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Disuse-induced deterioration of bone strength is not stopped after free remobilization in young adult rats

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Abstract

The effect of unilateral hindlimb immobilization and subsequent free remobilization on mechanical properties of femur was examined in young adult rats. Right hindlimb of 17 weeks old male rats was immobilized for 2 weeks. Rats were sacrificed either directly after immobilization (E0) or after 4 weeks of free remobilization (E4). Mechanical properties in three-point bending as well as dry mass (m_{dry}), geometry, apparent density (d_{app}), and mineralization of dry bone tissue were measured post mortem in right and left femora of experimental rats (E0, E4) and in right femora of age-matched controls (C0, C4). Differences between right femora of experimental and control animals and between right and left femora of experimental animals were analyzed. After immobilization only d_{app} in E0 was significantly lower than in C0. Side-to-side differences in E0 were present only in m_{dry} and d_{app}. Surprisingly, 4 weeks after remobilization the differences between experimental and control femora were more pronounced. Mineralization, d_{app}, maximum bending moment (M_{max}), yield bending moment (M_y) and stiffness of the right femur were lower in E4 than in age-matched C4. Side-to-side differences in remobilized rats (E4) were still significant for m_{dry} and d_{app}. Additionally, the medullary area was larger, and M_{max}, M_y, stiffness and work to failure were lower in the right femur than in the left. It is concluded, that the processes of bone deterioration initiated during immobilization do not cease immediately after resumption of normal mechanical loading.

Keywords: Apparent density; Immobilization; Mineralization; Rat femur; Three-point bending

1. Introduction

Mechanical loading is essential for the normal functioning of bone tissue. Immobilization results in imbalance of bone metabolism followed by rapid bone loss and impairment of bone mechanical function (Kiratli, 1996). According to Frost's theory of structural adaptation to mechanical usage (Frost, 1990), decreased mechanical usage depresses longitudinal growth and bone modeling-dependent bone gain, and stimulates bone remodeling-dependent bone loss. Mechanisms controlling gains and losses of bone mass are similar in young and aged rats and humans (Frost and Jee, 1992; Jee et al., 1997), so in experiments concerning the problem, rat models are popular.

Unloading-induced depression of bone formation was established on periosteal surfaces of cortical bone and in cancellous bone tissue, both in intensively growing (Chen et al., 1992) and in adult rats (Dehority et al., 1999; Li et al., 1990; Li and Jee, 1991; Maeda et al., 1993). The overactive remodeling with increased resorption was found in cancellous bone (Chen et al., 1992; Ijiri et al., 1995; Li et al., 1990; Maeda et al., 1993) and on endocortical envelope in diaphyses of long bones (Inman et al., 1999; Li and Jee, 1991; Maeda et al. 1993). The change of bone remodeling rate was followed by a decrease of bone mass, density (Ijiri et al., 1995; Li et al., 1990; Li and Jee, 1991; Maeda et al., 1993) and mechanical strength (Inman et al., 1999).

The processes of bone resorption and formation are uncoupled during a transient phase of immobilization. At a steady state, bone mass and metabolic activity stabilize on a level adequate to the lower bone mass and loads (Frost, 1990; Jee et al., 1997). The transient phase lasts longer in adult (Ijiri et al., 1995; Li et al., 1990; Li and Jee, 1991) than in intensively growing animals (Chen et al., 1992). The question arises whether termination of unloading, before a new steady state is established, would stop the bone loss.

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Another fundamental question is whether the immobilization-induced osteopenia is temporary and reversible or nonrecoverable. The fact that the time needed for recovery is longer than the time needed to produce osteopenic changes is proved both in intensively growing (Kannus et al., 1996; Trebacz and Baj, 1999; Tuukkanen et al., 1992) and in adult animals (Kaneps et al., 1997, Maeda et al., 1993). Moreover, the potential for complete and permanent recovery of an immobilized bone is questionable (Kannus et al., 1996; Maeda et al., 1993; Tuukkanen et al., 1992).

In the present work a unilateral hindlimb immobilization was applied to adult, but still growing rats. The immobilization was terminated after 2 weeks, sufficiently for initiation of substantial changes, but before a new steady state in bone turnover, was established, both in cancellous and in cortical tissue (Chen et al., 1992; Ijiri et al., 1995; Jee et al., 1997; Li et al., 1990; Li and Jee, 1991). The aim of the study was to evaluate effects of a relatively short period of hindlimb immobilization on mechanical properties, density and geometry of femur and, what seems more interesting, to examine the ability of bone to recover during free remobilization.

