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A time course of bone response to jump exercise in C57BL/6J mice

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Abstract Exercise, by way of mechanical loading, provides a physiological stimulus to which bone tissue adapts by increased bone formation. The mechanical stimulus due to physical activity depends on both the magnitude and the duration of the exercise. Earlier studies have demonstrated that jump training for 4 weeks produces a significant bone formation response in C57BL/6J mice. An early time point with significant increase in bone formation response would be helpful in: (1) designing genetic quantitative trait loci (QTL) studies to investigate genes regulating the bone adaptive response to mechanical stimulus; and (2) mechanistic studies to investigate early stimulus to bone tissue. Consequently, we investigated the bone structural response after 2, 3, and 4 weeks of exercise with a loading cycle of ten jumps a day. We used biochemical markers and peripheral quantitative computed tomography (pQCT) of excised femur to measure bone density, bone mineral content (BMC), and area. Four-week-old mice were separated into control ($n = 6$) and jump groups ($n = 6$), and the latter groups of mice were subjected to jump exercise of 2-week, 3-week, and 4-week duration. Data (pQCT) from a mid-diaphyseal slice were used to compare bone formation parameters between exercise and control groups, and between different time points. There was no statistically significant change in bone response after 2 weeks of jump exercise as compared with the age-matched controls. After 3 weeks of jump exercise, the periosteal circumference, which is the most efficient means of measuring adaptation to exercise, was increased by 3% ($P < 0.05$), and total and cortical area were increased by 6% ($P < 0.05$) and 11% ($P < 0.01$), respectively. Total bone mineral density (BMD) increased by 11% ($P < 0.01$). The biggest changes were observed in cortical and total BMC, with the increase in total BMC being 12% ($P < 0.01$). Interestingly, the increase in BMC was observed throughout the length of the femur and was not confined to the mid-diaphysis. Consistent with earlier studies, mid-femur bone mass and area remained significantly elevated in the 4-week exercise group when compared with the control group of mice. The levels of the biochemical markers osteocalcin, skeletal alkaline phosphatase, and C-telopeptide were not significantly different between the exercise and control groups, indicating the absence of any systemic response due to the exercise. We conclude that a shorter exercise regimen, of 3 weeks, induced a bone response that was greater than or equal to that of 4 weeks of jump exercise reported earlier.

Key words mechanical loading · bone remodeling · inbred C57BL · time course · bone density · jump exercise · femur

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Introduction

Physical activity or mechanical loading plays an important role in determining peak bone density. Physical activity creates loads on bone that have the potential to initiate bone formation and increase bone density [1–7]. Conversely, lack of mechanical loads results in rapid bone loss, as seen in immobilization studies [8–10]. A minimum effective strain (MES) [11–14], which is above average daily levels, is required to generate signals that are communicated to cells in bone tissue for adaptive bone formation through modeling. Physical activity levels above this MES can stimulate adaptive bone formation. Animal studies of bone adaptation have shown that mechanical regulation of bone due to physical activity is dependent on a combination of factors, including: (1) strain distribution; (2) strain magnitude; (3) number of repetitions; and (4) frequency [11–14].

We have previously reported [1] that mechanical loading, in the form of jump exercise, increases periosteal bone formation and bone strength in C57BL/6J mice, but not in C3H/HeJ mice. We used jump training as a model of exercise instead of treadmill exercise because jump exercise was found to be more effective in increasing limb bone mass in rodents [15]. In a previous study, 20 jumps/day for 4 weeks, which produced a bending strain of 176 N/mm^2 [1], was effective in gener-

ating the signal for adaptive bone response through modeling. The main aim of this study was to explore changes in femoral structural parameters after 2, 3, and 4 weeks of jump exercise. Data on the earliest bone response could provide useful information for mechanistic studies at the cellular level to investigate the initial stimulus to which bone tissue adapts by increased bone formation. In addition, an earlier time point than 4-week loading, if such elicited a significant bone formation response, would save time and effort in our genetic quantitative trait loci (QTL) studies to investigate genes regulating the bone adaptive response to mechanical stimulus. The secondary objective of this study was to evaluate the response to jump exercise by: (1) using peripheral quantitative computed tomography (pQCT) to measure changes in bone density, mass, and area; and (2) using biochemical markers to study systemic changes in bone metabolism. The pQCT would offer a less cumbersome and more precise tool to study bone response to loading as compared with bone histomorphometric measurements, which were used in an earlier study (1).

