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Short-term healing kinetics of cortical and cancellous bone osteopenia induced by unloading during the reloading period in young rats

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Abstract We investigated the short-term recuperation of bone mass during skeletal reloading after a period of unloading in young rats. One hind limb of 4-week-old rats was either unloaded irreversibly by sciatic neurectomy, or unloaded reversibly by external fixation. Other animals were sham-operated. After 9 days, the fixation-unloaded limbs were reloaded for 1–3 weeks and were compared with the hind limbs of age-matched unloaded (neurectomized) and sham-operated controls. Cortical and cancellous bone mass was measured using ashing and histomorphometry. Cortical bone mass (expressed as femoral dry and ash weight and tibial cortical bone area) was reduced in both unloaded groups and was accompanied by production of hypomineralized bone, as shown by a reduction in the percent ash of the dry weight. Cancellous bone mass (expressed as bone area and surface at the tibial metaphysis) was also reduced in both unloaded groups. Cortical bone mass deficit was greater in the fixation group than in the neurectomy group. Thereafter it increased in the neurectomy group despite a normal longitudinal growth rate, but returned to age-matched values in the reloaded group by 3 weeks. The changes in tibial cancellous bone mass were more pronounced but followed a similar pattern and normalized by 2 weeks. These data demonstrate that total unloading produced by external fixation causes a greater degree of bone mass deficit than partial unloading (produced by neurectomy); the rate of bone loss during unloading in the rat hind limb is more rapid than its recovery during reloading; and cancellous bone recuperates during the reloading phase faster than does cortical bone.

Key words Bone mass · Unloading · Reloading · Histomorphometry

Introduction

Mechanical unloading causes bone loss, as has been documented in human beings following bedrest [7, 10, 12, 18, 19] or spaceflight [14], and has been investigated in numerous animal models. Rat models include nerve sectioning [1, 5, 6, 15, 20–22], limb suspension [9, 13], external fixation [17] and spaceflight [4]. We [5, 20–22] and others (e.g. [18]) have shown that unloading-induced osteopenia in rats is relatively rapid and probably involves both increased bone resorption and decreased bone formation. However, the degree of recuperation the skeleton can achieve following such osteopenia has not been much studied [8]. In humans, spaceflight experiments suggest that recovery may not be complete after "recovery" periods far longer than the unloading period [14]. Human bedrest experiments have also shown that recovery is incomplete and may take much longer that the time needed to induce osteopenia [7, 10]. In dogs recovery from fixation-induced osteopenia was also partial and took a very long time [2]. In adult rats, bone mass did not return to age-matched values during 2 weeks of reloading following a 2-week period of unloading through suspension [13]. In another experiment, cortical bone mass did not recover during 8 weeks of reloading after a 1-week unloading via casting [16]. Maeda et al. [11] did not find a full restoration of cancellous bone volume following 6 weeks of reloading after a 6-week unloading period via external fixation in adult rats. Most of these studies were of longer duration and were performed in adult rats. The purpose of this study was to test the short-term kinetics of bone mass changes during a reloading phase following a short period (9 days) of unloading in young, rapidly growing rats.

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Materials and methods

All animal procedures were conducted according to the rules of the Animal Care Committee of the Tel-Aviv University School of Medicine. Four-week-old male Sprague-Dawley rats were anaesthetized with 10% chloral hydrate i.p. and were divided into three groups (6 animals each). In group 1, one hind limb was unloaded irreversibly by sciatic neurectomy (removal of a 3-mm segment of the sciatic nerve through a lateral femoral incision). In group 2, one hind limb was unloaded reversibly via external fixation by inserting it into a polystyrene tube that was sutured to the skin. Rats in group 3 were sham-operated by cutting through the skin and underlying muscles and suturing, and these animals served as controls. In all rats, the contralateral limb was left intact. Monitoring the animals during the unloading period revealed that while no loads were applied to the limbs inserted into the tubes (which were therefore totally unloaded), rats were occasionally using neurectomized limbs for short periods while eating. These limbs were therefore partially unloaded. Animals in group 2 were reloaded after 9 days by removing the polystyrene tube. Rats in all three groups were sacrificed 0, 1, 2 and 3 weeks after reloading of group 2 was started. After the rats were killed, both hind limbs were removed and fixed in 40% ethanol. After recording of their maximal length, femora were defleshed, dried at 60 °C for 24 h and defatted in ether for 24 h to determine the dry, fat-free weight (mg/mm bone length). Femora were then ashed at 700 °C for 24 h to determine the corrected mineral content [ash weight divided by bone length (mg/mm)]. The operated tibiae were defleshed, dehydrated with increasing concentrations of ethanol and embedded in methylmethacrylate [20–22]. They were first sectioned transversally at the tibio-fibular junction with a Polycut microtome (Reichert Jung) at 6 μm thickness. Using a computerized digitizer (Summagraphics, Seymour, Conn.) the cross-sectional areas of both the periosteal and the endosteal surfaces were measured and cortical bone mass was expressed as a percentage of the periosteal surface. Tibiae were then sectioned frontally through the proximal metaphysis, deplastified in warm xylene (40 °C, 6 h), stained with Masson's trichrome and used for histomorphometric analysis of the secondary spongiosa extending 0.9–1.8 mm distal to the epiphyseal growth plate. Sections were viewed through a Visopan microscope (Reichert, Austria) and all trabecular profiles were traced onto acetate paper at ×300. Cancellous bone area and surface were determined using the same digitizer. All trabeculae in contact with the cortices were excluded from the measurements. Non-paired *t*-tests were used to test the significance of the differences between the operated limbs of the different groups of rats, and paired *t*-tests were used for within-animal comparisons.