2. Materials and methods

Adult male Wistar rats were used in the study. After 2 weeks of acclimatization to vivarium conditions, 17 weeks old animals with mean body weight 412 g (S.D. = 26 g), were randomly divided into four groups, ten animals per group. There were two control groups (C0, C4) and two study groups (E0, E4). Right hindlimbs of the study animals were immobilized against the abdomen using bandages and padded tape as previously described by Li et al. (1990). The fixation was checked daily. All animals were housed in typical wire cages (35 × 50 cm wide, 20 cm high), five animals per cage. They were fed standard laboratory chow and water ad libitum. After 2 weeks, the rats from the groups E0 and C0 were sacrificed. Rats from the group E4 were released from tapes and bandages and allowed moving freely for next 4 weeks. One day after the remobilization they used all legs while moving. At the end of 4th week, rats from the groups E4 and C4 were killed. All rats were killed by cardiac puncture under ether anesthesia. The experiment was approved by the committee of ethics for animal experiments at the Medical University of Lublin.

Both femora were removed and outer surfaces of the bones were cleaned mechanically from soft tissues. The length of femur and the minimum and maximum external diameters of the middle part of femoral shaft were measured with calipers. The total cross-section area of the midshaft was calculated assuming the ellipsoidal shape. A mean apparent density (d_w) of whole intact femora with marrow in situ was estimated using a hydrostatic method.

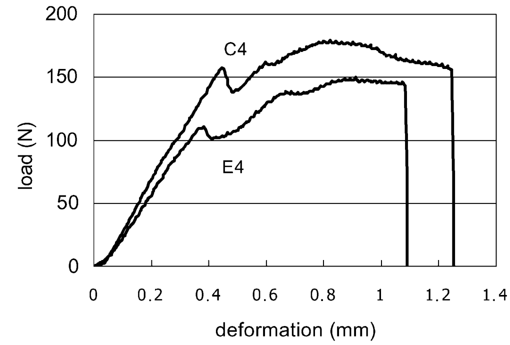

Fig. 1. The load–deformation curves for the right femur of an experimental rat after 4 weeks of hindlimb remobilization (E4) and of the right femur of an age-matched control rat (C4).

Right femora were tested mechanically in three-point bending using a Lloyd LRX Testing Machine.[1] The span of supports was 15 mm and deformation rate 2 mm/min. The load–deformation curve was analyzed (Fig. 1). The maximum bending moment, yield bending moment, work to failure and stiffness (the slope of the linear part of the curve) were calculated from the plot. The internal maximum and minimum diameters of broken femora were measured with calipers and medullary area in the fracture site was calculated, assuming the ellipsoidal shape. During measurements bones were kept wet in saline solution.

After mechanical testing all pieces of the broken femora were dried in 105°C for 24 h and weighed (dry weight, m_d). Before drying marrow was removed from the medullary cavity. Finally, bones were ashed in a muffled furnace at 630°C for 24 h and weighed again (m_a). Mineralization of the dry bone tissue ($\%M$) was calculated as: $\%M = 100(m_a)/m_d$.

The Student's t-test was used to analyze differences in mean values of measured parameters between the immobilized and the control rats (E0 vs. C0) and between the remobilized and the control (E4 vs. C4). Right-to-left differences in unilaterally immobilized groups (E0, E4) were analyzed using paired t-test. The effect of aging during 4 weeks of remmobilization (E0 vs. E4 and C0 vs. C4) was analyzed using Student's t-test. In all tests p-value <0.05 was considered significant. The statistics were calculated for $n = 10$ in each group, with the exception of mechanical parameters in E4, where $n = 9$, because one left femur was destroyed by uncontrolled impact load during testing.

[1] All mechanical tests were performed in the Institute of Agrophysics, Polish Academy of Sciences, Lublin.

3. Results

During 2 weeks of right hindlimb immobilization, the experimental rats lost weight, while at the same time, the control animals were still growing (Table 1). However, the experimental rats gained the weight rapidly during 4 weeks of free remobilization, so at the end of the experiment there was no significant difference between the experimental and the control groups, despite the continued increase of the weight of control animals.

