The Morphological Measurements with a Micro CT and the Stress Analyses of the Adaptive Remodeling by Applied Mechanical Stimuli in Rat Caudal Vertebrae*

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To observe the remodeling process in bone adapted to mechanical stimuli, a microcomputed tomography (micro CT) system with a high spatial resolution was utilized. The rat fifth caudal vertebrae (C5) were subjected to daily axial or offset loads. Morphological alterations of C5 were measured periodically and non-invasively with the micro CT between daily mechanical stimuli for 14 days. The finite element (FE) models were built from the binary cross-sectional images of cortex and trabecular bone architecture of C5. Von Mises stresses were calculated by applying the contact pressures from the rigid-body spring model (RBSM) analyses to the FE-models as boundary conditions. The transformations of cortex and the increases of the cortical area were observed in the loaded rats. FE-analyses in the offset loaded rat indicated that von Mises stress distributed uniformly under the analytical offset loaded condition. This fact suggests that the C5 would adapt to the offset loads applied externally by morphological alterations.

**Key Words**: Biomechanics, Adaptive Structure,Finite Element Model, Bone Remodeling, Mechanical Stimulus, Microcomputed Tomography, Stress Analysis, Stress Distribution

1. Introduction

It has been proposed that the mechanical environment is one of the determinants of modeling or remodeling in bone(1,2). Von Meyer(3) and Culmann had started to observe the cancellous bone architecture related to the mechanical environment. They stated that the trabeculae were reoriented through remodeling so that they aligned with the new principal stress trajectories when trauma or pathology changed the external loads on the bone. Most of studies about mechanical adaptations have been restricted to the effects of disuse, exercise and overuse of bone for a long time. This was primarily attributable to the fact that the experimental systems, in which bone could be isolated from its normal mechanical environment and could be exposed to the quantifiable stimuli, had not yet been established. More recently, some researchers have proposed animal models, where the alteration of bone morphology has been evaluated when the artificial stimuli were quantitatively subjected to the bone. These animal models are roughly classified into two different types of strategies. One is that the compressive force was applied using pins inserted into the bone through the skin(4)-(5). To stimulate bone non-surgically in another models, the bending or compressive force was subjected to the long bone by pressing soft pads on the skin(6)-(9). Both studies have focused on the influences of the specific mechanical factors; peak strain magnitude, strain density, strain frequency, strain rate, number of load cycles, and
duration of loading. In those previous studies, tissue sectioning, gene expression assay and histological analysis by soft X-ray were performed for animals sacrificed periodically through the experiments. The alterations of bone were discussed statistically. In the present study, we have observed the same site periodically and non-invasively with a microcomputed tomography (micro CT) in the same animal applied with mechanical stimuli.

Noninvasive measuring equipment for bone morphology has made remarkable progress such as a quantitative computed tomography (QCT)\textsuperscript{(14)}, micro CT\textsuperscript{(15)}\textsuperscript{(16)}, synchrotron CT\textsuperscript{(17)} and micro magnetic resonance imaging (micro MRI)\textsuperscript{(18)}. Many studies have been reported where the trabecular bone architectures were measured with such equipment. However, those measurements have been carried out \textit{ex vivo} in most studies except for the work of Kinney et al.\textsuperscript{(17)}, who used synchrotron CT. Some equipment has a quite enough spatial resolution to describe trabecular bone architecture of small animals, like rats, whose trabecular width averages under 100 μm and trabecular separation averages about 150 μm. The spatial resolution has a great influence on the accuracy of the measured feature of the trabecular architecture. If both resolution and trabecular width has the same dimension, an error of one pixel will be detected as a 100 percent error in the measured trabecular width. We developed a micro CT system that could measure the trabecular architecture of rats non-invasively with an adequate resolution, which was at least 1/3rd of the dimension of the interested features\textsuperscript{(19)}.

