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Original Article

Hylonome

Bone mass gained in response to external loading is preserved for several weeks following cessation of loading in 10 week C57BL/6J mice

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Abstract

Objective: Dynamic loads lead to increases in bone mass. How long these gains are maintained after cessation of loading, however, is not fully understood. **Methods:** A long term study was performed in which skeletal changes were monitored by pQCT every 2-4 weeks (wks) for a 12 wk period after application of external loading using four-point bending device on 10 wk old female C57BL/6J mice. **Results:** 2 wks of loading caused 15-40% increase in bone parameters (vBMD, cross sectional area (CSA)) and bone strength (yield load, maximum load and toughness). Positive correlations between these two parameters ($r = 0.72$ to 0.88, $p<0.05$) suggest that the changes in bone parameters induced by loading are responsible, in part, for the increase in bone strength. Once loading is terminated the bone response did not continue. The vBMD gained by loading was significant for a period of 5 wks and returned to the levels of controls at 12 wks. The CSA though declined but was still significantly elevated at 12 wks. Bone strength showed no difference between loaded and non-loaded bones at 12 wks. **Conclusion:** Our results show that external loading increased bone mass, was maintained for several weeks after termination of last loading.

Keywords: Density, Bone fate, Physical Exercise, Rehabilitation, Mice

Introduction

Mechanical loading (ML) is an effective stimulator of bone formation. Past studies using animal and human models have shown that loading increases bone formation while non-loading, such as prolonged bed rest, immobilization, and space flight, results in the increase of bone loss[1-9]. We, and others, using inbred strains of mice, have reported that ML causes increases in the volumetric bone mineral density (vBMD) when measured by peripheral quantitative computed tomography (pQCT)[2,3]. Thus, physical exercise has been used as a strategy to maintain BMD and prevent osteoporotic fractures in men and women.

Clinical studies in young and postmenopausal women subjected to treadmill exercise have shown that exercise induced benefits, such as increases in vBMD and bone mineral content (BMC), are eventually lost if exercise is ceased completely[10]. Similar data was presented by Vuori et al. when reporting that unilateral leg presses done four times a week, for 12 months increased bone mass, but returned to pre-training levels with only 3 months of retirement from exercise[11,12]. Another study involving gymnasts also showed that bone density gained by long term loading declines followed by off season (unloading)[13]. Thus, data from various independent studies in humans suggests that exercise induced bone mass benefits erode over time. Animal studies using a rat model have shown that increased femoral bone density, gained through treadmill exercise, resulted in a decreased bone formation rate after deconditioning[14,15]. However, the issue of how deconditioning affects bone mass maintenance in mechanosensitive mouse model is not well understood.

We, and others, have previously shown that compared to all other mouse strain, C57BL/6J (B6), a low bone density mouse, responds well to ML. We have reported that 2 wks of ML by four-point bending causes a 10-15% increase in tibia vBMD

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14. ABSTRACT

Objective: Dynamic loads lead to increases in bone mass. How long these gains are maintained after cessation of loading, however is not fully understood. Methods: A long term study was performed in which skeletal changes were monitored by pQCT every 2-4 weeks (wks) for a 12 wk period after application of external loading using four-point bending device on 10 wk old female C57BL/6J mice. Results: 2 wks of loading caused 15-40% increase in bone parameters (vBMD, cross sectional area (CSA)) and bone strength (yield load, maximum load and toughness). Positive correlations between these two parameters (r= 0.72 to 0.88 p<0.05) suggest that the changes in bone parameters induced by loading are responsible, in part, for the increase in bone strength. Once loading is terminated the bone response did not continue. The vBMD gained by loading was significant for a period of 5 wks and returned to the levels of controls at 12 wks. The CSA though declined but was still significantly elevated at 12 wks. Bone strength showed no difference between loaded and non-loaded bones at 12 wks. Conclusion: Our results show that external loading increased bone mass, was maintained for several weeks after termination of last loading.

