114 <sup>a</sup>	26
Yield load (N)	252	26	172 <sup>a</sup>	30	176 <sup>a</sup>	22	174 <sup>a</sup>	34	173 <sup>a</sup>	22	173 <sup>a</sup>	19
Fmax (N)	256	24	174 <sup>a</sup>	30	177 <sup>a</sup>	22	175 <sup>a</sup>	34	174 <sup>a</sup>	22	175 <sup>a</sup>	18
<i>Micro-CT</i>												
BMD (g/cm <sup>3</sup> )	1.051	0.055	0.952 <sup>a</sup>	0.065	0.980	0.042	0.945 <sup>a</sup>	0.078	0.946 <sup>a</sup>	0.089	0.970 <sup>a</sup>	0.069
BV/TV (%)	44.93	1.83	35.40 <sup>a</sup>	3.33	39.43 <sup>a,b</sup>	3.74	38.50 <sup>a</sup>	3.70	39.71 <sup>a,b</sup>	3.83	38.47 <sup>a</sup>	4.27
Tb.N	294	31	196 <sup>a</sup>	27	222 <sup>a</sup>	41	211 <sup>a</sup>	28	223 <sup>a</sup>	27	213 <sup>a</sup>	26
N.Nd	352	34	228 <sup>a</sup>	34	265 <sup>a</sup>	56	241 <sup>a</sup>	29	259 <sup>a</sup>	27	247 <sup>a</sup>	34
Tb.Sp (mm)	0.212	0.013	0.233 <sup>a</sup>	0.011	0.232 <sup>a</sup>	0.019	0.224	0.011	0.226	0.013	0.231 <sup>a</sup>	0.016
Tb.Th (mm) add	0.043	0.012	0.056	0.017	0.059	0.017	0.062	0.016	0.054	0.017	0.053	0.013
Tb.N/Nd	2.35	0.08	2.28	0.09	2.33	0.11	2.24 <sup>a</sup>	0.08	2.28	0.10	2.26	0.09
Ct.Wi (mm)	0.289	0.036	0.304	0.028	0.317	0.040	0.310	0.044	0.317	0.039	0.309	0.036

<sup>a</sup> Differs from Non-Ovx.  
<sup>b</sup> Differs from Ovx.  
<sup>c</sup> Differs from 70 and 90 Hz-v-1 ×/d.

**Table 2**  
Horizontal (h) WBV applied once a day for 15 min for 30 days in Ovx rats. Body weight (BW), uterus weight (UW), and results of serum, ashing, biomechanical and micro-CT analyses (Exp. 2).