Results

Longitudinal bone growth was not altered by unloading or reloading, so that femoral length did not differ between the various groups of rats at any time point examined (data not shown).

After 9 days of unloading, osteopenia was present in both neurectomy and fixation groups. This was reflected in reduced femoral dry weight and ash weight (Figs. 1, 2). However, the reduction in bone mass was greater in the fixation group than in the neurectomy group. During the subsequent 3 weeks, bone mass remained depressed in the neurectomy group. In the fixation group it was still lower than in the neurectomy group for the first 2 weeks, but increased dramatically during the 3rd week, reaching values the same as in the age-matched control group. In addition, the percentage ash weight of the dry weight was reduced in both unloaded groups after 9 days of unloading (Fig. 3), suggesting impaired mineralization. While this phenomenon persisted in the neurectomy group for the next 3 weeks, it gradually disappeared in the fixation group, in which the results equalled age-matched values after 2 weeks of reloading. Analysis of the ash weight of all limbs together indicated that the neurectomized and fixated femur invariably had a lower bone mass than the contralateral femur, but also that bone mass in the contralateral femora of neurectomized or fixated limbs was greater than in those of the sham-operated limbs at all time points, probably because of overloading (Fig. 4).

Since the two variables, dry and ash weight, combine cortical and cancellous bone, we measured each type separately in the tibia by histomorphometry. Cancellous bone area (Fig. 5) and surface (Fig. 6) were markedly reduced during the 9 days of unloading. Again, the reduction in bone mass was more pronounced in the fixation group than in the neurectomy group. While cancellous bone mass in the neurectomy group remained diminished throughout the experimental period (and finally reached about 50–60% of normal values), both measures returned to normal values in the fixation (reloaded) group by 2 weeks.

Similarly, cortical bone area was reduced in the two unloaded groups after 9 days of unloading (Fig. 7). Again, bone mass deficit was larger in the fixation group than in the neurectomy group. During the next 3 weeks, cortical bone deficit increased in the neurectomy group but returned to age-matched (control) values in the fixation group by 3 weeks.

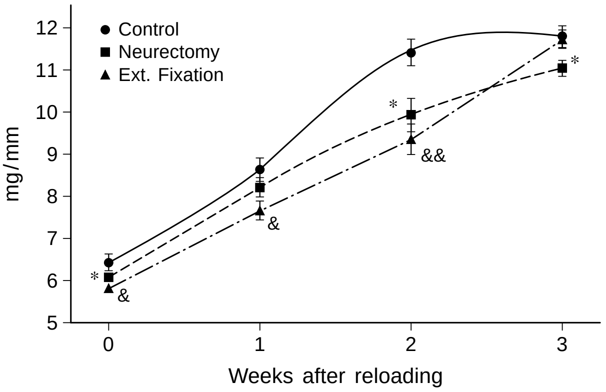
Discussion

Unloading for 9 days caused osteopenia, as expected. This was reflected in a reduction in dry and ash weight (measuring both cortical and cancellous bone), in cancellous bone area and surface, and in cortical bone area. The magnitude of the bone deficit in the neurectomy group was similar to that observed in our previous studies [20–22]: approx. 10% of ash weight and approx. 45% of cancellous bone area. In addition, similar to our previous studies, unloading caused a diminuton of the percent ash of dry weight, suggesting reduced mineralization of bone formed during the experimental period. All these variables were depressed to a greater extent in the fixation group than in the neurectomy group. Since the animals in the former group were experiencing total unload-