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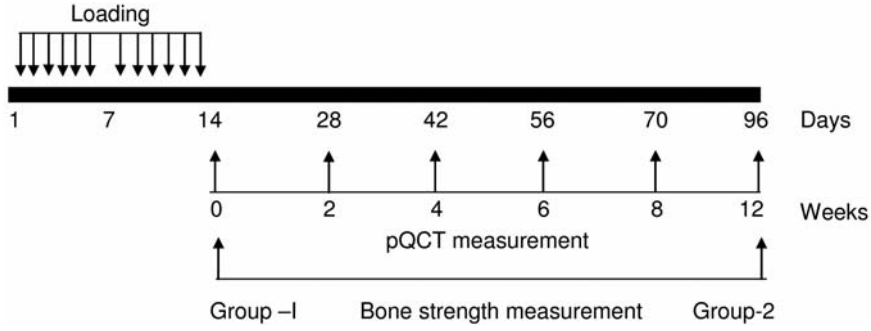

Figure 1. A schematic representation of the study design. (a) In group-I, mice were euthanized on day 14 after *in vivo* pQCT measurement of bone parameters, followed by tibias were collected and stored at 4ºC for mechanical testing. (b) In group-II, mice were euthanized on day 96 after *in vivo* pQCT measurement of bone parameter, followed by tibias were collected to perform mechanical testing.

and a 30-40% increase in cross-sectional area in 10 week old female B6 mice[3]. Thus, in this mechanosensitive B6 mouse, there is a robust increase in both vBMD and bone size after 2 wks of four-point bending, but, how long this newly formed bone, induced by four-point bending is maintained after cessation of loading is still unclear. Furthermore, to our knowledge, no study has examined whether bone mass or bone strength gained by four-point bending is maintained or lost after termination of loading and hence, the present study was carried out to address the above issue.

Materials and methods

Mice

10 Female C57BL/6J were purchased from the Jackson Laboratory (Bar Harbor, Me). All mice were housed under standard conditions of 14-hour light and 10-hour darkness, and had free access to food and water. The body weights of these animals measured before the initial loading were 18.32±0.79 grams. The experimental protocols were in compliance with animal welfare regulations and approved by our local IACUC.

In vivo loading model and peripheral quantitative computed tomography (pQCT) measurements

At 10 wks of age, two groups (n=5/group) of female B6 mice were subjected to ML using the four-point bending device as described previously[3]. The loading protocol consists of a 9.0 Newton (N) force (9N produce 3682 micro strain) at a frequency of 2 Hz for 36 cycles, performed daily under inhalable anesthesia (5% Halothane and 95% oxygen). The loading procedure was repeated for 6 days/week with 1 day of rest for 2 wks. The right tibia was used for loading and the left tibia as contralateral internal control in each mouse (From onwards in this manuscript, we will call right tibia as loaded bone and left tibia as non-loaded bone). In group-I, mice were euthanized by carbon dioxide inhalation on day 14 after *in vivo* pQCT measurement of bone parameters. Tibias were collected and stored at 4ºC for mechanical testing. In group-II, mice were subjected to 2 weeks of mechanical loading followed by *in vivo* pQCT measurement at the end of loading and at 2, 4, 6, 8 and 12 weeks after loading. Mice were euthanized at 12 week after *in vivo* pQCT measurement of bone parameter. Tibias were then collected to perform mechanical testing. A schematic representation of the study design is shown in Figure 1.

Peripheral quantitative computed tomography (pQCT) measurements

To measure loading induced changes in bone parameters in the loaded and non-loaded bone, we used pQCT (Stratec XCT 960M, Norland Medical System, Ft. Atkinson, WI) as described previously[3,16]. The resolution of pQCT scan is 70 micron. *In vivo* pQCT measurements were performed at immediate (0-time point), 2, 4, 6, 8, and 12 wks after the last loading regimen.