Parameters	Non-Ovx		Ovx		30 Hz-h-1×/d		50 Hz-h-1×/d		70 Hz-h-1×/d		90 Hz-h-1×/d	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Initial BW (g)	239	12	247	15	242	13	237	10	231	6	247	10
Final BW (g)	275	16	333 <sup>a</sup>	15	317 <sup>a</sup>	23	323 <sup>a</sup>	15	313 <sup>a</sup>	14	317 <sup>a</sup>	14
UW (mg)	550	131	119 <sup>a</sup>	17	116 <sup>a</sup>	19	116 <sup>a</sup>	18	116 <sup>a</sup>	18	126 <sup>a</sup>	13
<i>Serum</i>												
Alp (U/L)	54.1	9.3	72.5 <sup>a</sup>	9.2	78.1 <sup>a</sup>	33.0	73.1 <sup>a</sup>	12.7	76.4 <sup>a</sup>	30.8	72.8 <sup>a</sup>	25.2
Oc (µg/L)	16.3	2.8	18.3	3.0	15.8	3.2	15.8	2.7	16.6	2.7	14.7	13.1
<i>Ashing</i>												
Organic content (%)	63.7	3.6	66.4	2.4	67.4	5.0	68.4 <sup>a</sup>	3.0	67.0	3.3	67.1	2.7
Mineral content (%)	36.3	3.6	33.6	2.4	32.6	5.0	31.7 <sup>a,b,c</sup>	3.0	33.0	3.3	32.9	2.7
Ca <sup>2+</sup> /PO <sub>4</sub> <sup>3-</sup>	1.72	0.06	1.65	0.04	1.47 <sup>a,b,c</sup>	0.02	1.51 <sup>a,b,c</sup>	0.07	1.61 <sup>a</sup>	0.03	1.60 <sup>a</sup>	0.01
<i>Biomechanics</i>												
Stiffness (N/mm)	129	29	99 <sup>a</sup>	19	98 <sup>a</sup>	18	87 <sup>a</sup>	5	89 <sup>a</sup>	13	88 <sup>a</sup>	13
Yield load (N)	214	31	181 <sup>a</sup>	38	169 <sup>a</sup>	47	161 <sup>a</sup>	18	181 <sup>a</sup>	22	172 <sup>a</sup>	38
Fmax (N)	233	36	191 <sup>a</sup>	38	182 <sup>a</sup>	46	171 <sup>a</sup>	20	185 <sup>a</sup>	22	178 <sup>a</sup>	32
<i>Micro-CT</i>												
BMD (g/cm <sup>3</sup> )	1.024	0.063	0.931 <sup>a</sup>	0.046	0.892 <sup>a</sup>	0.100	0.949 <sup>a</sup>	0.040	0.931 <sup>a</sup>	0.037	0.933 <sup>a</sup>	0.040
BV/TV (%)	43.86	3.13	36.73 <sup>a</sup>	3.33	35.79 <sup>a</sup>	2.29	38.07 <sup>a</sup>	2.34	38.46 <sup>a</sup>	2.67	37.73 <sup>a</sup>	2.84
Tb.N	277	28	221 <sup>a</sup>	24	208 <sup>a</sup>	23	221 <sup>a</sup>	35	225 <sup>a</sup>	20	220 <sup>a</sup>	24
N.Nd	339	37	262 <sup>a</sup>	33	247 <sup>a</sup>	29	266 <sup>a</sup>	44	269 <sup>a</sup>	27	262 <sup>a</sup>	34
Tb.Sp (mm)	0.216	0.009	0.230 <sup>a</sup>	0.013	0.237 <sup>a</sup>	0.009	0.224	0.015	0.224	0.011	0.231 <sup>a</sup>	0.012
Tb.Th (mm)	0.040	0.016	0.046	0.011	0.040	0.009	0.042	0.009	0.044	0.009	0.043	0.008
Tb.N/Nd	2.40	0.06	2.32 <sup>a</sup>	0.07	2.33 <sup>a</sup>	0.06	2.35	0.07	2.34	0.03	2.33 <sup>a</sup>	0.08
Ct.Wi (mm)	0.270	0.023	0.303	0.022	0.287	0.035	0.306 <sup>a</sup>	0.040	0.293	0.015	0.302	0.037

<sup>a</sup> Differs from Non-Ovx.

<sup>b</sup> Differs from Ovx.

<sup>c</sup> Differs from 70 and 90 Hz-h-1×/d.

significantly higher in all Ovx groups compared to the Non-Ovx rats in both experiments. Oc levels did not differ ( $P > 0.05$ ) among the groups (Tables 1 and 2).

In Experiment 3, Alp levels were higher in all Ovx groups compared to the Non-Ovx rats; however, the difference did not reach a significant level in the 70 Hz-v-2×/d group. Oc levels were higher in the Ovx

**Table 3**  
Vertical (v) or horizontal (h) WBV applied twice a day for 15 min for 30 days in Ovx rats. Body weight (BW), uterus weight (UW), results of serum, ashing, biomechanical and micro-CT analyses (Exp. 3).

