Effects of various mechanical loading on osteogenetic processes of tibial cancellous bone in rats

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Abstruct

The purpose of this study was investigated the effects of various mechanical loading on osteogenetic processes of tibial cancellous bone in rats.

Sixty eight male Wistar rats, aged seven weeks were used as materials, and rats were assigned to sixteen groups, divided as follows: (1) 30% of the maximum jump height (E30-4, 7, 14, 21 days), (2) 45% of the maximum jump height (E45-4, 7, 14, 21 days), (3) 90% of the maximum jump height (E90-4, 7, 14, 21 days), (4) control groups (CO-4, 7, 14, 21 days). And, rats were jumped 100 times/day, 5 days/week for each experimental periods. Rats in each group were euthanized at the end of experimental period, tibiae were excised from them and those specimens were observed morphometrically.

BV/TV of E45 and E90 showed high value at experimental start early period, and recognized increasing Tb.Th in E45-4, and Tb.N in E90-4. The number of TRAP-positive cells were in all experimental groups at same periods. And, BV/TV and Tb.Th showed significantly high value in E90-21 than CO-21.

The present study indicate that the different strength exercise advance osteogenetic processes in early stage, but eventual bone structure is similar to low strength exercise.

Keywords: bone structure, jump training, mechanical loading

Introduction

It is said that mechanical stress is an important in increase of bone volume and maintenance of bone structure. In many studies with rats materials, the effects of exercise on the increase bone volume and maintenance of bone structure, for example, jump

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training[1-3], climbing exercise [4,5], running exercise [6-8], and swimming[9,10]. It is said that jump training was increased bone volume and improved bone strength compared with aerobic exercise like running exercise [11-13]. It is known that jump number and height contribute to increase in bone volume and improve of bone strength[14]. However, the effects of various mechanical loading on osteogenetic processes of tibial cancellous bone in rats is still unclear. The aim of this study was investigated the effects of various mechanical loading on osteogenetic processes of tibial cancellous bone in rats.

1. Materials and Methods

Animals and sampling

Sixty eight male Wistar rats, aged seven weeks were used as materials, and rats were assigned to sixteen groups, divided as follows: (1) 30% of the maximum jump height (E30-4, 7, 14, 21 days), (2) 45% of the maximum jump height (E45-4, 7, 14, 21 days), (3) 90% of the maximum jump height (E90-4, 7, 14, 21 days), (4) control groups (CO-4, 7, 14, 21 days). EX group performed jumping exercise to mention later during each experimental period. After the experiment period, I euthanized rats by carbon dioxide absorption. I exfoliated the skin of the rat hind leg, by removing as much as possible the soft tissue, were excited tibia and gastrocnemius muscles after confirmation by the death. Thereafter, left tibia length measured by calipers, and left gastrocnemius muscle weights were measured by electronic force balance immediately. Tibiae were immersed in karnovsky containing 4% paraformaldehyde and 5% glutaraldehyde or 4% paraformaldehyde immediately, and fixed treatment went, after I did sagittal fracture inside and outside basis the center of the tibia by a dental hand motor.

Jumping exercise

According to the methods of jump exercise of Umemura et al[15]. Jumping height was measured at the experimental starts, and 1 and 2 week from experimental starts. And, rats were jumped 100 times/day, 5 days/weeks for each experimental periods.

Histological, Enzyme histochemical analysis

Fixed specimens were decalcified by 8% EDTA (pH 7.2~7.4), were dehydrated, were cleared, and were embedded in paraffin wax, and made the perfect serial sections (thickness of about 4 μm).

Paraffin sections were deparaffinized, rinsed in distilled water, and incubated with a solution containing 0.01% naphthol-AS-MX-phosphate, 0.06% Fast Red violet LB salt, 50nm sodium tartrate, and 0.2m acetate buffer (pH 5.2) for 15-30 min at 37℃. Sections were counterstained with Mayer's haematoxylin. The stained specimens were observed by light microscope, and counted the positive cells (n=4). Measuring range was same as bone morphometric analysis to mention later.