Two weeks of immobilization of right hindlimb resulted in a decrease of mean density of right (-3%, $p = 0.0003$) and left (-1.4%, $p = 0.0275$) femora of the experimental rats (E0) in respect to the controls (C0). The mass and size of femur as well as mineralization of dry bone tissue were not significantly affected (Table 2). There were also no significant differences between the groups E0 and C0 considering mechanical parameters in three-point bending (Table 3).

After 4 weeks of free remobilization, the apparent density of the right femur in the experimental rats (E4) was still significantly lower (2.4%; $p = 0.0065$) than in the age-matched control (C4), the bones were also significantly less mineralized (1.4%; $p = 0.0417$). Moreover, the differences in mechanical parameters between E4 and C4 groups appeared (Table 3). Maximum bending moment, yield bending moment and stiffness in experimental rats were significantly lower than in the control by 11.3% ($p = 0.0162$), 14.4% ($p = 0.0167$) and 7.4% ($p = 0.0188$), respectively.

The results of statistical analysis of side-to-side differences between right (immobilized) and left (free) femora of the experimental rats are given in Table 4. Just after 2 weeks of immobilization, geometry of right and left femora did not differ significantly. However, the

Table 1
Body weight (g)[a]

	Before immobilization (17 weeks old rats)	After immobilization (19 weeks old rats)	After remobilization (23 weeks old rats)
Control	412 (26)	443 (30)	488 (44)
	$n = 20$	$n = 20$	$n = 10$
Experimental	411 (26)	370 (24)***	470 (25)
	$n = 20$	$n = 20$	$n = 10$

[a] Mean (standard deviation).
***Significantly different from the control, $p \leqslant 0.001$ by Student's t-test.

Table 2
Measures of mass and geometry of femora[a]

		After immobilization (19 weeks old rats)	After remobilization (23 weeks old rats)
Dry mass (g)	Control right	0.686 (0.053)	0.786 (0.057)
	Experimental right	0.663 (0.053)	0.741 (0.061)
	Experimental left	0.692 (0.062)	0.770 (0.067)
Length (mm)	Control right	39.8 (1.2)	41.4 (0.8)
	Experimental right	39.9 (0.8)	40.8 (0.8)
	Experimental left	39.8 (0.9)	40.7 (0.8)
Midshaft cross-section total area (mm^2)	Control right	12.17 (0.89)	13.15 (1.02)
	Experimental right	12.59 (1.02)	13.17 (1.21)
	Experimental left	12.36 (1.06)	13.24 (1.36)
Medullary cross-section area (mm^2)	Control right	4.66 (0.34)	5.46 (0.47)
	Experimental right	4.90 (0.42)	5.72 (0.50)
	Experimental left	4.84 (0.49)	5.42 (0.56)
Wet apparent density (g/cm^3)	Control right	1.542 (0.026)	1.594 (0.030)
	Experimental right	1.496 (0.020)***	1.556 (0.025)*
	Experimental left	1.520 (0.020)*	1.577 (0.021)
Mineralization of dry tissue (%)	Control right	68.4 (0.6)	69.2 (1.2)
	Experimental right	67.9 (0.7)	68.2 (0.8)*
	Experimental left	68.2 (0.7)	68.6 (0.7)

[a] Mean (standard deviation).
***Significantly different from the control right, $p \leqslant 0.001$ by Student's t-test.
*Significantly different from the control right, $p \leqslant 0.05$ by Student's t-test.

Table 3
Results of mechanical testing of femora in three-point-bending[a]

		After immobilization (19 weeks old rats)	After remobilization (23 weeks old rats)
Maximum bending moment (N m)	Control right	0.545 (0.047)	0.657 (0.081)
	Experimental right	0.563 (0.058)	0.583 (0.035)**
	Experimental left	0.548 (0.061)	0.637 (0.055)
Yield bending moment (N m)	Control right	0.368 (0.023)	0.473 (0.068)
	Experimental right	0.370 (0.032)	0.405 (0.045)**
	Experimental left	0.371 (0.059)	0.446 (0.045)
Stiffness (N/mm)	Control right	349 (36)	390 (19)
	Experimental right	342 (26)	361 (30)*
	Experimental left	320 (44)	384 (25)
Work to failure (mJ)	Control right	137.9 (21.7)	131.1 (23.9)
	Experimental right	126.3 (17.1)	133.8 (19.1)
	Experimental left	121.2 (28.7)	157.9 (43.3)

[a] Mean (standard deviation).
**Significantly different from the control right, or $p \leqslant 0.01$ by Student's t-test.
*Significantly different from the control right, or $p \leqslant 0.05$ by Student's t-test.