Fig. 1 Changes caused to femoral dry weight of the operated limb by unloading or reloading (* P <0.05 for neurectomy vs control, & P <0.05 for fixation vs control, && P <0.01 for fixation vs control)

Fig. 2 Changes caused to femoral ash weight of the operated limb by unloading or reloading (* P <0.05, ** P <0.01 for neurectomy vs control, & P <0.05, && P <0.01 for fixation vs control)

Fig. 3 Changes caused to femoral % ash weight of the dry weight of the operated limb by unloading or reloading (* P <0.05 for neurectomy vs control, & P <0.05, && P <0.01 for fixation vs control)



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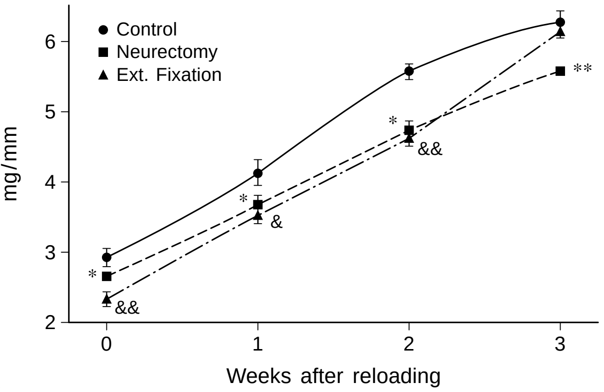

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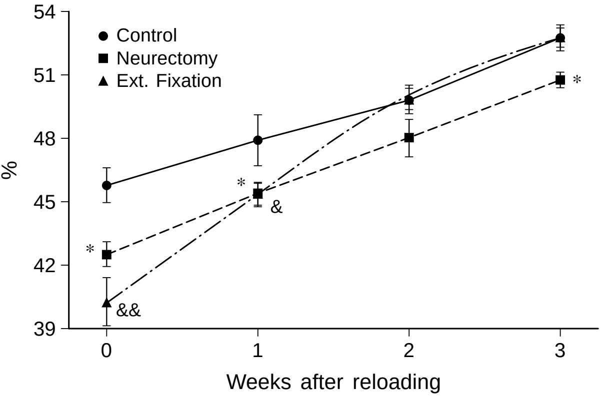

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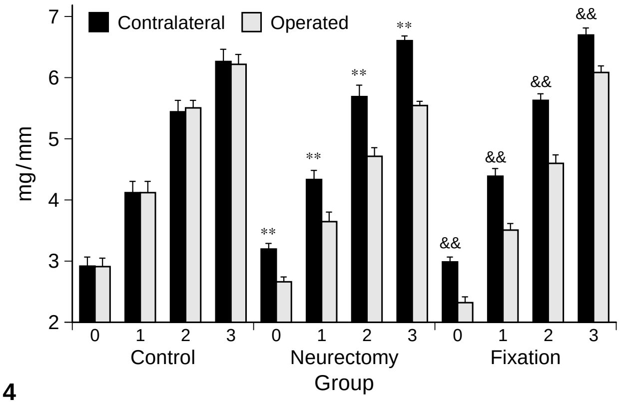

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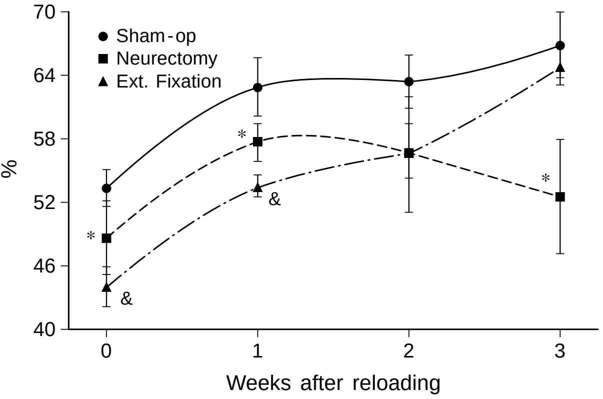

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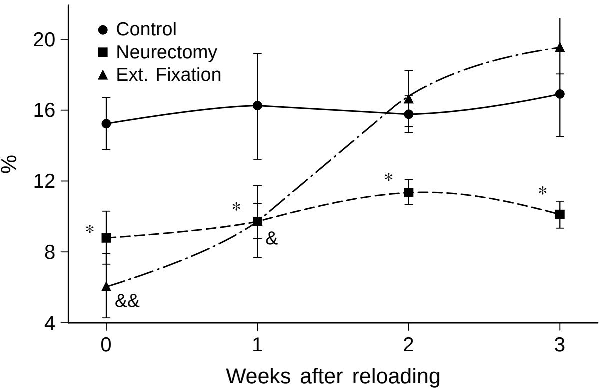