Furthermore, the other specimens embedded in rigolac resin without decalcification were ground until thickness of about 100 micrometers. Those specimens were stained by Toluidine blue and observed with light microscope and performed bone morphometric analysis to mention later.

Bone morphometric measurement analyses

We prepared a rigolac resins that it is similar to mention above, and stained by Toluidine blue and measured each parameters using their specimens. The parameters were measured: bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). Measuring range was defined as the interior of the proximal tibial metaphysis 1.0 mm to 1.5 mm and 0.5 mm from the cortical bone (Fig. 1).

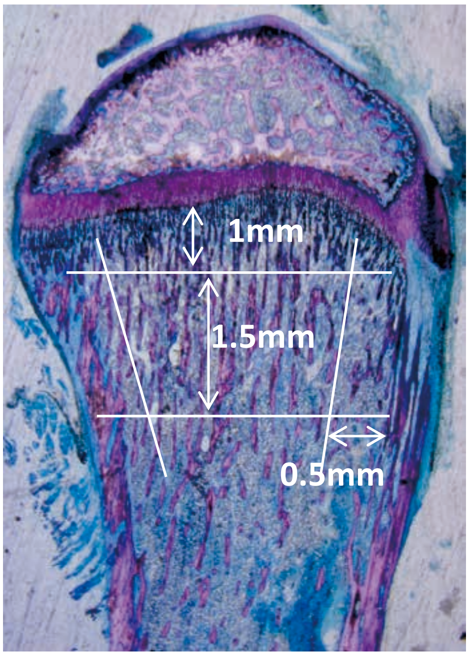

Fig 1. Bone histomorphometric measurement ranges in tibia

Statistical analysis

All data are expressed as means ±SD, assayed by IBM SPSS Statistics 21.0 software (IBM, Armonk, NY, USA), and compared by a one-way analysis of variance followed by

Tukey's post hoc analysis. The level of statistical significance was set at $p<0.05$.

2. Results

Body weight, tibia length and muscle weight

Table 1. Results of Body weight, tibia length and muscle weight in each group

		4days	7days	14days	21days
Body weight (g)	CO	242 ± 8.29	290 ± 15.43	370 ± 17.68	387.5 ± 15
	E30	278.75 ± 7.5	298.5 ± 14.01	332.5 ± 13.23	360 ± 44.16
	E45	296.25 ± 17.97*	307.5 ± 12.58	336.25 ± 28.39	362.5 ± 35.24
	E90	273.5 ± 10.6	306.25 ± 8.54	328.33 ± 40.49*	370 ± 29.44
Tibia length (mm)	CO	3.46 ± 0.06	3.67 ± 0.09	3.96 ± 0.12	4.05 ± 0.02
	E30	3.83 ± 0.08	3.89 ± 0.06	3.95 ± 0.08	4.03 ± 0.08
	E45	3.76 ± 0.15	3.86 ± 0.06	3.97 ± 0.12	4.08 ± 0.04
	E90	3.83 ± 0.13	3.86 ± 0.02	3.94 ± 0.08	4.03 ± 0.05
Muscle weight (g)	CO	1.3 ± 0.06	1.64 ± 0.19	1.97 ± 0.19	2.13 ± 0.17
	E30	1.78 ± 0.05	1.89 ± 0.22	2.15 ± 0.15	2.34 ± 0.13
	E45	1.79 ± 0.22	1.92 ± 0.07	2.25 ± 0.28	2.43 ± 0.29
	E90	1.78 ± 0.04	2.03 ± 0.25	2.09 ± 0.11	2.6 ± 0.21*

＊ : $p<0.05$ vs. CO groups

Table 1 shows body weight, the tibia length, and the weight of gastrocnemius muscles of rats in each group. Body weight was significantly higher in E45-4 than CO-4, whereas E90-14 showed a significantly low value in comparison with CO-14. Tibia length wasn't recognized difference in each group. Gastro weight was showed significantly high value in E90-21 compared with CO-21.