Parameters	Non-Ovx		Ovx		35 Hz-v-2×/d		70 Hz-v-2×/d		35 Hz-h-2×/d		70 Hz-h-2×/d	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Initial BW (g)	250	13	247	14	249	12	246	11	243	13	244	10
Final BW (g)	279	9	341 <sup>a</sup>	23	341 <sup>a</sup>	20	328 <sup>a</sup>	17	338 <sup>a</sup>	23	327 <sup>a</sup>	20
UW (mg)	627	166	121 <sup>a</sup>	19	132 <sup>a</sup>	55	126 <sup>a</sup>	41	125 <sup>a</sup>	25	120 <sup>a</sup>	22
<i>Serum</i>												
Alp (U/L)	69.2	12.1	84.3 <sup>a</sup>	13.9	87.1 <sup>a</sup>	16.7	81.7	13.2	86.7 <sup>a</sup>	17.4	86.6 <sup>a</sup>	16.5
Oc (µg/L)	16.7	2.7	21.4 <sup>a</sup>	4.3	19.3	3.3	18.3	4.1	17.0 <sup>b</sup>	3.8	17.3 <sup>b</sup>	4.1
<i>Ashing</i>												
Organic content (%)	65.0	5.6	66.1	4.6	71.2 <sup>a</sup>	4.8	72.5 <sup>a,b</sup>	5.2	69.2	5.0	66.5 <sup>c</sup>	4.8
Mineral content (%)	35.0	5.6	33.9	4.6	28.8 <sup>a</sup>	4.8	27.5 <sup>a,b</sup>	5.2	30.8	5.0	33.5 <sup>c</sup>	4.8
Ca <sup>2+</sup> /PO <sub>4</sub> <sup>3-</sup>	1.58	0.10	1.59	0.08	1.57	0.05	1.58	0.05	1.58	0.05	1.61	0.07
<i>Biomechanics</i>												
Stiffness (N/mm)	126	29	96 <sup>a</sup>	16	107	40	102	21	100	26	105	19
Yield load (N)	240	34	188 <sup>a</sup>	43	197	48	192 <sup>a</sup>	40	187 <sup>a</sup>	23	176 <sup>a</sup>	47
Fmax (N)	255	26	194 <sup>a</sup>	46	206 <sup>a</sup>	52	198 <sup>a</sup>	37	190 <sup>a</sup>	25	187 <sup>a</sup>	40
<i>Micro-CT</i>												
BMD (g/cm <sup>3</sup> )	1.404	0.046	1.320 <sup>a</sup>	0.050	1.334 <sup>a</sup>	0.053	1.326 <sup>a</sup>	0.058	1.326 <sup>a</sup>	0.032	1.322 <sup>a</sup>	0.042
BV/TV (%)	69.30	12.69	49.81 <sup>a</sup>	11.72	53.93 <sup>a</sup>	16.07	48.66 <sup>a</sup>	11.19	43.40 <sup>a</sup>	13.72	51.63 <sup>a</sup>	12.34
Tb.N	212	49	203	25	199	26	183	39	180	45	211	34
N.Nd	254	61	241	36	235	34	215	45	213	63	251	40
Tb.Sp (mm)	0.237	0.022	0.225	0.022	0.225	0.011	0.225	0.015	0.230	0.019	0.219	0.011
Tb.Th (mm)	0.069	0.039	0.025 <sup>a</sup>	0.010	0.032 <sup>a</sup>	0.014	0.027 <sup>a</sup>	0.008	0.021 <sup>a</sup>	0.009	0.028 <sup>a</sup>	0.012
Tb.N/Nd	2.35	0.09	2.33	0.10	2.32	0.03	2.31	0.06	2.28	0.21	2.33	0.07
Ct.Wi (mm)	0.239	0.026	0.247	0.024	0.240	0.033	0.230	0.038	0.247	0.035	0.226	0.034

<sup>a</sup> Differs from Non-Ovx.

<sup>b</sup> Differs from Ovx.

<sup>c</sup> Differs from 70 Hz-v-2×/d.

non-treated group compared to the Non-Ovx and 35 and 70 Hz-h-2×/d groups (Table 3).

3.4. Biomechanical analysis

Vibration applied once a day had no effect on biomechanical parameters in Experiments 1 and 2 (Tables 1 and 2). The Non-Ovx rats had significantly higher stiffness, yield load, and Fmax levels compared to all Ovx rats, including the WBV rats. In Experiment 3, significantly lower stiffness levels were observed in the non-treated Ovx group, whereas those of the WBV Ovx rats did not differ from those of the Non-Ovx rats (Table 3). Yield loads were lower in all Ovx rats compared to the Non-Ovx rats, with the exception of the 35 Hz-v-2×/d group (Table 3). Fmax levels were significantly lower in all Ovx rats compared to the Non-Ovx rats (Table 3).