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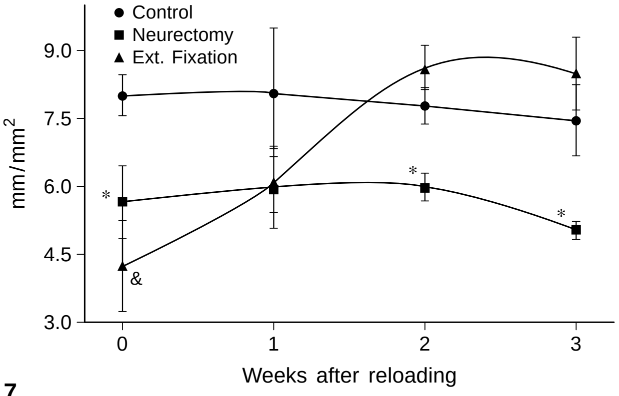
Fig. 4 Changes in femoral ash weight of both the operated and contralateral limbs in the control, unloaded and reloaded groups (** $P < 0.01$ for neurectomized vs contralateral limb, && $P < 0.01$ for fixation vs contralateral limb)

Fig. 5 Changes caused to tibial cortical bone area of the operated limb by unloading or reloading (* $P < 0.05$ for neurectomy vs control, & $P < 0.05$ for fixation vs control)

Fig. 6 Changes caused to tibial cancellous bone area of the operated limb by unloading or reloading (* $P < 0.05$ for neurectomy vs control, & $P < 0.05$, && $P < 0.01$ for fixation vs control)

Fig. 7 Changes caused to tibial cancellous bone surface of the operated limb by unloading or reloading (* $P < 0.05$ for neurectomy vs control, & $P < 0.05$ for fixation vs control)



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ing of the operated limb, as opposed to partial unloading in the latter, we interpret this finding as "unloading-dose-dependent" osteopenia; That is to say the greater the degree of the unloading, the more severe the osteopenia.

The mass of both cortical bone and cancellous bone returned to age-matched values in this study. This is in contrast to the studies by Maeda et al. [11], Tuukkanen et al. [16] and Sessions et al. [13], who reported incomplete recovery of bone mass. The difference may lie in the fact that we used rats which were younger than those in any of the other studies and possibly capable of better recuperation. Another factor may be the longer unloading period used by Maeda and Sessions, which may require a longer recovery period. Finally, Tuukkanen et al. present bone deficit as a difference in ash weight between the operated and the contralateral limb of each rat and show that a residual deficit still exists after 4–8 weeks of reloading. Such a difference, however, may be caused by increased bone mass in the contralateral limb, which had been overloaded during the unloading period, rather than by reduced bone mass in the reloaded limb. We tested this possibility in our study by calculating the corrected ash weight in the contralateral limb and indeed found it to be increased in both the neurectomy and the fixation groups throughout the experimental period. Thus, calculating bone mass deficit in this study by the difference between the operated and the contralateral limb would yield an incomplete recovery (a 9% deficit remaining after 3 weeks of reloading).

The kinetics of bone mass recovery differed between cortical bone and cancellous bone. It took cortical bone mass 3 weeks to normalize and cancellous bone mass only 2 weeks. Both these rates are slower than the rate at which bone mass was lost (9 days). It is known that cancellous bone exhibits greater physiological activity than cortical bone, thus putting it in a better position to respond to changes in the mechanical environment [3]. Indeed, we always find cancellous bone to be more responsive to unloading, demonstrating a 40–50% loss as against a 10–15% loss of cortical bone mass during the same unloading period (this study) [20–22].

Undoubtedly, in this model the healing of unloading-induced osteopenia is very much affected by the continuing growth of the animals. These animals are growing rapidly at this age, so that the kinetics of bone mass healing must be greatly accelerated. This puts such data in contrast with data recorded in experiments using humans and adult animals such as dogs and nongrowing rats. Nevertheless, it must be pointed out that the effect of unloading itself is powerful enough for the bone mass deficit to be maintained and even worsened in the neurectomy group, despite normal bone elongation and normal body weight gain (data not shown).

In summary, our data demonstrate that in young, rapidly growing rats total unloading produced by external fixation causes a greater amount of bone mass deficit than partial unloading (produced by neurectomy) and that the rate of bone loss during unloading is greater than its recovery during reloading. Cancellous bone recuperates during the reloading phase faster than does cortical bone.

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