Materials and methods

Animals and treatment

All animal protocols used in this study had prior approval of the Animal Subjects Committee at the Chukyo University Graduate School of Health and Sport Sciences.

Four-week old C57BL/6J mice were obtained from Japan SLC (Hamamatsu, Japan) and acclimatized for 1 week on a 12-h light/dark cycle, with food (standard chow) and water available ad libitum. Mice were randomly assigned to either the jump-exercise or the non-exercised control groups (n = 6–7 mice/group). Details of the jump-exercise protocol have been described in the previous report [1]. In brief, each mouse in the jump groups was placed at the bottom of a special cage, 10 cm wide, 10 cm deep, and 25 cm high. The jumping exercise was initiated by applying an electrical current (80 volts) to the wire floor of the cage. Each mouse was housed individually and jumped from the floor of the cage to catch the top edge of the cage with its forepaws. The mouse was returned to the floor of the cage to repeat the procedure. The electrical current we used had an automatic turn-on phase and turn-off phase. We placed the mice on the stimulus plate at the turn-off phase and, after the first 3 days, most of the mice could jump before the turn-on phase. Each mouse in the three jump groups jumped ten times per day, 5 days a week, for 2, 3, and 4 weeks. The control mice were largely unstressed.

At the end of the experiment, blood was collected and femurs were dissected from both hind limbs. Serum was separated and kept at −70°C until analyzed for biochemical markers. Femurs were kept moist and frozen at −70°C. Frozen serum and femurs were analyzed at the J.L. Pettis Memorial Veterans Medical Center for biochemical analysis and bone density measurements, using peripheral quantitative tomography (pQCT).

Peripheral quantitative computed tomography (pQCT)

Bone density, mass, and area data were obtained from excised femurs (n = 6), and values were expressed as means ± SD. Two different thresholds were used to analyze the pQCT scans. The high threshold analysis gives the most accurate area results. The low threshold gives the most accurate mineral content analysis. The bones were scanned nine times, covering the entire length of the femur with each slice separated by 11% of the femur length. Slice 5, which is the mid-shaft slice, was used for all calculations and comparisons. Data from individual slices were used to plot total and cortical bone mineral content (BMC) (Fig. 1). Data from individual slices were compared between jump and control groups of mice by analysis of variance (ANOVA) and post-hoc test. The precision of the repeated pQCT measurement at the mid-diaphyseal femur showed a coefficient of variation (CV) of less than 3% for all the parameters studied in this investigation.

Measurements of biochemical markers of bone turnover

Mouse C-telopeptide enzyme-linked immunosorbent assay (ELISA)

The C-telopeptide measurements were performed with a mouse C-telopeptide ELISA, described earlier [16]. The sensitivity of the ELISA was 0.3 ng/ml. The average within-assay CV was less than 7%; the average between-assay CV was less than 14%.

Mouse osteocalcin radioimmunoassay (RIA)

The osteocalcin measurements were performed with a mouse osteocalcin radioimmunoassay (RIA), described earlier [17]. The sensitivity of the RIA was 15 ng/ml. The average within-assay CV was less than 10%; the average between-assay CV was less than 15%.

Skeletal alkaline phosphatase assay

Alkaline phosphatase was measured in serum by a kinetic method [7], using *p*-nitro-phenylphosphate (PNPP) as substrate and 15 mM L-phenylalanine to inhibit intestinal alkaline phosphatase. The L-phenylalanine inhibition assay exhibited intraassay (n = 10) and interassay (n = 8) CVs of 1.9% and 3.8%, respectively. The assay can detect less than 10 mU/ml of alkaline phosphatase in mouse serum.