Mechanical properties of bone

Tibiae were stored frozen in gauze moistened with PBS and thawed in PBS at 4°C. The anterior-posterior diameter (AP.Dm) and lateral-medial diameter (LM.Dm) were measured with calipers. The tibiae were tested by three-point bending using the Instron DynaMight testing system (Model 8840; Instron, Canton, MA, USA) as previously described[17]. Each tibia was placed on two immovable supports which were 5 mm apart. An initial 1.0 N was applied on the tibia at a position of 2.10 mm away from the tibia-fibular junction to prevent the rotation of the bone due to the shape of the tibia and to be consistent in breaking region between the bones. Furthermore, this area corresponds to the 4 mm loading region[3,16]. It was then loaded from this midpoint at a constant rate (2 mm/minute) to the point of fracture. Load displacement curves were used to calculate yield load (P_y), maximum load (P_{max}) and toughness (Ut). Cross sectional moment of inertia was calculated from the measured anterior-posterior diameter and lateral-medial diameter and from the average cortical thickness obtained by pQCT analysis as previously described[18,19]. The number of mice used for this experiment is n=5 of which one mice had a technical problem in breaking the bone, and was excluded from the study. Thus, data from only 4 mice were used for this analysis.

Statistical analysis

Data are presented as Mean ± Standard error (SE). Loading induced changes in skeletal parameters were determined by calculating percent changes in the loaded versus non-loaded bones of the same animal to avoid any inherent variation between animals. One-way ANOVA (Newman-Keuls Post Hoc test) was used to evaluate the influence of time on the loaded and non-loaded bones. Correlation matrices (values for bone parameters and mechanical properties of respective mouse were used for correlation) were used to determine whether there is any association between changes in skeletal parameters and mechanical properties. Standard t-test was used to compare skeletal changes between loaded and non-loaded bone. We used STATISTICA software (StatSoft, Inc version 7.1, 2005) for our analysis and the results were considered significantly different at $p<0.05$.

Results

Bone anabolic response after cessation of loading

Two wks of four-point bending increased bone mineral content (BMC) by 45% in the loaded bone compared to corresponding non-loaded bone. The increased BMC was maintained (10-45%) throughout the 12 week cessation period after last loading (Figure 2a). The increased BMC in the loaded bone is caused by both bone size and vBMD changes. Bone size, as reflected by periosteal circumference (PC), was increased by 18% after two wks of ML and was significantly different from corresponding non-loaded bone throughout the entire study (Figure 2b). vBMD was increased by 14% after two wks of ML and remained high compared to non-loaded bones only until 4 wks after cessation of exercise (Figure 2c).

ANOVA analysis revealed that absolute values of non-loaded bones for PC were not different between the post loading time points while in the loaded bones, PC values of 6, 8 and 12 wks were different ($p=0.03$) from 0, 2 and 4 wks post loading time points (Figure 2b). Similarly, for vBMD, the absolute values of loaded bones showed no significant different between the post loading time points while the absolute values of non-loaded bones at 6, 8 and 12 week were significantly different ($p<0.05$) from 0, 2 and 4 week post loading time points as evident from ANOVA analysis (Figure 2c). In the case of BMC, the values of loaded bone were not different between the post loading time points while in the non-loaded bones, the values of 12 week were significantly different from 0, 2, 4, 6 and 8 wks of post loading time points (ANOVA analysis).

Figure 3 shows percent changes in loading induced increases in cross sectional area (bone size), cortical area, cortical thickness and vBMD as a function of time after cessation of last loading. Mechanical loading induced gains in all four parameters declined with time but at different rates. The vBMD and cortical thickness gained after two weeks of loading in the loaded bone was significant over non-loaded bones for a period of 5-6 weeks and then returned to the levels of control at 12 weeks. However, the gain in the CSA and cortical area after two weeks of loading though declined but still was significantly elevated at 12 weeks.

