Abstract: This study examined the effects of a bite-jumping appliance combined with low-intensity pulsed ultrasound (LIPUS) stimulation on the mandibular condyle of growing rats using micro CT (mCT) and histological examinations. Twelve Wistar rats were divided into three groups of four individuals each: Group 1 was an untreated control group, Group 2 received bite-jumping appliances, and Group 3 received bite-jumping appliances and LIPUS stimulation (15 min/day, 2 weeks) to the temporomandibular region. We measured the length and three-dimensional bone volume of each rat's mandibular condyle using mCT. The condylar cartilage was observed after the rats had been sacrificed. There was no significant difference in condylar sagittal width among the groups. The bite-jumping appliance combined with LIPUS stimulation increased the condylar major axis, mandibular sagittal length and condylar bone volume to a greater degree than use of the bite-jumping appliance alone. Histological examination demonstrated hypertrophy of the condylar cartilage layers, the fibrous layer and hypertrophic cell layer of the rats treated with bite-jumping appliances combined with LIPUS stimulation in comparison to rats treated with bite-jumping appliances alone. (J Oral Sci 58, 415-422, 2016) Keywords: orthodontics, orthopedics, functional appliance, LIPUS.

Introduction
In clinical orthodontics, functional devices such as bite-jumping appliances and activators are widely used for correction of Class II malocclusion (1). However, there is some controversy about the effectiveness of such appliances. Tulloch et al. (2) investigated the effectiveness of early (preadolescent) and later (adolescent) treatment for children with severe Class II malocclusion using either headgear or a functional appliance. Comparison of previously treated children and untreated controls after the second phase revealed that early treatment had little effect on subsequent treatment. Pancherz et al. (3) followed patients for 32 years after therapy with a banded Herbst appliance and reported that only 64% of the subjects had a stable sagittal jaw relationship. On the other hand, Xiong et al. (4) investigated changes in mandibular morphology of adult rats using fixed bite-jumping devices and reported that bite jumping of the mandible in adult rats induced changes in the size and angulation of the condylar process. According to Franchi et al. (5), treatment of Class II malocclusion with functional appliances followed by fixed appliances produced a significantly greater increase in total mandibular length and mandibular ramus height during the pubertal peak. Abtahi et al. (6) reported that functional appliances enhanced mandibular growth in rabbits, especially when combined with mechanical stimulation such as irradiation with low-level lasers, which increased bone formation in the condylar region and was accompanied by an increase...
in cartilage thickness and fibrous tissues. Currently, a number of mechanical stimulation therapies are available to promote bone growth, including lasers, shortwave, micro-vibration devices, and low-intensity pulsed ultrasound (LIPUS). Nishimura et al. (7) reported that micro-vibrations with a resonance frequency of 60 Hz applied through the periodontal membrane to the teeth promoted tooth movement. However, for promotion of mandibular growth, it is difficult to administer sufficient micro-vibration from the skin surface. The deep-reaching effect of LIPUS has been demonstrated in the orthopedic field, where LIPUS administered at the skin surface has been shown to promote healing of bone fractures (8). In the orthodontic field, Okşayan et al. (9,10) have reported that the use of a bite-jumping appliance can stimulate condylar growth and increase sagittal mandibular advancement in growing rats. Furthermore, they reported that use of an intraoral appliance with low-level laser therapy stimulated condylar growth and increased mandibular advancement. El-Bialy et al. (11) have reported that LIPUS stimulation promoted mandibular growth in rabbits, and Khan et al. (12) found that treatment with growth hormone and LIPUS application led to a significant increase of hemimandibular bone volume and surface area. Kaur et al. (13) also reported that basic fibroblast growth factor (bFGF) and LIPUS had an additive effect on mandibular growth. In contrast, Ogurtan et al. (14) described that LIPUS stimulation had no effect on the anterolateral growth plates of rabbits. Therefore, the growth-promoting effect of LIPUS stimulation in the epiphyseal region remains uncertain. In addition, in a number of these previous studies (11,14), morphological examinations were performed using cephalometric radiographs or computed tomography (CT) images after the rats had been sacrificed. In vivo microCT (mCT) is a recently developed technique that allows multiple longitudinal observations to be made in the same individual (15). mCT can yield clear bone images of small animals with an exposure time of only 17 s, whereas ‘traditional’ CT requires several minutes (16).