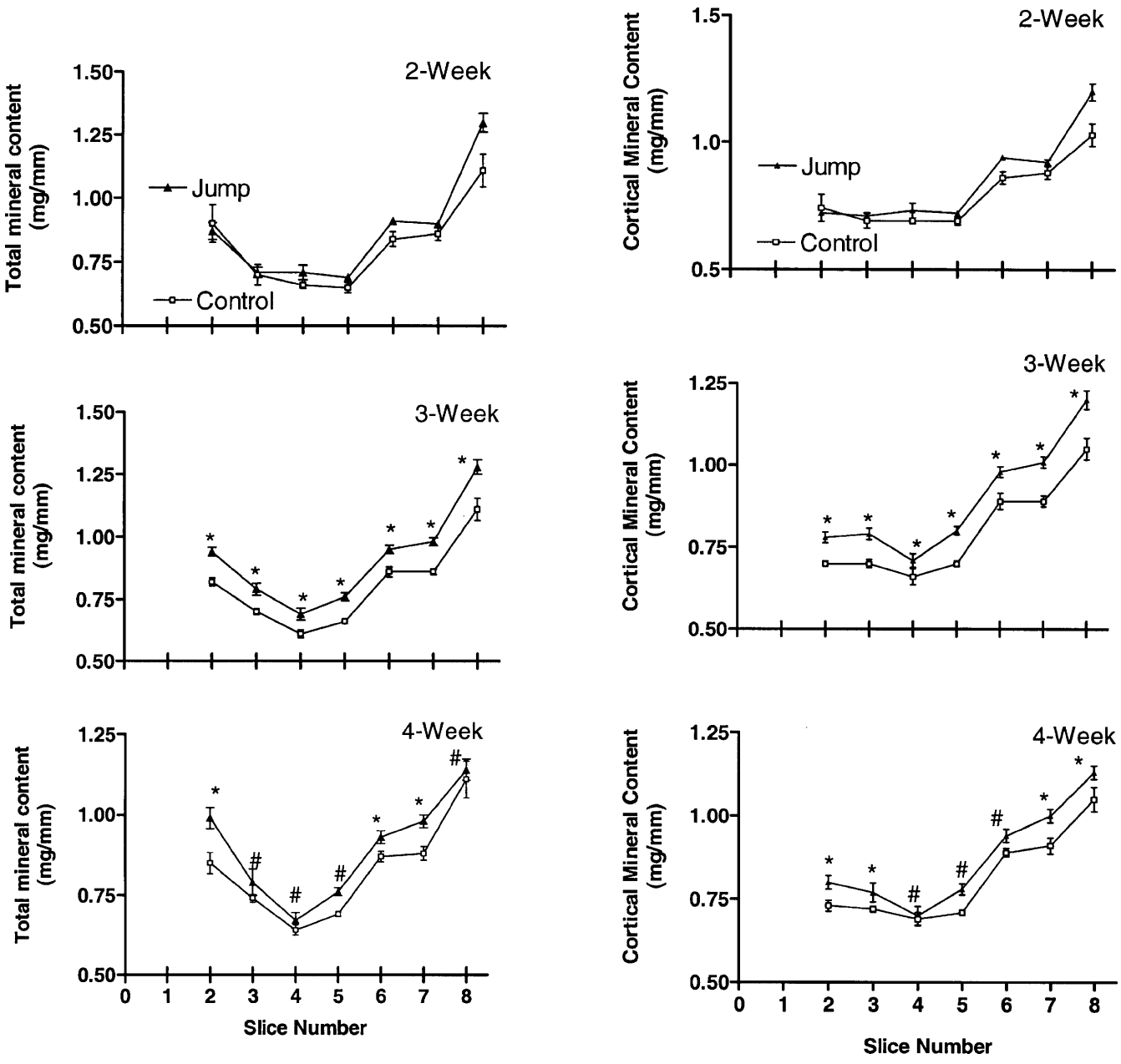

Fig. 1. Effects of 2, 3, and 4 weeks of jump exercise on cortical and total bone mineral content measured by peripheral quantitative computed tomography (pQCT) at nine different slices of the femur. Each slice was separated by 11% of the femur length, with slice 1 starting at the distal end. The data show (slices 1 and 9 were omitted due to large variation) that the increase in bone mineral content occurred throughout the length of the femur. Each data point represents the mean ± SEM ($n = 6$). The P value for both cortical and total bone mineral content of control vs jump group was <0.0001 by analysis of variance (ANOVA) for the 3-week and 4-week jump-exercise groups. The P values for comparison of individual slices in control and jump groups of mice by post-hoc tests are $^{\#}P > 0.05$ and $^{*}P < 0.05$