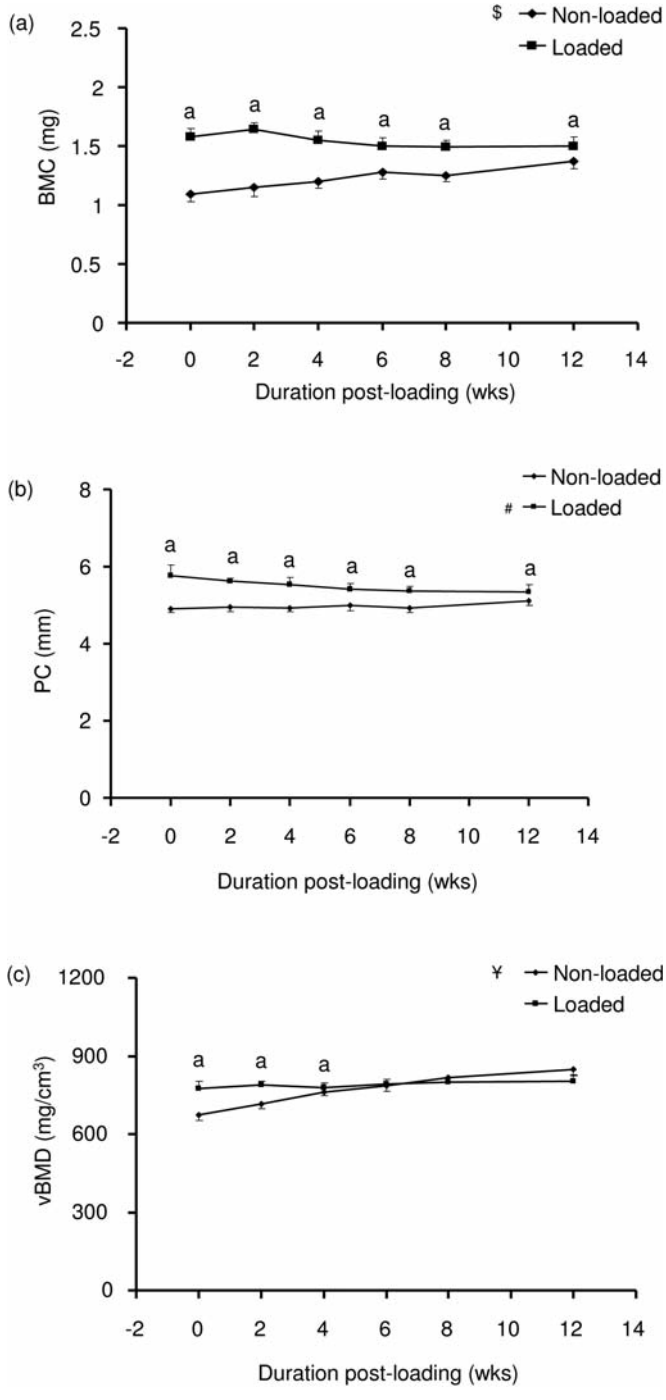

Figure 2. Changes in bone parameters measured at different time points after cessation of loading. The y-axis corresponds to absolute changes in bone parameters in response to 12 days four-point bending on 10 week female C57BL/6J mice. The x-axis corresponds to various time points. (a) Bone mineral content (BMC), (b) Periosteal circumference (PC) and (c) total volumetric Bone mineral Density (vBMD). Values are mean ± SE, [a]$p<0.05$ vs. non-loaded bone, N=5. [#]PC loaded values of 6-, 8- and 12-week are different from 0-, 2-, and 4-week ($p<0.05$, ANOVA, Newman-Keuls Post Hoc Test). [¥]vBMD non-loaded values of 6-, 8- and 12- week are different from 0-, 2-, and 4-weeks ($p<0.05$, ANOVA, Newman-Keuls Post Hoc Test). [$]BMC non-loaded values of 12 week are different from 0-, 2-, 4-, 6- and 8-weeks ($p<0.05$, ANOVA, Newman-Keuls Post Hoc Test).