The purpose of this study was to evaluate computationally the alteration of the stress distribution by using a finite element analysis (FEA) based on the micro CT images when the fifth caudal vertebrae (C5) of rats were subjected to the mechanical loading regime. The morphological alterations of C5 were measured non-invasively and periodically using a micro CT system. This micro CT system has a high spatial resolution with a range variable from 26 μm/pixel to 260 μm/pixel, and is able to describe the trabecular bone architecture in small animals. Axial loads or offset loads were applied to C5 using pins inserted into C4 and C6\textsuperscript{(20)}\textsuperscript{(23)}. We discuss the relationships between the responses of bone and the forms of applying loads. In addition, the stress distributions in C5 under adaptation were calculated by using a FEA together with a rigid-body spring model (RBSM). FE-models were made by extracting the cortex and trabecular morphometry from the micro CT images. Contact pressures on the intervertebral joint surfaces of C5 were estimated from RBSM. The von Mises stress distributions were calculated using these contact pressures as boundary conditions for FEM.

2. Methods

2.1 Experimental systems of mechanical stimulation to rat C5 and morphological measurements with a micro CT

Sprague-Dawley female rats were used for objects. They were purchased in the week preceding the experiment and provided food and water \textit{ad libitum}. Three rats that became 18 weeks old were divided as follows. (I) Axial loaded rat. (II) Offset loaded rat. (III) Non-loaded rat as control. Stainless steel pins with a 1.2 mm diameter were inserted into the transverse axes of C4 and C6 through the skin of every rat before the experiment. The positions to be inserted pins were determined using an X-ray fluoroscopy and marked with the fine stainless steel wires wound around the rat’s tail. Pins were driven with a hand-held drill and transfixed into the center of the vertebral body length. We confirmed the success of insertion with using the X-ray fluoroscopy again. These preparations were made under general anesthesia of pentobarbital sodium (30 mg/kg). These pins were used for the loading to C5.

The median plane of C5 was measured non-invasively using a micro CT system (MCT-1250SMF, HITACHI MEDICO, Tokyo, Japan) on the next day. The spatial resolution of the micro CT increases with geometric magnification. A higher resolution can be obtained for the specimen positioned closer to the X-ray source. The resolution was determined to be 167 μm/pixel for this \textit{in vivo} measurement because the dimension of the rat body restricted the distance from measured C5 to the X-ray source. The rat’s tail was settled on the rotating platform of the micro CT by means of a fixing jig. The position of vertebral body was arranged to get the longitudinal axis of C5 parallel to the measured plane of the micro CT when C5 was observed along ventral/dorsal direction by means of X-ray fluoroscopy. Since the median plane lies between bilateral processes of cranial side of C5, the hollow site between these was adjusted to the measured plane through the cranial/caudal observation. This procedure enabled to determine the correct slice position to get median plane of C5. It took about nine minutes to get a cross-sectional image. The exposing time of X-ray was less than two minutes in the measurement. Therefore, we thought that the radiation had no measurable effect on experimental animals.

The rats were settled on a loading apparatus for mechanical stimulation after the micro CT measurements (Fig. 1). The maximum load in each cycle was controlled by feedback with strain gauges. The C5 of
(I) and (II) rats were subjected, with pins inserted into C4 and C6, to a daily loading period of 3600 consecutive 1.0 Hz load cycles of sinusoidal-wave-like compressive stimuli whose magnitude was 5.0 N. In the case of (I), pins were loaded normally on the same plane. In the case of (II), the pin, which was inserted into C4, was displaced for 4 mm toward the ventral direction and was reciprocated along cranial/caudal direction on that plane (Fig. 2). The animals were anesthetized with pentobarbital sodium for about 70 minutes during measurements and loading. The control rat was also anesthetized with the same amount of anesthesia as the loaded rats. Applying the load of 5 N produced a longitudinal strain of about 25 με on the cortical surface of C5. This measurement was carried out for other two rats, which were the same age and almost the same weight to the loaded rats, by sticking strain gauges directly on the vertebral surface.