The purpose of the present study was to investigate the growth-promoting effect of LIPUS stimulation on the rat mandibular condyle with combined use of a bite-jumping appliance. Here, the condyles were measured longitudinally using mCT and the condylar cartilage was observed after the rats had been sacrificed. As described by Kuroki et al. (17), three-dimensional changes in the volume of the condyle were examined to compensate for any errors in two-dimensional linear measurement. Linear measurements and three-dimensional bone volume analysis using mCT allowed objective evaluation of time-dependent changes in condylar growth.

Materials and Methods

Animals
Twelve male Wistar rats (aged 8 weeks; body weight at the beginning of the experiment = 210-260 g) were used in this study. The animals were divided into three groups of four rats each. Group 1 was an untreated control group (control group). Group 2 rats received a bite-jumping appliance (BJ group). Group 3 rats received a bite-jumping appliance and LIPUS stimulation of the temporomandibular region (BJ+LIPUS group). The experiments were approved by the Animal Experimentation Committee of Nihon University School of Dentistry, Japan (AP14D016).

Bite-jumping appliance
Bite-jumping appliances were fixed to the upper and lower incisors of animals in the experimental groups, as described by Xiong et al. (4). The appliance produced a vertical displacement of 1-2 mm and a 4-mm anterior advancement of the mandible (Fig. 1).

LIPUS stimulation
In the BJ+LIPUS group, the right and left temporomandibular regions were stimulated using an ultrasonic generator (BR-Sonic Pro; Ito Chou-tampa Co. Ltd., Tokyo, Japan). LIPUS was applied with an average spatial intensity of 30 mW/cm², frequency 3.0 MHz, and a duty cycle of 20% after application of medical ultrasound gel to the shaved skin surrounding the temporomandibular joint (18). The temporomandibular region was stimulated with LIPUS for 15 minutes/day for 14 days under anesthesia with sodium pentobarbital (50 mg/kg body weight).
mCT analysis

An in vivo mCT examination was performed on days 0, 3, 5, 7, and 14 (R_mCT2 system; Rigaku Co., Tokyo, Japan). CT images were obtained at ×2.0 magnification with an X-ray energy of 90 kV, an X-ray intensity of 100 mA, and a voxel size of 100 × 100 × 100 µm for 17 s, as described by Takenouchi et al. (19). Images from 512 frames were reconstructed using three-dimensional viewer software (I-View-R; Rigaku Co.). A fixed work plane for linear measurements was assigned to provide consistency. The basal plane was defined with three points: the right and left most inferior points of the lower border of the angular process (Go) and the center point of the right and left posterior-inferior points of attachment of the digastric muscle (Me, Fig. 2). The work plane was defined by including the upper edge point of the coronoid process (Cp point) and a perpendicular line (the Cl line) from the upper edge of the condyle (Cd) to the basal plane. Changes in the condylar sagittal width and the major axis were measured according to the methods described by Xiong et al. (4). First, a virtual line (c) was defined via the superior notch (a) and the inferior notch (b), and then a line (d) parallel to line c was drawn at the mesial position 1 mm from the condylar apex (Fig. 3). The intersection point of line d and the condylar superior border, B: The intersection point of line d and the condylar inferior border, C: The posterior point of the mandibular foramen, D’: The midpoint between A and B, D: The intersection point of the C-D’ extension line and the outer contour of the condyle, E: The posterior-inferior point of attachment of the digastric muscle.

Fig. 2 Definition of the working plane of a sagittal CT cross-section.

Fig. 4 A diagram of the region of interest used for the subtraction method (ROI: 2.0 × 2.0 × 4.0 mm).