Bone morphometric analysis

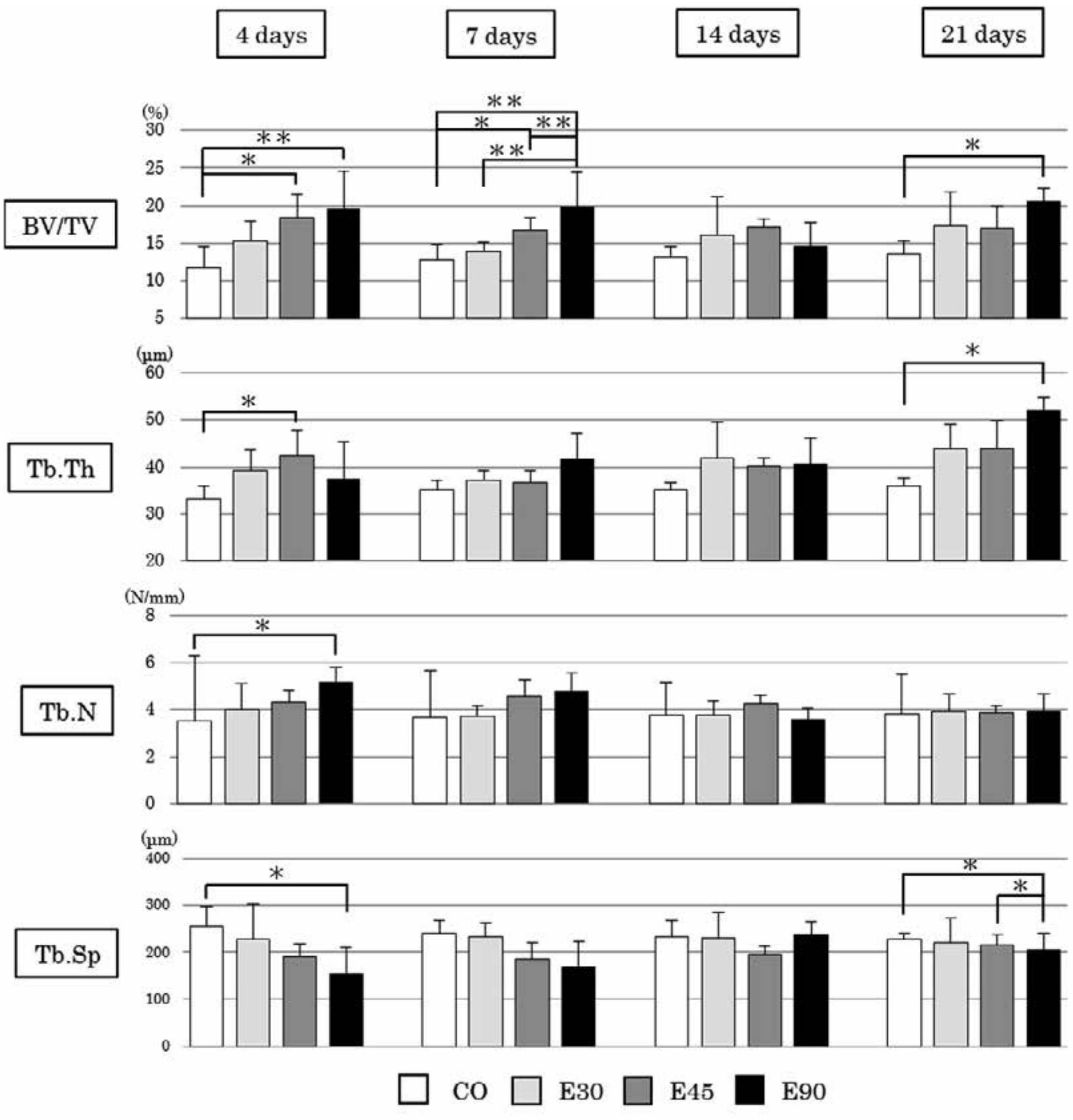

Fig. 2 Result of bone morphometric analysis in each groups.
＊: p<0.05, ＊＊: p<0.01

Figure 2 shows the results of bone morphometric analysis of the secondary cancellous bone in the proximal tibia.