These micro CT measurements and mechanical stimulation were repeated daily for 14 days. Then, binary cross-sectional images of caudal cortex were obtained after using an edge-enhanced smoothing filter and thresholded processes\(^{(26)}\). The periodic changes of cross-sectional area of cortex were compared in three rats.

2.2 Stress analyses with rigid-body spring models and finite element models

The animals were sacrificed by overdose of pentobarbital sodium at the end of the measurements in vivo and loading protocol, and then C4, C5 and C6 were removed. Next, the median plane of C4 and C5 was measured ex vivo with the micro CT. The spatial resolution of 35 μm/pixel was selected in these measurements. Binary cross-sectional images of caudal cortex and trabecular bone architecture were obtained using the same edge-enhanced smoothing filter and thresholded processes. The outward appearances of the cortical cross-sectional images were digitized using a digitizer (MITABLET-II KD3030, GRAPHTEC, Japan). Those features were used for constructing two-dimensional RBSMs\(^{(26)}\). The compressive and shear springs, with a stiffness ratio of 0.001, were applied normally to the intervertebral joint surfaces between C4 and C5, and the extension springs, equivalent to the ligaments or capsule, were applied to the dorsal and ventral sides of these models. When the reaction forces of each spring were calculated, a unit compressive load was applied to the center of gravity on the longitudinal axis of C4. The contact pressures applied to the intervertebral joint surface of C5 were calculated after multiplying each reaction force and 5.0 N together. These RBSM were adopted about (I), (II) and (III) rats fitting the longitudinal axis of C4 with that of C5 under the analytical axial loads applied, and translating the longitudinal axis of C4 into the ventral direction of 2 mm under the analytical offset loads applied (Fig. 3). The rotation around the center of gravity and the
movements along the cranial/caudal and ventral/dorsal direction were free in C4 while both rotation and movements were fixed in C5. The distance between C4 and C5 was determined from the X-ray fluoroscopy of each tail of rats.

Next, the FE-models for the cortex with and without trabecular bone were built from the binary cross-sectional images of cortex and trabecular bone architecture of C5. The trabeculae that lost the connectivity with around cortex or trabecular bone were excluded from models. FE-models consisted of about 4,800~5,500 elements. FE-analyses about C5 were performed, and von Mises stresses were calculated using the contact pressures from the RBSM analyses for the boundary conditions. The displacement of the bottom of the dorsal cortex was fixed while the fixed cranial/caudal displacement and free ventral/dorsal were conditioned at the bottom of the ventral cortex in these analyses. We used 11.0 GPa of Young’s modulus and 0.3 of Poisson ratio (μ = 0.3). The FE-analyses were performed using a COSMOS/M (Structural Research and Analysis Corporation, Santa Monica, CA).

3. Results

It was possible to carry out the periodic measurements in vivo of the morphological alterations of the median plane in C5 cortex using the micro CT. Figure 4 shows the micro CT images of C5 measured with the resolution of 167 μm/pixel before and after the experiment. No morphological difference was found in the axial loaded rat, the offset loaded rat and the control rat before applying mechanical stimuli. The control rat without loading had very little morphological alteration in cortex after 14 days. On the other hand, the transformations of cortex and the formations of new bone were observed in the axial loaded rat and the offset loaded rat. Especially, applying the offset loads enhanced to form spur-like bones. Such transformations became visually significant on the fifth day from the onset of mechanical loading.

Figure 5 shows the changes in cross-sectional area of cortex through the experiment obtained from binary images of the median plane of C5. The increasing ratio of the cross-sectional area in the axial loaded rat, the offset loaded rat and the control rat were 22.5, 20.0 and 6.5 percent respectively on the 14th day. These indicate that an appositional modeling was probably activated by mechanical stimulation in the loaded rat. However, few differences in the ratio of increase were found between the axial loaded rat and the offset loaded rat.

The median plane images of C5 measured ex vivo after the experiment are shown in Fig. 6. Regions with low CT density were observed at the dorsal and ventral periosteum in the axial loaded rat and the offset loaded rat. It is likely that those mean the

Fig. 4 Loading direction and morphological alterations of cross section in C5. The control rat without loading showed very little morphological alterations in the cortex after the experiment. The transformations of the cortex and the formations of new bone were observed in the loaded rat.