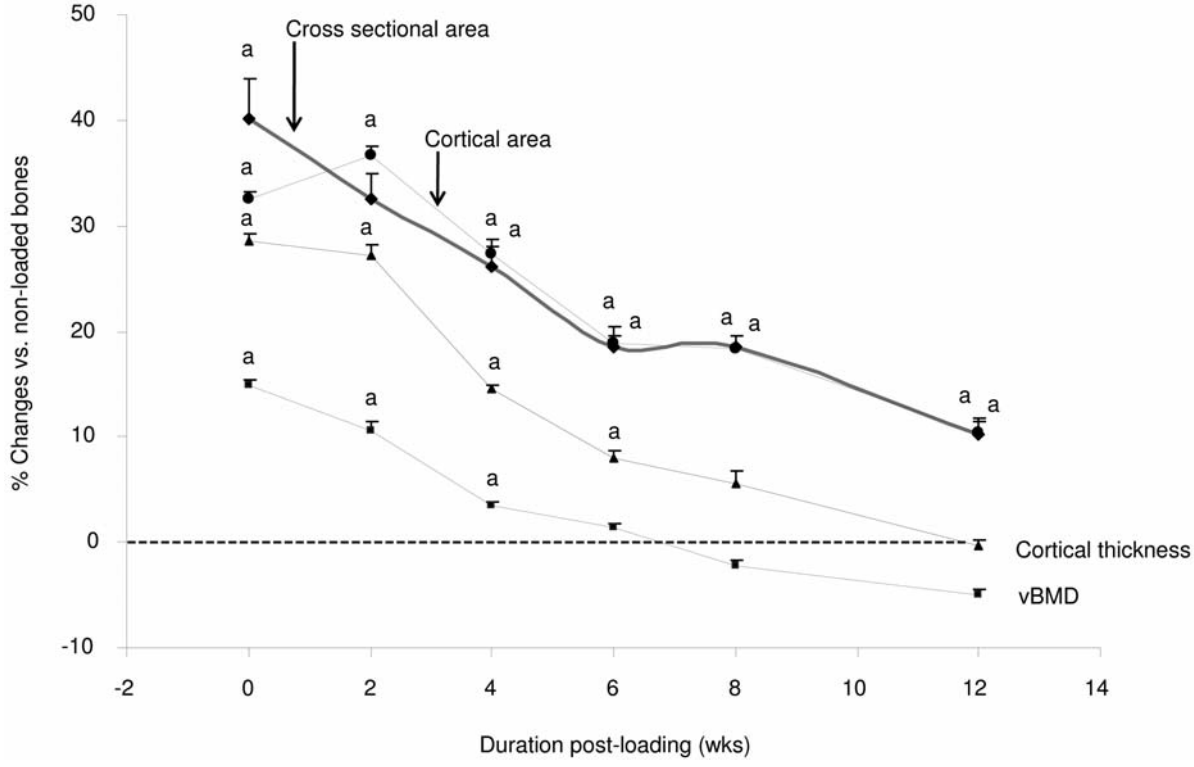

Figure 3. Changes in CSA and vBMD measured at different time points after cessation of loading. The y-axis corresponds to percent change and x-axis corresponds to duration post-loading (weeks). CSA, Cross sectional area and vBMD, total volumetric bone mineral density, values are mean ± SE, [a]p<0.05 vs. non-loaded bone, N=5.

Parameters	**0 week**		**12 week**	
	Non-loaded bone	Loaded bone	Non-loaded bone	Loaded bone
Yield load (N)	14.48±0.42	20.34±0.84*	17.64±0.72	16.44±0.13
Maximum load (N)	18.54±0.67	24.05±0.58*	21.93±0.39	21.05±0.17
Toughness (N/mm^2)	2.17±0.03	3.30±0.10*	2.03±0.02	1.94±0.14

Abbreviations: N, Newton
*Values are mean ± SE with four mice per group, *p<0.05 vs. non-loaded bones.*
0- and 12-week mechanical parameters of loaded bones are significantly different (ANOVA, Newman-Keuls Post Hoc test).
0- and 12-week mechanical parameters of non-loaded bones are not significantly different, p=0.07 (ANOVA, Newman-Keuls Post Hoc test).

Table 1. Changes in mechanical properties of bone measured at 0- and 12-weeks after the cessation of last loading.