3.5. Micro-CT

In Experiment 1, the analysis revealed significantly lower total BMD, Tb.N, and N.Nd in all Ovx rats compared to the Non-Ovx rats (Table 1). The exception was BMD in the 35 Hz-v-1×/d group, which did not differ from that of the other groups. BV/TV was higher in the 35 Hz- and 70 Hz-v-1×/d groups compared to the Ovx non-treated group, but it was still lower than in the Non-Ovx rats. Trabecular separation values in the 50- and 70 Hz-h-vibrated rats, did not differ from those of the other groups. Tb.N/Nd was significantly lower in the 50 Hz-v-1×/d group compared to that of the Non-Ovx group. Tb.Th and Ct.Wi were not affected by ovariectomy or by vibration treatments (Table 1).

In Experiment 2, total BMD, BV/TV, Tb.N, and N.Nd were lower in all Ovx groups compared to the Non-Ovx group (Table 2). Tb.Sp in the 50 Hz- and 70 Hz-h-1×/d rats did not differ from those of the other groups. Tb.Th did not differ among the groups. Ct.Wi was thicker in the 50 Hz-h-1×/d group compared to the Non-Ovx group (Table 2).

In Experiment 3, total BMD, BV/TV, and Tb.Th were lower in all Ovx groups, regardless of the vibration treatments (Table 3). Other trabecular and cortical parameters did not differ among the groups (P > 0.05).

3.6. Ashing

In Experiment 1, all Ovx rats had significantly lower mineral content and higher organic content compared to the Non-Ovx rats. The molar ratio of calcium to orthophosphate was higher in the 70- and 90 Hz-vibrated rats than in the Non-Ovx, non-treated Ovx, and 35 Hz-v-1×/d groups (Table 1).

In Experiment 2, mineral content of the vertebral body was lower and organic content was correspondingly higher in the 50 Hz-vibrated rats than in the Non-Ovx rats. Ca<sup>2+</sup>/PO<sub>4</sub><sup>3-</sup> ratio was lower in all of the vibration treatment groups and the lowest in the 30- and 50-Hz vibrated groups compared to other groups (Table 2).

In Experiment 3, v-vibration applied two times per day diminished the mineral content, whereas h-vibrations had less of an effect. Organic content was higher in the v-vibrated groups. No differences in Ca<sup>2+</sup>/PO<sub>4</sub><sup>3-</sup> ratio were observed among the treatment groups (Table 3).

Correlation analyses of ashing and biomechanical parameters revealed positive relationships between mineral content and biomechanical parameters in all experiments (Table 4). Organic content correlated negatively with stiffness, yield load, and Fmax. In Experiment 3,

the relationships between yield load and organic and mineral contents were not significant (Table 4).

3.7. Expression of bone genes

In Experiment 1, the expression of the Coll1α1 gene was upregulated in all Ovx groups, reaching a significant level in the 70 Hz-v-1×/d group (Fig. 3). The Rankl gene was downregulated in the 90 Hz-v-1×/d group compared to the non-treated Ovx group, whereas the Opg gene did not differ among the groups (Fig. 3). No significant differences in Alp, Trap, Oc, or Igf-1 gene expression were detected (data not shown).

In Experiment 2, Coll1α1 gene expression was significantly higher after 30 and 70 Hz of h-vibrations compared with the Non-Ovx rats (Fig. 3). The expression of the Opg gene was lower in the 90 Hz-vibrated group compared to the Non-Ovx group. No significant differences in Rankl, Alp, Trap, Oc, or Igf-1 gene expression were detected among the groups (data not shown).

In Experiment 3, no significant differences in the expression of bone formation (Opg, Coll1α1, Alp, Oc, Igf-1) or resorption (Trap, Rankl) markers were found (Fig. 3; other data not shown).

4. Discussion

In the present study, the effectiveness of horizontal and vertical WBV in a wide range of frequencies and two different application regimes (1× or 2×/d) in the amelioration of osteopenic spine properties was compared. Transmission of the vibration displacement and magnitude while the rats were standing on four limbs was below 20%, whereas frequency was transmitted at 100%. Vibration transmissibility during bipedal stance of rats has been reported to be around 70% (Holguin et al., 2011). Stimulation of rat backs while in the quadrupedal stance, as in the present study, with high-frequency, low-magnitude vibrations have been found to be favorable for bone tissue (Holguin et al., 2011; Judex et al., 2007).