BV/TV was significantly higher in E45-4, 7 and E90-4, 7 than CO. Furthermore, BV/TV of E90-7 was significantly higher than E30-7 and E45-7. But, didn't significantly differ among the experimental group at 21 days after the experiment start.

Tb.Th was significantly higher in the E45-4 than the CO-4, but wasn't recognized significantly differ in the E30-4 and E90-4. E45-4 showed the high value compared with E90-4. Thereafter, there were no differences in each groups at 7 and 14 days, but E90-21 showed high value compared with CO-21.

Tb.N was significantly higher in E90-4 than CO-4, but no significant difference after experimental period, and showed similar value at 21days later.

Values of Tb.Sp was lower in E90-4 compared with CO-4. Thereafter, recognized the variance of value of Tb.Sp during experimental period, and significantly lower value in E90-21 compared with CO-21 and E45-21.

TRAP positive cells

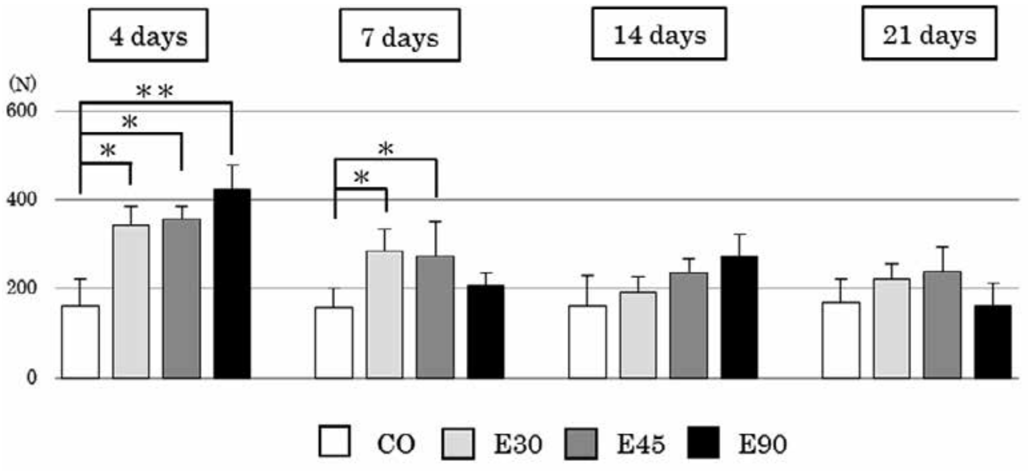

Fig. 3 Result of the number of TRAP positive cell in each groups.
＊: $p<0.05$, ＊＊: $p<0.01$

Figure 3 shows the results of the number of TRAP-positive cell of the secondary cancellous bone in the proximal tibia. The number of TRAP positive cells in all EX group were significantly increased than CO in experimental started 4days, and E30 and E45-7 showed high value compared with CO-7. However, no significantly difference comparison with CO after experimental period.