Bone mechanical properties after termination of loading

In order to determine if the increase in skeletal parameters reflects an increase in bone mechanical properties, we performed a bone breaking study on the loaded and non-loaded bones of mice immediately after the loading and at 12 wks after cessation of loading. We found that immediately after 2 wks of loading, yield load, maximum load and toughness were significantly increased by 42%, 29% and 42%, respectively in the loaded bones compared to non-loaded bones (Table 1). However, at 12 wks, there was no difference in the above parameters between loaded and non-loaded bones (Table 1). Furthermore, we also performed a correlation analysis between these mechanical properties vs. vBMD, CSA and cortical thickness to elucidate whether increase in bone mechanical properties is due to an increase in the changes in bone parameters, measured by pQCT. The results show a significant correlation between vBMD, area and cortical thickness with yield load, maximum load and toughness (r= 0.72 to 0.88, p<0.05).

Discussion

In this study, the choice of inbred strain of mice, the loading device and regimen used were based on previous findings[3,16]. Two wks of four-point bending on the right tibiae when compared to left tibiae resulted in a significant increase in bone parameters, such as BMC, CSA and vBMD, as shown in Figure 1. The magnitude of increase in skeletal parameters after 2 wks of loading is consistent with our previous study[3]. However, in this study, we show that this increase in CSA (40%) and vBMD (15%) gained immediately after 2 wks of loading, did not continue over time after the cessation of last external loading. The decrease in the difference in CSA between non-loaded and loaded bones over time raises a question of whether this is due to loss of bone mass acquired during loading or due to the influence of growth in the loaded or non-loaded bones. The findings from our study illustrate that the reduction in CSA difference between non-loaded and loaded bones over time was purely due to a gradual loss in gained bone and not due to growth as evident from ANOVA analysis. In the case of vBMD, we found a significant increase in vBMD with advancing age (i.e. between 12 and 22 wks) by ANOVA in the non-loaded but not in the loaded bone. It is possible that vBMD increased in the non-loaded bone is to compensate for the increased body weight during this period while in the loaded bones this did not occur as the vBMD is sufficiently increased due to the four-point bending. It remains to be determined whether the lack of vBMD difference between the loaded and non-loaded bones at 12 wks after cessation of external loading is due to a difference in bone accretion rates between non-loaded and loaded bones between 12 and 22 wks of age and/or due to partial loss of ML-induced newly formed bone after cessation of loading in the loaded tibia.

In the pQCT analysis of bone parameters using the lower threshold (180-730), we found that the magnitude of increase in periosteal circumference (PC) was 18% ± 7.0 after the last loading and was significant when compared with 7.2% ± 1.7 using the higher threshold (730-730). This is due to rapid accumulation of low mineralized bone at the periosteal surface. This is most prevalent immediately after the last day of loading, as seen by the red color using the pQCT threshold indicator (Figure 4). Consequently, we found a significant increase in CSA immediately after the last day of loading, accompanied by an increase in vBMD. Over time, we found that the periosteal circumference in the loaded tibia tends to reduce gradually. As a result, the magnitude of difference between loaded and non-loaded tibia reduced at 12 wks after cessation of loading, resulting in no difference in the periosteal circumference of loaded tibia between lower (PC, 9.13% ± 3.9) and higher threshold (PC, 9.8% ± 1.2). These findings, suggest that the newly formed bone undergoes remodeling at the periosteal site to cortical bone over time which leads to a reduction in bone size in order to accommodate the loading induced changes in the bone shape. During this remodeling process, we found that the rate of loss in gained CSA (bone size) after cessation of loading was 10% every 2 wks for 8 wks. These data, suggest that the positive effects of ML on CSA were