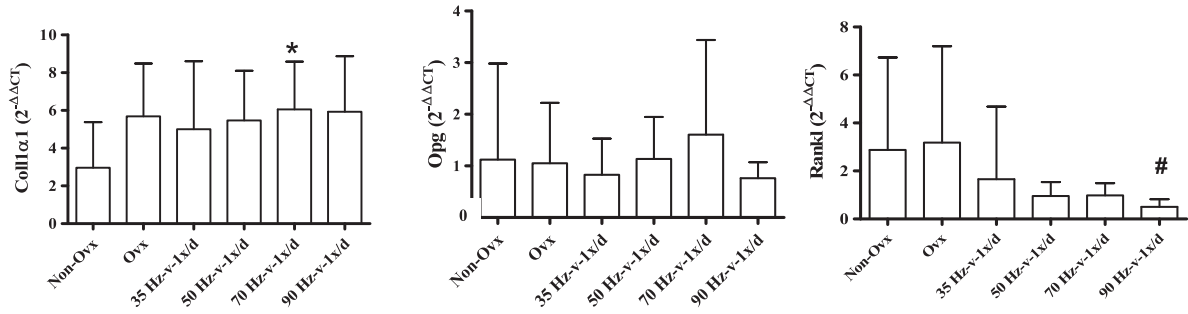
In the present study, the lumbar spine was found to be sensitive to ovariectomy, whereas the effects of the vibration treatments were not always clearly distinct. The biomechanical test showed an improvement in the stiffness of the vertebral body after all vibration treatments were applied twice a day, compared with the Non-Ovx group (Exp. 3). The elastic deformation phase of the compression test was assessed by stiffness, and the end of the elastic phase was evaluated by yield load, which was also improved after the 35 Hz-v-2×/d vibration treatment. However, the vibration treatments did not counter the effects of ovariectomy, and the biomechanical parameters did not differ from those of the non-treated Ovx group. Exposure to the vibration treatments once a day did not change the biomechanical properties of the bone.

The relationships among mineral content, organic content, and biomechanical parameters have been investigated intensively (Currey, 1999; Vuong and Hellmich, 2011). Changes in mineral content can have a pronounced effect on bone biomechanics. In the present study, mineral content and organic content correlated with stiffness, yield load, and Fmax of the vertebral body. Organic content was generally higher and mineral content was lower in the Ovx animals than in the Non-Ovx rats. The effect of vibration treatment on mineral content was only seen in the 2×/d-v-vibrated animals, in which it was diminished. In contrast, the Ca<sup>2+</sup>/PO<sub>4</sub><sup>3-</sup> ratio changed significantly after v- and h-vibrations were applied once a day, and the effects of v- and h-vibrations

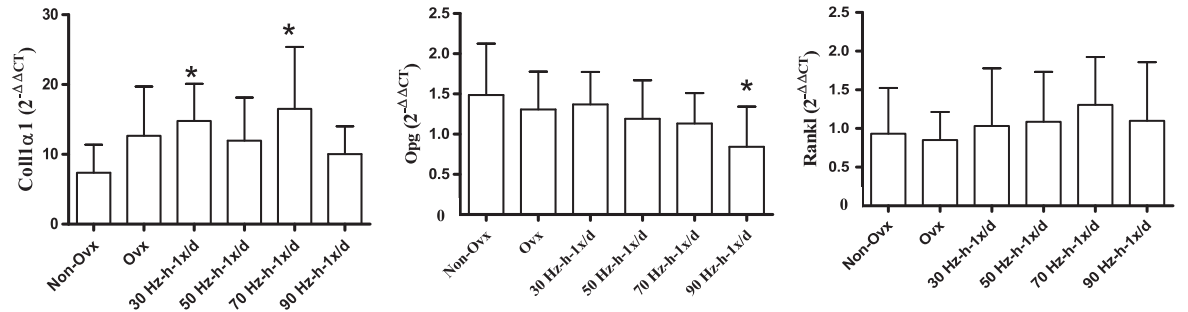
**Table 4**  
Correlations of ashing parameters (organic content [OC] and mineral content [MC]) and biomechanical properties assessed by Pearson's coefficient (r), two-tailed p-value.