Fig. 5 Changes in cross-sectional area of cortex through the experiment. These areas were calculated from binary images of the median plane in C5.

Fig. 6 Micro CT images of the median plane of C5 measured ex vivo after the experiment. Regions with low CT density were observed at the dorsal and ventral periosteum in loaded rats (arrows).
formations of woven bone in which mineralization is immature. The following RBSM and FE-analyses were carried out except for those regions.

The representations of von Mises stress distributions in the FE-models containing the cortex and the trabecular bone are shown in Fig. 7. The contact pressures calculated from RBSM are also indicated as arrows in this figure. In the case of the axial loaded rat and the control rat under the analytical axial loading conditions, the stress levels in the cortical sites were low and nearly uniform as shown in Figs. 7(a) and (c), although the stress concentrations were seen in the trabecular bone sites. The offset loaded rat showed high stress sites in the cortex as well as the trabecular bone in Fig. 7(b), which was the result under the analytical axial loading condition. On the other hand, the stress distributions in the axial loaded rat and the control rat, in the case of the offset loading condition applied analytically, showed much asymmetry as in Figs. 7(d) and (f). Since the center of the applied load was translated toward the ventral direction in these analyses, high stress regions were seen on the left side of the figures, also known as the ventral. It is noticed that the asymmetry was small in the offset loaded rat as seen in Fig. 7(e), and the stress distribution almost uniform relative to other rats.

Figure 8 demonstrates the results of analyses using models without trabecular bone. Compared with the results of models containing the cortex and the trabecular bone, high stress sites were seen in all cases. It indicates that the trabecular bone architectures have an important role in the load carrying systems. In the case of the axial loading condition applied analytically, the stress levels in the offset loaded rat of Fig. 8(b) were higher than those in others as shown in Figs. 8(a) and (c). The asymmetry of the stress distributions was relatively large in the offset loaded rat of Fig. 8(e), as well as in the axial loaded rat and the control rat as Figs. 8(d) and (f) under the analytical offset loading conditions show.

4. Discussion

The micro CT used in this study has a high spatial resolution enough to describe the trabecular bone architecture. The method was established to measure the caudal vertebrae of rats non-invasively under an anesthetic, although the spatial resolution was limited in the measurements in vivo. Since the same site of the same animal could be periodically observed to evaluate the alteration of bone, for proper statistical
Fig. 8 Von Mises stress distributions as the results of FE-models without trabecular bone.

(a): an axial loaded rat under the axial loading boundary condition applied. (b): an offset loaded rat under the axial loading boundary condition applied. (c): a control rat under the axial loading boundary condition applied. (d): an axial loaded rat under the offset loading boundary condition applied. (e): an offset loaded rat under the offset loading boundary condition applied. (f): a control rat under the offset loading boundary condition applied. Arrows indicate the contact pressure calculated from RBSM.

analysis it was not necessary to sacrifice many animals. Double-labeled fluorescence has been traditionally used for observations of turnover in bone. However, it is impossible to know the rate of bone resorption in this method because the initial label would disappear due to resorption. Both formation rate and resorption rate can be obtained by periodic observations using the micro CT. Considering the recent progress and spread of noninvasive measuring equipment, the establishment of this experimental system will be of great significance.