Table 4
Significance of differences between the right (immobilized) and the left (free) femur in experimental rats[a]

	E0 (after immobilization)		E4 (after remobilization)	
	p-value	R/L (%)	p-value	R/L (%)
Dry mass	0.0190	95.8	0.0190	96.2
Length	n.s.	100.2	n.s.	100.2
Total cross-section area	n.s.	101.9	n.s.	99.5
Medullary area	n.s.	101.2	0.0074	105.5
Wet apparent density	<0.0001	98.4	0.0002	98.7
Mineralization	n.s.	99.6	n.s.	99.4
Maximum moment	n.s.	102.7	0.0007	91.5
Yield moment	n.s.	100.0	0.0013	90.8
Stiffness	n.s.	106.9	0.0505	94.0
Work to failure	n.s.	104.2	0.0334	84.7

[a] p-values calculated from paired t-test, n.s.—$p > 0.05$; R/L—right femur vs. left femur in percent.

mass and the mean apparent density of the right femur were significantly lower, though the mineralization of dry bone tissue was the same in both femora. The differences, although reduced, were still significant after 4 weeks of free remobilization. Moreover, the medullary area increased in the right femur with respect to the left. The mechanical parameters of the right and left femora (Table 4) did not differ significantly after immobilization. Surprisingly, after 4 weeks of free remobilization the side-to-side differences in mechanical parameters were much more pronounced. All analyzed parameters were significantly lower in the right femur than in the left.

Considering the fact that during the remobilization rats were still growing (Tables 1 and 2), the effect of aging was analyzed both in the experimental and in the control rats (Table 5). During 4 weeks of remobilization there was a significant increase in dry mass, mean apparent density and length of both femora in the experimental rats and of the right femur in the control animals. The cross-sectional total area of femur midshaft changed neither in the control nor in the experimental groups.

Mineralization of dry bone tissue increased only in the control rats. There was also a significant increase of medullary area in the control and in both femora of the experimental rats. Mechanical parameters of femur changed significantly during the remobilization both in the control rats and in left, loaded leg in the experimental rats, except for work to failure in the control animals. The mechanical properties of the right, previously unloaded, femur in the experimental rats did not change during the remobilization. Summarizing the differences between the groups E0 and E4, it can be stated that during 4 weeks of remobilization, the experimental rats were still growing, the body weight as well as mass, length and mean apparent density of both femora increased. However, this growth was accompanied by the improvement of mechanical parameters in the left, loaded femur only.

Table 5
Effect of aging (19 weeks old vs. 23 weeks old) in the control (C0, C4) and the experimental rats (E0, E4)[a]

	C0 versus C4 right femur	E0 versus E4 right femur	E0 versus E4 left femur
Dry mass	0.0007	0.0069	0.0146
Length	0.0014	0.0362	0.0381
Total cross-section area	n.s.	n.s.	n.s.
Medullary area	0.0004	0.001	0.0270
Wet apparent density	<0.0001	<0.0001	<0.0001
Mineralization	0.0026	n.s.	n.s.
Maximum moment	0.0006	n.s.	0.0050
Yield moment	<0.0001	n.s.	0.0079
Stiffness	0.0113	n.s.	0.0049
Work to failure	n.s.	n.s.	0.0500

[a] p-values calculated from Student's t-test, n.s.—$p > 0.05$.

4. Discussion

The experiment presented in this paper, concerns effects of immobilization on mechanical properties of femora in young adult rats. The study was focused on the examination of ability of bone to recover during free remobilization.

After 2 weeks of hindlimb immobilization a drop of mass and mean density of immobilized femora was found which was consistent with results of previous experiments both on growing (Kannus et al., 1996; Li et al., 1990; Martin, 1990; Trebacz and Baj, 1999) and adult rats (Ijiri et al., 1995; Inman et al., 1999; Li and Jee, 1991; Maeda et al., 1993). Mechanical parameters were not affected, though in experiments on young, intensively growing rats 2 weeks of unloading resulted in a significant decrease of mechanical strength of bone (Martin, 1990; Peng et al., 1994; Trebacz and Baj, 1999). In young adult rats a significant decrease of bone strength was found after 6 weeks of limb immobilization (Inman et al., 1999), and in young adult dogs markedly lowered mechanical properties were found after 16 weeks of immobilization (Kaneps et al., 1997).