Fig. 3 Condylar sagittal width (A-B), sagittal major axis (C-D), and mandibular sagittal length (E-D). a: Superior notch, b: Inferior notch, c: Virtual line from a to b, d: A line parallel to c was drawn at the mesial position 1 mm from the condylar apex, A: The intersection point of line d and the condylar superior border, B: The intersection point of line d and the condylar inferior border, C: The posterior point of the mandibular foramen, D’: The midpoint between A and B, D: The intersection point of the C-D’ extension line and the outer contour of the condyle, E: The posterior-inferior point of attachment of the digastric muscle.
Histological examination
Mandibular condyles were stained with hematoxylin and eosin to observe the condylar cartilage (19). After the rats had been sacrificed, the mandibles were removed from the cranium and fixed with 10% paraformaldehyde. The samples were decalcified for 4 weeks at 4°C in 10% paraformaldehyde containing 10% EDTA. The samples were then subjected to ethanol dehydration, cleared with xylene, and embedded in paraffin wax for tissue sectioning. The middle of the mandibular condyle, which included the mandibular foramen, was sliced sagittally at a thickness of 3 μm. After the sections had been deparaffinized and rehydrated, they were stained with hematoxylin and eosin and mounted with Malinol (Muto Pure Chemicals, Tokyo, Japan).

Statistical analysis
The Scheffe test was used for analysis of comparisons of condylar length and volume among the control, BJ, and BJ+LIPUS groups using the SPSS software package (ver. 16.0 for Windows; SPSS Inc., Chicago, IL, USA). P values of <0.05 were considered to indicate statistical significance. Effect sizes and power values (1-β) were also calculated.

Results
There were no significant differences in body weight among the BJ+LIPUS, BJ and control groups during the experimental period (Fig. 5).

Linear measurements
Changes in condylar width in the groups are shown in Fig. 6. No significant differences among the groups were evident during the period of the experiment. Changes in condylar sagittal major axis length among the groups are shown in Fig. 7. No significant differences among the groups were evident at day 3. The condylar sagittal major axis length was increased significantly in the BJ group after day 7 relative to the control group. The condylar sagittal major axis length in the BJ+LIPUS groups during the experiment. The BJ group received the bite-jumping appliance. The BJ+LIPUS group received the bite-jumping appliance and LIPUS stimulation of the temporomandibular region.
group was increased significantly after day 5 relative to the control group. The condylar sagittal major axis length in the BJ+LIPUS group was increased significantly relative to the BJ group on day 14. Changes in mandibular sagittal length among the groups are shown in Fig. 8. The mandibular sagittal length in the BJ+LIPUS group was increased significantly after day 7 relative to the control group. The mandibular sagittal length in the BJ+LIPUS group was increased significantly relative to the BJ group on day 14.

**Bone volume analysis**

Increases in condylar bone volume are shown in Fig. 9.

| Table 1 Each calculation for effect size and power (days 7, 14) |
|---------------------------------|-----------------|-----------------|
|                                 | day 7 | day 14 |
| Rate of condylar width          | effect (f) | 0.40 | 0.51 |
|                                 | power (1-β) | 0.36 | 0.54 |
| Rate of condylar major axis     | effect (f) | 0.98 | 0.83 |
|                                 | power (1-β) | 0.99 | 0.93 |
| Rate of mandibular sagittal length | effect (f) | 0.51 | 0.71 |
|                                 | power (1-β) | 0.54 | 0.83 |
| Rate of condylar bone volume    | effect (f) | 0.76 | 0.74 |
|                                 | power (1-β) | 0.88 | 0.87 |

There were no significant differences among the groups at day 3. The condylar bone volume was increased significantly after day 5 in the BJ+LIPUS and BJ groups relative to the control group, and that in the BJ+LIPUS group was increased significantly relative to that in the BJ group on day 7. Comparative mCT superimposed images of the sagittal condyle among the control group and the BJ+LIPUS group using the subtraction method at 14 days are shown in Fig. 10. Power analysis of the linear measurements and bone volume analysis are shown in Table 1.
**Histological examination**

Typical histological images of the condylar cartilage layer in each group at day 14 are shown in Fig. 11. The condylar cartilage layers in the BJ+LIPUS and BJ groups were hypertrophic in comparison with the control group. The fibrous layer and hypertrophic cell layer in the BJ+LIPUS group were thickened in comparison with those in the BJ group.