Correlations	OC-stiffness		OC-yield load		OC-Fmax		MC-stiffness		MC-yield load		MC-Fmax	
	r	p	r	p	r	p	r	p	r	p	r	p
Exp. 1 (n = 85)	-0.297	0.006	-0.409	0.0001	-0.413	<0.0001	0.297	0.006	0.409	0.0001	0.413	<0.0001
Exp. 2 (n = 84)	-0.260	0.017	-0.312	0.004	-0.319	0.003	0.260	0.017	0.312	0.004	0.319	0.003
Exp. 3 (n = 79)	-0.427	<0.0001	-0.211	0.06	-0.225	0.04	0.427	<0.0001	0.211	0.06	0.225	0.04

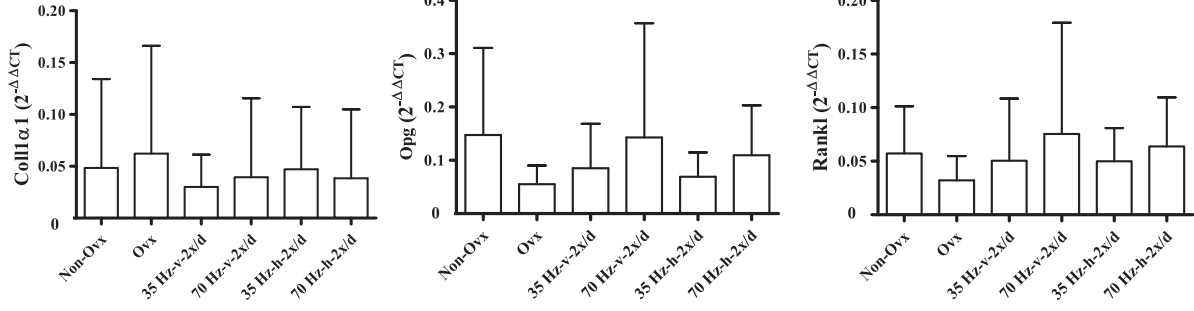
## Experiment 1



## Experiment 2



## Experiment 3



**Fig. 3.** Relative mRNA expression levels of Col1 $\alpha$ 1, Opg, and Rankl genes in Experiments 1, 2, and 3 (mean  $\pm$  SD). At least 12 replications per treatment group were conducted. (\*): differs from Non-Ovxx, (#): differs from Ovxx.

were different. The higher  $\text{Ca}^{2+}/\text{PO}_4^{3-}$  ratio after v-vibrations at high frequencies (70 and 90 Hz) supported the positive effect of vibrations on the lumbar spine, whereas reduction of the ratio after h-vibration at lower frequencies (30 and 50 Hz) seemed to worsen the effect of Ovxx. Decreased calcium and phosphorus levels have previously been reported in the femurs of Ovxx rats (Prabhakara Reddy and Lakshmana, 2003). In the present study, only a tendential decrease in the  $\text{Ca}^{2+}/\text{PO}_4^{3-}$  ratio was observed in Ovxx rats.

According to the daily stress stimulus theory of bone remodeling, daily tissue level stress stimuli are expressed as the linear sum of all daily loading events (strain, magnitude, cycle number) (Beaupr e et al., 1990). On the other hand, Qjin et al. (1998) reported a nonlinear dependence of loading intensity and cycle number in the maintenance of bone mass and morphology and suggested that frequency or strain rate must also play a critical role in the mechanisms by which bone responds to mechanical strain. In the present study, a daily 15-minute vibration at 70 Hz did not produce similar results as a 30-minute vibration (2 $\times$ /d) at 35 Hz did, even though the cycle number was 63,000 per day after both treatment regimes. It is still unknown whether the vibration stimuli induced by external devices are similar to the mechanical loading induced by physical exercises.

One of the hypotheses of the effect of WBV on bone is the activation of muscle, which mechanically loads the bone (Fritton et al., 2000; Rubin et al., 2006). Another proposed mechanism through which WBV exerts an effect on bone tissue is that WBV signals become amplified within bone tissue by stress-generated fluid flow, thus activating bone cells that act as mechanosensors (Fritton et al., 2000; Rubin et al., 2006).

Similar to the biomechanical test, the level of bone markers in serum changed only after 2 $\times$ /d vibration exposure (Exp. 3). The normalization of Oc levels was observed after h-vibration treatments in Ovxx rats. Enhanced serum Oc and Alp levels have been reported in Ovxx rats, indicating increased bone turnover due to estrogen deficiency (Kalu, 1991; Hauschka et al., 1989).