Statistical analysis

Comparison of biochemical markers of bone metabolism between different groups was performed by the Mann-Whitney test (with a two-tailed P-value of <0.05 accepted as significant difference), because biochemical markers do not show normal distribution. All comparisons of pQCT femur structural data were performed on slice 5 by analysis of variance (ANOVA). ANOVA was used to reveal the differences among the control and the three jump-exercise groups. Post-hoc comparisons were performed by the Neuman-Keuls method and were used to determine the differences between specific means. All data are reported as means and standard deviations.

Results

As compared with the control group of mice, mice in the jump-exercise groups after 2, 3, and 4 weeks of exercise had no significant differences in body weight or longitudinal growth (length) of the femur, which was consistent with our previous findings [1]. The body weights were: 2-week control, 16.9 ± 1.1 g; 2-week jump group, 16.6 ± 0.7 g; 3-week control, 17.9 ± 1.1 g; 3-week jump group, 18.2 ± 0.5 g; 4-week control, 17.9 ± 1.0 g; and 4-week jump group, 17.5 ± 0.9 g. The femur lengths were: 2-week control, 13.7 ± 0.75 mm; 2-week jump group, 14.1 ± 0.18 mm; 3-week control, 14.4 ± 0.41 mm; 3-week jump group, 14.3 ± 0.19 mm; 4-week control, 14.0 ± 0.61; and 4-week jump group, 14.0 ± 0.20 mm.

Changes in mid-femur periosteal expansion and cross-sectional parameters are shown in Table 1. Periosteal circumference was increased by 3% ($P < 0.05$) after 3 weeks of exercise and remained higher in the 4 week exercise group (Table 1). Cortical area was increased by 11% ($P < 0.001$) after 3 weeks of exercise and was 8.4% higher than that in the controls in the 4-week exercise group ($P < 0.01$). Total area also increased, by 6.2% ($P < 0.01$), after 3 weeks of exercise, and remained elevated after 4 weeks. Cortical thickness was elevated by 8% ($P < 0.01$) and 5% (P, not significant) in the 3-week and 4-week exercise group, respectively. Although there was a time-related increase in endosteal circumference, the P values did not reach statistically significant levels.

Changes in total bone mineral density (BMD) and total and cortical bone mineral content (BMC) in the control and jump exercise groups are shown in Table 2. Changes for total and cortical BMC and total BMD between control and exercise groups did not reach significance for the 2-week exercise period. Maximum increases in total BMD and both total and cortical BMC were observed in the 3-week exercise group. Total BMD and total BMC were increased by 11.2% ($P < 0.01$) and 12.5% ($P < 0.01$), respectively, in the 3-week exercise group compared with the control group. Total BMC and total BMD values remained significantly elevated in the 4-week exercise group, and were 11% ($P < 0.01$) and 5% ($P < 0.05$), respectively, higher as compared with the control group, ANOVA values for the increase in total BMD and total BMC for treatment and weeks of exercise are shown in Table 3.

Figure 1 shows the plots for individual slices of total and cortical bone mineral content. The cortical and total BMC were significantly increased in the 3-week and