**Discussion**

Various animals have been used in studies of maxillofacial growth. Experimental animals such as dogs or small pigs show significant growth differences among individuals (20), require a prolonged growth period, and are costly to maintain. In the present study, Wistar rats were used because rats mature rapidly and their body size is recordable with mCT (21). Örset et al. conducted a morphometric study of normal maxillofacial growth in rats using CT (22), and reported that the temporomandibular joints of humans and rats were anatomically similar (23). The mandibular condyles in both humans and rats act as both a growth site and a functional site affecting craniofacial morphogenesis and masticatory function. Test and control groups were supplied powdered feed to reduce eating dysfunction, as reported by Kawai et al. (24). As there were no significant differences in body weight among the control, BJ, and BJ+LIPUS groups throughout the experimental period, we concluded that the animals had received appropriate nutrition and been fed correctly.

There is some controversy regarding the effects of LIPUS in growing animals. Oyonarte et al. (25) reported that LIPUS stimulation produced histological changes in the condylar cartilage of growing rats, whereas Ogurtzan et al. (14) concluded that LIPUS stimulation had no effect on the antebraichial growth plates of rabbits. These differences in outcome may have been related to differences in the target tissues examined, and the previous studies involved morphological examinations of cephalometric radiographs or CT images after the rats had been sacrificed. In the present study, changes in condylar length along the sagittal width and major axis were recorded longitudinally using in vivo mCT, according to the method described by Xiong et al. (4). Additionally, changes in the three-dimensional bone volume of the condyle were examined to improve measurement precision (17). Measurements of objects using two-dimensional images and plain X-ray films are of insufficient accuracy, and three-dimensional measurements are indispensable for high-precision measurements.

The present study employed a method described by Oyonarte et al. (25) that was modified in certain respects. In their study, the right and left condyles of animals in the test group were stimulated simultaneously using two probes; however, LIPUS stimulation on one side might have affected the other side also. In our study, the BJ+LIPUS group was stimulated with LIPUS and compared with both the BJ group and a control group in terms of changes in both condylar length and bone volume. Rats in the test groups were fitted with bite-jumping appliances, which produce a vertical and anterior displacement of the mandible. A bite-jumping appliance produces the same effect as a functional appliance when comparing orthodontic and orthopedic approaches for treatment of Class II malocclusion. The devices were strongly bonded to prevent removing during the experiment.

Assessment of linear growth demonstrated no significant differences in condylar sagittal width among the groups. However, the condylar sagittal major axis in the BJ+LIPUS group and BJ group was increased significantly relative to the control group after day 5 and day 7, respectively. Use of a functional appliance with LIPUS stimulation is likely to affect condylar growth in the major axis but would not affect the width. The condylar major axis in the BJ+LIPUS group was significantly increased on day 14, and mandibular sagittal length showed the same tendency as the condylar sagittal major axis. This result supports the findings of El-Bialy et al. (26), who reported that LIPUS enhanced mandibular growth in growing baboons, especially when anterior mandibular jumping appliances were used in combination. The condylar bone volume-as determined using the subtraction method-in both the BJ+LIPUS and BJ groups was increased significantly after day 5 relative to the controls. The condylar bone volume in the BJ+LIPUS group and the BJ group was increased significantly relative to the control group after day 5 and day 7, respectively. The voxel change in the BJ+LIPUS group was +77.8 voxels, while that in the BJ group was +63.1 voxels. This increase in condylar bone volume matched the results of linear measurements of changes in the condylar major axis. The results of mCT measurements suggested that the condyle grew in a longitudinal direction, possibly being related to the growth in condylar length and width (Enlow’s “V” principle) (27). The subtraction method employed in this study objectively demonstrated that bite-jumping appliances enhance mandibular growth in growing rats, especially when combined with LIPUS stimulation.

Our histological examinations showed that the condylar cartilage layers in the test groups were hyperto-
The present study has thus examined the effect of a bite-jumping appliance combined with LIPUS stimulation on the growth of rat mandibular condyles using mCT and histological examination. Our major findings were as follows. 1. The bite-jumping appliance in combination with LIPUS stimulation increased the volume of condylar bone to a greater degree than the bite-jumping appliance alone. This increase was attributable principally to changes in the condylar major axis. 2. Histologically, the fibrous and hypertrophic cell layers were hypertrophic in the animals that had received the bite-jumping appliance combined with LIPUS stimulation, as compared with animals fitted with the bite-jumping appliance alone. These findings suggest that the bite-jumping appliance enhances mandibular growth in growing rats, especially when combined with LIPUS stimulation.

Acknowledgments
This study was supported by a Grant-in-Aid for Scientific Research (24593111) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References