Mineral apposition rate

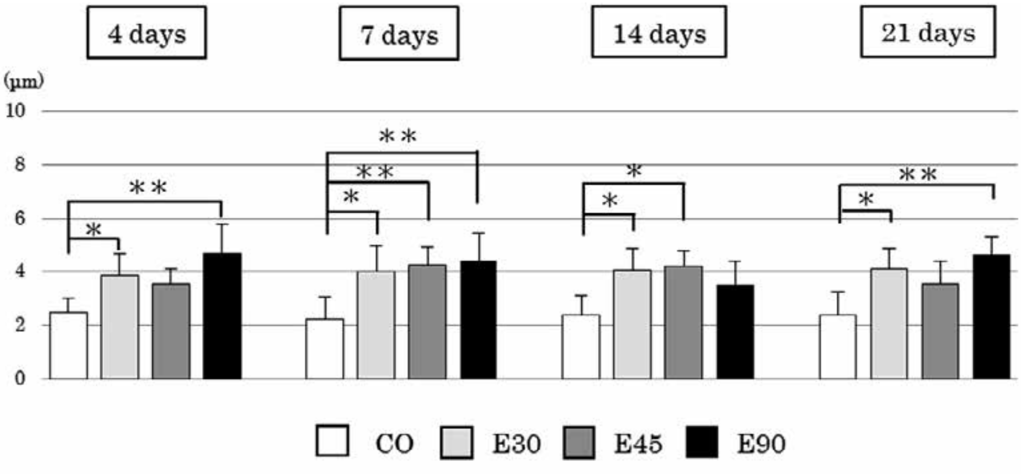

Fig. 4 Result of mineral apposition rate in each groups.
＊: $p<0.05$, ＊＊: $p<0.01$

Fig 4 shows the result of the mineral apposition rate (MAR) of the secondary cancellous bone in the proximal tibia. Mineral apposition rate was significantly increased in all experimental groups in E30, E45-7, 14, and E90-4, 21 than CO. But, it wasn't recognized significantly differ among all experimental groups.

Discussion

In this study, our objective was to investigate the effects of various mechanical loading on osteogenetic processes of tibial cancellous bone in rats.

Rubin and Lanyon[16] said that high strength exercise results in significant effects on the bone. In this experiment, BV/TV in E45 and E90 were significantly increased than CO in the start of the experiment early, and E90 was significantly higher than the other experimental groups in 7 days. These results suggested that high strength jump exercise were increased bone volume at early stage. Although, we didn't observed differences between experimental groups in 21 days later. Nagasawa et al. [14] were investigated the effects of different strength (30, 40 and 50 cm) jump training (40 times/day, 5 days/week, 4 weeks) in rat tibia, and reported that bone mass and bone strength weren't differences between the 40 cm and 50 cm jumping exercise groups. According to Nagasawa's report and our results, it is suggested that low strength exercise was gradually increased bone volume.

Ju et al. [17] were investigated the influence of jump training on femoral bone volume in rats, and found that jump training increased Tb.Th and bone volume. In our results, BV/TV and Tb.Th were significantly higher in E90 than CO in 21 days. The study in which a performed high strength drop-jump was reported increasing of Tb.Th and bone mineral density[18]. Also, Notomi et al. [19] reported that 8 weeks resistance training caused increasing of Tb.Th and bone mineral density. According to the experiment of mechanical loading on tibia and fibula in mouse, were recognized increasing Tb.Th in more high strength experimental groups[20]. According to these and our results, it is understood that high BV/TV in high strength exercise group depends on the Tb.Th.

Tb.Th of E90 was lower value compared with CO at early stage, on the other hand, Tb.N of E90 was significantly increased than CO. There are reports that running exercise was increased bone volume accompany make increased Tb.Th[21, 22]. Increasing Tb.N by continuous stimulation exercises (running, swimming…etc), but no evidence about Tb.Th. It is suggested that mechanical stress on bone by sustainable exercise (running and swimming…etc) is low. On the other hand, jump training particularly high strength exercise such as E90, confer the more impact on bone, and increasing Tb.Th. Moreover, changes with growth on cancellous bone in spine[23], femur[24], and tibia[25], increasing of Tb.N and Tb.Th according to growing. Actually, in this experiment, Tb.N was no significantly differ during 7-10 weeks, but bone volume increased by Tb.Th increasing. These facts suggested that increasing Tb.Th is necessary for resistance to large mechanical stress, and dispersed on surrounding cortical bone. Tb.N of E90-4 show high value compared with another groups, but Tb.Sp of E90 in same period, was lower than the other groups and it was low generally afterwards. Thus, high Tb.N and narrow Tb.Sp in E90 at early stage, it meaning the high BV/TV. The results of TRAP staining, recognize increasing number of TRAP-positive cells, but markedly decreased after experiment. This, in order to remodeling suitable direction for disperse mechanical stress arrangement bone trabecular, accompanied with increasing mechanical stress, and it is thought to be increased trabecular thickness after obtained such a suitable arrangement direction.