Table 1. Cross-sectional parameters and area of mid-shaft femur

	2-Week		3-Week		4-Week	
Parameters	Control	Jump	Control	Jump	Control	Jump
Periosteal circumference (mm)	4.23 ± 0.08	4.24 ± 0.052	4.22 ± 0.08	4.35 ± 0.083*	4.19 ± 0.04	4.33 ± 0.11*
Endosteal circumference (mm)	3.27 ± 0.06	3.23 ± 0.08	3.22 ± 0.06	3.27 ± 0.10	3.17 ± 0.06	3.25 ± 0.103
Cortical thickness (mm)	0.15 ± 0.01	0.16 ± 0.006	0.16 ± 0.003	0.17 ± 0.007**	0.16 ± 0.01	0.17 ± 0.005[a]
Total area (mm^2)	1.43 ± 0.05	1.43 ± 0.033	1.42 ± 0.05	1.50 ± 0.059**	1.40 ± 0.03	1.48 ± 0.075**
Cortical area (mm^2)	0.53 ± 0.03	0.60 ± 0.019	0.59 ± 0.013	0.66 ± 0.026**	0.59 ± 0.03	0.64 ± 0.028**

* $P < 0.05$; ** $P < 0.01$
Values are means ± SD
[a] NS, Not significant

Table 2. Bone mineral content and bone density parameters of mid-shaft femur

	2-Week		3-Week		4-Week	
Parameters	Control	Jump	Control	Jump	Control	Jump
Cortical mineral content (mg/mm)	0.69 ± 0.04	0.72 ± 0.02	0.71 ± 0.03	0.80 ± 0.02**	0.71 ± 0.014	0.78 ± 0.04*
Total mineral content (mg/mm)	0.65 ± 0.05	0.69 ± 0.03	0.68 ± 0.04	0.76 ± 0.04**	0.69 ± 0.02	0.76 ± 0.03**
Total bone mineral density (mg/cc)	409 ± 30	434 ± 6	425 ± 19	461 ± 8**	445 ± 13	465 ± 17*

* $P < 0.05$; ** $P < 0.01$
Values are means ± SD

Table 3. *P* values for analysis of variance (ANOVA) and Newman-Keuls post-hoc tests

	ANOVA Treatment	ANOVA Weeks	Post-hoc; week 2 vs week 3 or week 4	Post-hoc; week 3 vs week 4
Periosteal circumference at mid-femur (mm)	$P = 0.046$	NS	NS	NS
Total bone mineral content (mg/mm)	$P = 0.03$	$P = 0.01$	$P < 0.014$	NS
Total bone mineral density (mg/cc)	$P = 0.004$	$P = 0.03$	$P < 0.03$	NS

Table 4. Biochemical parameters in jump-exercise groups compared with control groups of mice

	2-Week		3-Week		4-Week	
Parameters	Control	Jump	Control	Jump	Control	Jump
Skeletal ALP (U/l)	152 ± 12	141 ± 34	175 ± 15	191 ± 24	164 ± 23	166 ± 23
Osteocalcin (ng/ml)	166 ± 32	168 ± 46	237 ± 60	200 ± 23	170 ± 58	141 ± 20
C-telopeptide (ng/ml)	4.4 ± 1.4	5.9 ± 1.8	5.2 ± 1.4	6.0 ± 1.7	6.2 ± 1.2	6.1 ± 1.8

Differences between control and jump groups were not significant
Values are means ± SD
ALP, Alkaline phosphatase

4-week jump groups, by ANOVA ($P < 0.0001$), as compared with the respective control groups of mice. The increases in cortical and total BMC in the 3-week jump group were statistically significant (by post-hoc test) for slices 2–8, as compared with the 3-week control group. Differences between the 4-week control and exercise groups for total BMC were not significant (by post-hoc test) for slices 3–5 and 8; similarly, differences in cortical BMC for slices 4–6 were not significant.

Although some parameters, such as cortical BMC and total BMC showed a tendency to be lower in the 4-week exercise group as compared with the 3-week exercise group, these changes were not significant by ANOVA (Table 3).

Serum levels of skeletal alkaline phosphatase, osteocalcin, and C-telopeptide are shown in Table 4. None of the biochemical markers showed any significant differences between control and jump groups of mice at any time point. Data on the 4-week group were consistent with our earlier findings [1] showing that no systemic markers were elevated after 4 weeks of jump exercise.