Mechanical stimuli with pins inserted into C4 and C6 were applied to C5. The disturbances brought by factors other than mechanical stimulation would be little because the C5, as observed bone, suffered no direct surgical interference. Pins were free on purpose in order to keep the mechanical environments physiologic between episodes of loading. Fixing of pins can isolate the bone from daily activities. However, there is a good possibility that undesired error of fixing would keep bone nonphysiological and might lead to pathologic states. Other animal models had been proposed to investigate the bone responses adapted to mechanical stimuli. However, they had some defects, and determining the relationship between the responses of bone and the conditions of mechanical stimulation proved difficult. Rubin and Lanyon established an experimental model where turkey ulna was functionally isolated by surgical treatment, and pins, inserted into each end of the preparation, were used for loading\textsuperscript{(10)}. However, the bone responses might have been influenced by surgical interference, or by inserting pins into observed bone itself, that is by factors except mechanical stimulation. Turner et al. developed other models without a surgical treatment at all, in which the rat tibia was loaded by pads through soft tissues using a four-point loading apparatus\textsuperscript{(10)-(12)}. This model is seen to be excellent in that undesirable factors except the mechanical stimuli influencing bone remodeling were completely removed. They related bone responses in the loaded animals to strains measured beforehand in other individuals. The indirect loading through soft tissues, which differ largely between individuals, might give rise to large differences between the actual strains generated in the loaded individuals and the calculated strains in others.

Bone remodeling is considered to be influenced by the level and distribution of the functional strains within bone. The strain parameters were measured in vivo with strain gauges set on the bone surface and they were corresponded to the remodeling phenomena.
in most of the studies. We measured the longitudinal strain of 25 με on the C5 surface by the axial load of 5.0 N in our study. This measurement was carried out by setting strain gauges directly on the site between ventral and lateral bone surfaces. The region where the strain of 25 με generated would not correspond with the ventral and dorsal cortical surfaces where the new bone was formed. The increases of cross-sectional area in C5 were observed in our study despite of the relatively small strain. There were no reports that the new bone formation was observed under such a small strain. Mosley et al. stated that the mechanical stimuli of 1 000 με or 2 000 με showed suppression in the rate of periosteal new bone deposition\textsuperscript{[13]}. This generated strain resembled the physiological strain level in the rat ulna of the weight-bearing bone. No discernible response was seen at 500 με. Similarly, the original bone area was retained at 1 000 με, and strains smaller than that were associated with a decrease in osteogenic activity in the studies using turkey ulna by Rubin and Lanyon\textsuperscript{[7]}. In addition, Chow et al. found a significant increase of the bone volume in rat C8 subjected to load of 150 N\textsuperscript{[21]} although the rat C8 was not weight-bearing bone. They stated that the load of 150 N limited the maximum strain to 700 με, which was similar to the strain observed during relatively gentle bone usage, for example, walking. They observed no increase under a load of 30 N, producing a strain of 140 με. However, it is impossible to simply compare our result with the previous studies, because there are some important differences in the experimental conditions. First, the caudal vertebrae were subjected to the different loading regime. The C8 in the studies of Chow et al. was applied to the loading with the rather short duration and cycles, for instance, single load of 300 cycles, daily loading with 30 cycles for 9 days. Our loading regime was much longer compared with their studies although our maximum load was small. Second, inserted pins were free between episodes of loading in our animal model, which was discussed above. Third, the caudal vertebrae do not experience the loads that support their own bodies. There is a remote possibility that the rat caudal vertebra undergoes the load of 5.0 N in her daily life, which is almost twice the load of the body weight of rat herself. Therefore, when the load of 5.0 N was sensed nonphysiological for the caudal vertebrae, the new woven bone would be formed in C5 responded to the even smaller strain.

The stress analyses brought some suggestions that bone morphological alterations by mechanical stimulation influenced the stress distributions within cortex and trabecular bone. As for the results against the analytical offset loading conditions, only the offset loaded rat did not show any high stress sites in the cortex and trabecular bone models. The stress distributions were asymmetrical between the dorsal and ventral sides in the axial loaded rat and the control rat. The analyses of the cortex models without trabecular bone showed similar stress distributions among three rats. This indicates that the adaptation against the offset loads mainly depends on the alteration in the trabecular architecture. Under the analytically axial loading conditions, the offset loaded rat showed high stress concentrations in the trabecular bone. These high stress sites did not appear when the trabecular bone would not alter its architecture. This is further evidence of the alteration in the trabecular architecture. It seems reasonable to suppose that the adaptation against the offset loads depends on the alteration in the trabecular bone where the metabolic rate is generally said to be high compared with the cortex. The vertebral body would adapt to the external mechanical load, as the stress concentrated sites in the trabecular bone were distributed, through the remodeling of its architecture.