Generally, it is said that the mechanical stress to bone was detected in osteocytes[26-28], and suppressed the bone resorption by exercise stimulation[29]. Running exercise (4 days/week, 8 weeks) was increased bone volume, and reduced serum of TRAP levels, eroded surface[8]. And, 2 weeks climbing exercise was reduced multinuclear osteoclasts[5]. Ju et al. reported that jumping exercise reduced eroded surface in rat femur. In the present study, it was recognized more TRAP-positive cell in E30 and E45-21 than CO-21, but not in E90. Although, there are few reports investigating the appearance osteoclast in early stage of experiment, and, in this study, there are a lot of osteoclasts comparison of CO at experimental start 4 days later. Some possibility is thought about from this result, microfracture of bone trabeculae be caused by excess mechanical stress. Osteocytes in bone trabecular which caused a microcrack died, and released RANKL[30]. And, RANKL induced differentiation and activation of osteoclasts in bone marrow[31]. That is, from these facts, it is suggested that increasing stimulation of RANKL accompanied with microcrack and osteocyte's death induced increasing the number of TRAP-positive cell. Although, in this study, we didn't perform histological examination, and we need further investigation.

The study performed jump exercise for mouse, and reported increasing mineral apposition rate in tibial cortical bone[32]. In this present study, mineral apposition rate of E30 showed significantly high value compared to CO, and E45-7, -14 and E90-4, -21 comparison with CO, similarly to E30. Yuki et al. [3] performed 4 weeks jumping exercise to growing rats, reported increasing of Runx2-positive reaction cells, and upregulation of mRNA levels of osterix and BMP-4 in bone marrow. Also, Kodama et al. [32] investigated the effect of jumping exercise on mouse tibia, and reported that jumping exercise made levels of serum IGF-1 increasing. Another, it has been reported that mRNA levels of IGF-1 in rat tibial cortical bone were increased by increasing mechanical stress with four-point bending model[33]. In the present study, show high BV/TV from the experiment start early days in EX, and it is suggested that increasing mechanical stress impact on differentiation and activation of osteoblast. In our previous study, we reported that TGF-β were dispersed into bone marrow by temporary bone resorption accompanied with immobilization[34]. It is suggested that factor of differentiation and activation of osteoblasts, like TGF-β and BMP, were released to bone marrow by osteoclast's bone resorption at early stage.

3. Conclusion

The different strength exercise advance osteogenetic processes in early stage, but eventual bone structure is similar to low strength exercise.

Committee of Animal Experiment and Ethics

This study was approved by committee of Animal Experiment and Ethics for the research, Graduate School of Welfare Society design, Toyo University.

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異なる強度の加重がラット脛骨の骨形成過程に及ぼす影響

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要　約

本研究では、異なる運動強度がラット脛骨の骨形成過程に及ぼす影響について検討することを目的とした。

材料として7週齢のWistar系雄性ラット68匹を用い、それらを無作為に実験群（EX）および対照群（CO）に分類した。さらにEX群は各ラットの体重および最大跳躍高が群間でほぼ均等に成るように3群（EX30、EX45およびEX90）に分類した。実験群には最大跳躍高の30、45および90%の跳躍運動を100回/日、5日/週の頻度で、4、7、14および21日間行わせた。実験期間終了後、各群のラットから脛骨を摘出して種々の標本を作製し、組織学的、酵素組織化学的および形態計測学的に観察、計測した。

E45およびE90は、実験開始初期の段階から高い骨密度を示し、E45-4では骨梁幅の増加、E90-4では骨梁数の増加が認められた。同様の時期には、TRAP反応陽性細胞数もCOと比較して有意に多数認められ、石灰化速度も上昇していた。実験開始21日後には、E90の骨密度および骨梁幅においてのみCOと有意差を認めた。

これらの結果から、異なる強度の運動を行わせた場合、高強度運動群では早期に骨形成が進められるが、最終的な骨構造は低強度運動と同様であることが理解された。