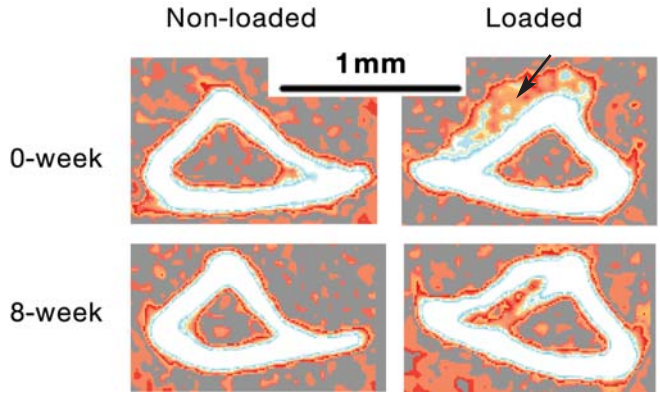

Figure 4. Cross sectional area of non-loaded vs. loaded bone. This figure shows the newly formed bone at the periosteal site in response to 2 weeks of loading and its fate over time after cessation of loading. The arrow corresponds to newly formed bone and the white color represents cortical bone.

maintained for several wks after the cessation of the last external loading and this is consistent with the earlier reports in rat and human model[20-22].

Since in our study, we found a significant increase in bone parameters in response to loading and because earlier reports have shown that an increase in bone parameters leads to an increase in bone mechanical properties, we anticipate a measurable difference in mechanical properties between the bones immediately after loading and at 12 wks. Accordingly, the findings from our study revealed that bone strength was significantly increased by 29 to 42% immediately after 2 wks of loading (Table 1) and this increase in bone strength is in part, mediated by an increase in vBMD and CSA as evident from our correlation analysis. However, at 12 wks after the cessation of loading, we found that there was no difference in the yield load, maximum load and toughness between the loaded and non-loaded bones (Table 1). This is because; the vBMD in the non-loaded bones increased with advancing age and the CSA decreased gradually in the loaded bones, together contributing for the loss of bone strength difference between the loaded and non-loaded bones at 12 wks.

Some of the limitations of this study are as follows: 1) in the past, reports by others have predicted that some of the skeletal changes induced by four-point bending are due to periosteal pressure. In our previous QTL study, we found that sham-loading neither increased periosteal bone formation nor caused changes in expression levels of bone marker genes, demonstrating that the skeletal changes induced by bending are not due to periosteal pressure[16] and this is also consistent with earlier reports[23]. This finding also applies to the present study since we used the same loading model and regimen. 2) In this study, we used 10 wk old mice for our four-point bending experiment. Since bones continue to grow beyond 10 wks although at much lower pace this raises a question whether growth has any effect on the response to loading. In the past,

we have performed four-point bending on three ages (10-, 16- and 36 wks) and reported that there was no significant difference in the bone response between the age groups for a given load. However, it remains to be determined whether the loss rate in bone mass after cessation of loading is different in young vs. old mice. 3) It is worth mentioning that the four-point bending method of loading also adds lamellar bone initially, however, as the duration of loading becomes larger, there is an increase in accumulation of bone forming osteoblast cells at the loading site. As a result, this gives rise to a large amount of low mineralized bone, so called woven bone over the lamellar bone. Our study, however, cannot rule out the contribution of woven bone vs. lamellar bone. 4) The four-point bending loading method applied in this study produced woven bone at the periosteal surface which is remodeled subsequently. Since the site of loading is at the mid diaphysis which contains no trabecular bone, this method of loading is not applicable to evaluate the loading response on lamellar bone. Future studies using axial loading model which induces both trabecular and cortical bone formation are needed to determine if axial loading induces woven bone or lamellar bone at the trabecular site. Since exercise induces lamellar bone formation in humans, it is likely that axial loading in mice will also promote lamellar bone formation in the metaphysis of tibia. 5) In the past, we have measured micro-cracks by histological analysis to rule out the possibility whether the changes on bone parameters induced by four-point bending are due to micro-crack induced healing. We found that there was no significant difference in the micro-crack areas between loaded and non-loaded bones of B6 mice. In the present study, we have used the same loading regimen and therefore, the response induced by four-point bending cannot be attributed to damaged induced bone formation. Furthermore, we did not observe any fracture as evident from X-ray and pQCT image[16].

In conclusion, our study shows that the positive effects of mechanical loading on bone were maintained for a substantial period of time after the cessation of the last external loading.

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