In contrast to protein markers, changes in the mRNA expression of bone markers were detected after 1 $\times$ /d vibration exposure. Decreased Opg gene expression after h-vibrations and Rankl expression after v-vibrations and increased Col1 $\alpha$ 1 gene expression were observed. As stated previously, gene expression did not correspond directly to protein synthesis levels (Vogel and Marcotte, 2012). In the present study, bone marker measurements were limited to one point in time, and it is unknown whether the changes in serum were predetermined earlier by the changes in gene expression. It can be suggested that the response

of bone to 15-min vibrations applied once a day occurred later, and therefore, the changes were still detectable at the mRNA level.

Micro-CT analysis is a modern method of bone analysis (Brouwers et al., 2010) that has many advantages compared to histological analyses. A previous study demonstrated that biomechanical parameters of the lumbar vertebral body were correlated with histomorphological bone parameters (Sehmisch et al., 2009b). In that study, Fmax was influenced by both cortical and trabecular thickness, whereas stiffness and yield load were influenced mainly by trabecular bone properties (Sehmisch et al., 2009b). In the present study, the biomechanical properties, BMD and BV/TV of the vertebral body, were diminished in Ovx rats, which correspond with the significant bone loss reported after Ovx in rats (Kalu, 1991; Komrakova et al., 2010). Ovx rats develop osteopenic changes within a few weeks after surgery (Kalu, 1991). In the present study, success of Ovx was confirmed by atrophied uteri. The WBV treatments improved BV/TV only in the 35- and 70 Hz-v-1×/d groups (Exp. 1), confirming the different effects of vibration types and application regimes.

Another implication proposed for WBV is its use to promote weight loss or decrease fat mass. A previous study reported that 12-week WBV reduced age-related increases in body fat accumulation and BW without affecting food consumption in female rats (Maddalozzo et al., 2008). In contrast, vibration for eight weeks in male rats caused an increase in BW (Naghii et al., 2011). In the present study, 30-day vibrations did not change the BW or food consumption of the Ovx rats. Thus, the increase in BW and food intake in rats, which is a well-known phenomenon observed after ovariectomy (Kalu, 1991; Komrakova et al., 2010), was not reversed by the vibration treatments. However, this finding also indicates that the vibration treatments were not invasive and well tolerated by the rats.

V-vibrations at lower frequencies (35 to 50 Hz) have been found to be most favorable for the extremities, whereas h-vibrations have shown negative effects, regardless of the vibration frequency (Komrakova et al., 2013). In the present study, the effect of vibrations on the lumbar spine was ambiguous. V-vibrations applied once a day affected BV/TV and  $\text{Ca}^{2+}/\text{PO}_4^{3-}$  favorably and decreased Rankl gene expression. When applied twice a day, v-vibrations diminished mineral content. H-vibrations (1×/d) reduced  $\text{Ca}^{2+}/\text{PO}_4^{3-}$  ratio and Opg mRNA level, whereas h-vibrations (2×/d) normalized serum OC levels. Many of the other measured parameters did not show any significant differences between the vibrated groups and the non-treated Ovx group.

Experimental and clinical studies that have evaluated WBV as ineffective are rare (Brouwers et al., 2010; Prisby et al., 2008; Merriman and Jackson, 2009; Abercromby et al., 2007). The latest reviews have shown that vibration treatments mostly affect leg muscles and have less of an effect on bone in women (Lau et al., 2011; Prisby et al., 2008; Merriman and Jackson, 2009). This finding should primarily result in increased mobility and a decrease in accidental falls in elderly people.

Vibration treatments in human studies have been applied one, three, and five times per week (Slatkovska et al., 2010). The findings of the present study indicate that changing the application frequency and the direction of vibration might influence spine response differently. However, we were unable to find any clearly beneficial or harmful effects of vibration regimes applied to the osteopenic lumbar spine.

The limitation of the present study is that it was conducted on rats, despite the differences in physiology and skeleton between rats and humans (quadruped vs. biped, parallel vs. perpendicular spine position, lack of Haversian remodeling etc.). In addition, all of the rats underwent tibia osteotomy. Although we observed in our preliminary experiments (unpublished data) that the spine properties were not influenced by osteotomy, we are unaware of whether the effects of the vibration interacted with osteotomy in these rats. A further limitation is the absence of histomorphometric analyses that can provide additional data on bone formation and resorption.

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