Although after 2 weeks of immobilization there was no noticeable change in the strength of femur, the effect of unloading appeared during free remobilization. After 4 weeks of remobilization strength, work to failure and stiffness were significantly lower in the remobilized femur than in the contralateral.

The mechanical properties of a whole femur in three-point bending depend on both the mechanical properties of bone tissue, and also on the size and shape of the bone. Variations of cross-sectional geometrical properties are mainly responsible for the differences in bending behavior of femur though an influence of density of cortical tissue is also proved (Martens et al., 1986; Strømsøe et al., 1995). Moreover, mechanical properties of cortical tissue are a function of porosity and mineralization, as was stated by Currey (1988) in a comprehensive study of bone samples from different species.

In the present study, 2 weeks of immobilization of a hindlimb influenced neither the external size of femur nor the medullary cavity area, also mineralization of bone tissue was not affected. During 4 weeks of remobilization the medullary area in the right, previously immobilized femur of experimental rats increased with respect to the left, probably because of immobilization-induced increase of endocortical resorption. Mineralization of bone tissue increased significantly in the control group, while in both femora in the experimental group, mineralization did not rise. This result may imply that maturation of bone tissue in experimental rats was slowed down or, considering very intensive gain of weight of these rats during remobilization, that mineralization of bone tissue did not "keep up" with the rapid growth.

These subtle changes of size and mineralization in unloaded bone initiated by immobilization, persisting and even increasing during first period of remobilization, resulted in significant differences in mechanical parameters of both femora.

Previous reports indicate that recovery of bone mass and morphological features following immobilization varies with duration of unloading, age and species of the animal, duration of remobilization, and type and intensity of exercise during the rehabilitation period (Table 6). Result of experiments on growing rats showed that neither free remobilization nor moderate exercises are sufficient for complete recovery of bone after immobilization. Moderate exercises did not increase the rate of recovery of bone during 9 weeks of remobilization (Tuukkanen et al., 1992). Moreover, the beneficial effects of intensified exercises in the first period of recovery were lost during the following period of free remobilization (Kannus et al., 1996). In adult rats, recovery of bone mass and morphological properties during free remobilization, without any exercises, was incomplete at least in the first period of the remobilization (Maeda et al., 1993; Ijiri et al., 1995).

Recovery of mechanical parameters was examined by Kaneps et al. (1997) in young adult dogs. They found a

Table 6
Recovery of bone after immobilization in different animal models

Age at start	Length of immobilization	Length of recovery	Exercise	Outcome at end of recovery	Refs.
Growing rats	2 weeks	9 weeks	Moderate	Incomplete recovery of bone ash	Tuukkanen et al., 1992
13 weeks, rats	3 weeks	29 weeks	10 weeks, intense	Incomplete recovery of bone density	Kannus et al., 1996
6 months, rats	6 weeks	6 weeks	None	Incomplete recovery of bone mass	Maeda et al., 1993
6 months, rats	18 weeks	20 weeks	None	Incomplete recovery of bone morphology	Ijiri et al., 1995
1–2 years, dogs	16 weeks	32 weeks	16 weeks, increasing	Incomplete recovery of cortical bone mass	Lane et al., 1996
1–2 years, dogs	16 weeks	32 weeks	16 weeks, increasing	Recovery of mechanical properties	Kaneps et al., 1997
17 weeks, rats	2 weeks	4 weeks	None	Mechanical properties decline	Present work

complete recovery of bone strength after 32 weeks of remobilization following 16 weeks of immobilization but the protocol of remobilization included 16 weeks of increasing training. However, even additional exercise failed to produce a complete recovery of cortical bone mass in the same animals (Lane et al., 1996).

In this study, a significant worsening of bone mechanical properties was found during 4 weeks of remobilization. There was also no considerable recovery of bone mass. Thus, despite the fact that immobilization was terminated during the early, active phase of disuse-induced bone loss, no recovery was noticed, what is more, free remobilization appeared to be somewhat harmful to bone properties.

Summarizing, the results obtained in the present study demonstrate that disuse-induced processes of bone deterioration last longer than immobilization and a resumption of previous loading is not sufficient to stop them. Changes initiated in bone during unloading persist within, at least, first period of remobilization; as a consequence the bone grows weaker despite the restoration of normal activity. Results of the study may be important for the treatment of post-traumatic osteopenia and particularly emphasize the importance of the first, early phase of rehabilitation.

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