Rats lack a secondary haversian remodeling in their cortex and are regarded as modeling animals. Therefore, the bone formation is not coupled to bone resorption, and bone formation and resorption in a specific site are independent each other. In addition, the endocortical bone forms less bone than absorbed and the bone formation rate is usually higher than the bone resorption rate at the periosteum, which follows the increase of inside and outside diameters in the cortex. However, there are some arguments questioning whether the remodeling is observed in the trabecular bone of rat\textsuperscript{[31]}. Baron et al. showed the evidence of sequential remodeling in the trabecular bone in rat caudal vertebrae by histological analysis\textsuperscript{[32]}. Erben suggested that the prevailing activity in cancellous bone of aged rats was remodeling in the lumbar vertebral body\textsuperscript{[33]}. This evidence of remodeling was proved by observations of scalloped reversal cement lines that indicated new bone formed on a site of previous resorption. In our study, the C5 in the offset loaded rat adapted to the applied offset loads and had no stress concentrations within bone as shown in Fig. 7(e). Figure 7(b) showed the high stress sites against the analytical axial loading conditions although the C5 in the offset loaded rat was considered to be strengthened by increasing the cross-sectional area of cortex. It is likely that the adaptation in C5 of the offset loaded rat was not progressed by the appositional bone modeling but by the bone remodeling accompanied by bone resorption.

Our study had some simplifications and assumption to be noted. First, the findings of this study are
restricted to the responses of only one rat in each group. Although it is true that measuring the same animals for multiple time points would reduce the number of animals required, some statistical analyses will be necessary to assess the reproducibility of the responses of the individual rat to the mechanical stimuli. Second, FE-analysis has performed only for the two-dimensional analytical model. It would seem that analysis in two-dimension was too simplify to evaluate the stresses in vertebral body whose trabecular bone had complicated three-dimensional connectivity. Some researchers calculated the tissue stresses in detailed three-dimensional architecture of bone samples. Large-scale three-dimensional models of the whole bone have some difficulties in analysis, that is, the difficulty in the determination of the boundary conditions, or the limitation of the computing ability. Even in our two-dimensional analysis, it was qualitatively indicated that the stress concentrations tended to distribute because of the mechanical adaptation of the stimulated vertebrae. Third, the combination method of RBSM and FEM was used to calculate the contact pressure distribution on the surface of the intervertebral joints. The merits of this method are its ease of modeling, short calculating, and improved convergence. According to Miura et al., results of the combination method were equivalent to those of conventional FEM for the two-dimensional two body contact problems simulating the non-conforming and conforming joints. The contact pressure that was accurate quantitatively in vivo could not be indicated in our study because analytical models were in two-dimensional. Therefore, only qualitative results were shown in RBSM and FEM-analyses.

Notwithstanding its limitations, it was demonstrated in our study that the computational analyses based on the micro-CT images could evaluate the alteration of the stress distribution in the rat caudal vertebra when it was subjected to the daily cyclic loading regime. These techniques would utilize for assessing the alteration of the stress distribution and the morphological changes related to the mechanical environment, and provide a better understanding of the adaptive remodeling processes in bone.

5. Conclusions

In concluding, we developed a method to periodically measure the rat caudal vertebrae during applied mechanical stimulation by using the micro CT. Since these measurements were carried out non-invasively, we could observe the morphological alterations in the same site of the same rat.

A marked increase of the cross-sectional area was observed in both the axial loaded rat and the offset loaded rat. The appositional generation of bone appears to be activated by mechanical stimuli.

A low stress concentration was seen in the offset loaded rat under the analytical offset loaded condition compared with other rats. The C5 responded to daily offset loading by progressively restructuring to adapt to the new mechanical conditions.

References