Discussion

The bone formation response to a mechanical loading cycle of 20 jumps/day for 4 weeks in the C57BL/6J mouse model has been well documented [1]. The elevated mid-femur bone mass and area after 4 weeks of jump exercise seen in the present study is consistent with earlier findings showing a similar response to jump exercise. However, we used a lower loading cycle, of 10 jumps per day, compared with the previous study that used a loading cycle of 20 jumps/day. We made this change because it has been recently reported that, in rats, a substantial bone formation response could be obtained in loading cycles of 10 jumps/day, and the higher loading cycles resulted in only a marginal increase in bone response [15]. The lower loading cycle used in this study will also suit the QTL studies where large-scale screening may be required.

Our results indicate that there were slight increases in cortical area and bone mineral content (BMC) after 2 weeks of exercise, as compared with the control group of mice. However, these changes did not reach statistical significance. It could be assumed that the bone mass and area in response to jump exercise of 2 weeks duration were below the detection limits of the methods (pQCT) used in this study.

After 3 weeks of jump training, periosteal circumference, which is the most efficient means of measuring adaptation to exercise, clearly showed a significant increase in mid-femur structural parameters assessed by pQCT. Changes in bone mass and area after 3-week exercise were either greater than or comparable to those in the 4-week exercise group. However, periosteal circumference, bone density, and total and cortical BMC remained elevated in the 4-week jump group when compared with the 4-week control group of mice. The 3% increase in periosteal circumference in the

4-week jump exercise group was consistent with our previous study [1], which showed about 2.2% higher periosteal perimeter after 4 weeks of jump exercise by histomorphometic analysis. Similarly, increases in cortical (8%) and total area (6%) after 4 weeks of exercise were consistent with the earlier findings observed at 4 weeks of exercise, which showed about 8% and 4% increases in cortical and total area, by histomorphometric analysis. Notably, we did not see any increase in bone mass and area after 4 weeks of jump exercise as compared with the 3-week jump-exercise group. It may be speculated that increases in bone density, mass, and area reached maximum levels after 3 weeks of loading and then the values plateaued, showing no more changes on further loading. However, in the absence of data on longer loading duration, this assumption needs confirmation.

A significant finding of this study was that the effect of jump exercise was most evident in the increase in total and cortical BMC measured by pQCT. Similar to other parameters, the highest increases in both cortical and total BMC were observed after 3 weeks of jump exercise. Our data on the cortical as well as the total BMC of nine slices (Fig. 1) indicate that the increase in BMC occurred throughout the entire length of the femur, and was not confined to the mid-shaft region of the femur. Comparisons of individual slices showed that the increases in total and cortical BMC were significant for the 3-week exercise group. In the 4-week exercise group, these differences did not reach the significance level for slices 3–6, mainly due to the larger variation in BMC in the jump group, and also because the post-hoc test used to test statistical significance involved several variables, thus increasing the stringency of the test. A less conservative test indicated that the total and cortical BMC in most of the slices were both significantly higher in the jump groups as compared with the controls. This increase in the BMC of the entire length of the femur was particularly interesting, because earlier studies explored only a small area at a specific bone site [1–3]; thus, it was not clear whether the increase in bone mass was localized in the mid-shaft region or whether was spread throughout the length of the femur. Data presented in this study (Fig. 1) amply illustrate that the relative amount of bone mineral comprising the middle 10–12 mm of the diaphysis was consistently 9%–10% higher in the jump groups of mice as compared with the control mice.

Consistent with our earlier study [1], the endosteal circumference did not change significantly at any time point, including the 4 weeks of jump exercise. In addition, we did not observe any systemic response in bone turnover markers. Therefore, biochemical markers may not be useful in detecting early bone response to mechanical loading.

In conclusion, the present investigation showed that a significant increase in bone mass and area at our sampling site occurred after 3 weeks of jump exercise, and, as expected, these parameters remained elevated after 4 weeks of jump exercise. The results of this study show that a relatively small number of jumps/day and a short duration of jump exercise can achieve a detectable bone response; therefore, longer periods of exercise may not be necessary for bone hypertrophy to develop in mice. In addition, the data presented in this study indicate that pQCT measurements can be, effectively used to quantitatively determine changes in bone mass and area in response to the mechanical loading induced by jump exercise.

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