Craniofacial development in rats fed on powder diet with the extraction of all upper molars

(上顎臼歯を抜歯し粉状食にて飼育したラットにおける頭蓋顔面の発達)

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Abstract

Growth of the craniofacial bone is a complex biological phenomenon induced by highly coordinated interaction between genetic and environmental factors. The purpose of this study was to confirm whether the environmental factors, namely powdered diet and extraction of all upper molars, have an influence on the cranium and mandibular bone size, bone volume, mineral bone density, widths between left and right condyles and gonions, and intra-arch widths at the first and third molars in rat mandibles.

Ten 5-week-old male Wister rats were fed on powdered diet after extraction of all upper molars (experimental group=5 rats) and pellet diet without extraction of molars (control group=5 rats) for 20 weeks. At 20 weeks of age, the animals were examined by Micro-computer tomography. The mean values of the measurements in the two groups were compared.

There was no significant difference between the groups in the cranial sizes. The vertical lengths were significantly shorter in the experiment group than the control group while no significant difference was observed in the mandibular horizontal lengths and widths at the left and right condyles and gonions. The mandibular volume and bone mineral density in the experimental group were significantly lower than that of the control group. The intra-arch width at the lower third molars was also significantly shorter in the experimental group than the control group.

Key words: Lower masticatory function, bone volume, bone mineral density, width of dental arch, Rats, in vivo Micro-CT

Introduction

Prediction of the occurrence of adult malocclusion while in childhood is quite important in pediatric dentistry. Growth of the craniofacial bone is a complex biological phenomenon generated by highly coordinated interactions between genetic and environmental factors. One of the most interesting theories on environmental factors is the "functional matrix hypothesis" of growth proposed by Moss (1).

The preference for soft, cooked foods in the diet, which constitutes one acquired factor, resulted in insufficient development of the masticatory organs, causing the size of jawbones to shrink between the prehistoric and contemporary eras (2). Seale (3) reported that maternal diet affected tooth crown width in baby mice, suggesting that tooth crown size in humans may have increased due to the shift in dietary contents in each era from nutritionally poor to nutritionally rich foods. This may have resulted in an imbalance between tooth size and jaw size, increasing the incidence of malocclusion. Numerous studies have shown a relationship between masticatory muscle function and craniofacial growth (4-7). Mastication has a marked influence on mandibular growth and development (8). The mandible is known to change shape with different diets; ramus height was found to be greater in rats fed a hard diet than in those on a soft diet, (9,10). That is, the muscles are assumed to provide an important mechanical stimulus for bone formation and subsequent structural alterations. Matsumura et al. (11) reported extreme decrease of mastication affects mandibular shape and size. However, this assumption about jawbone and dental widths are still controversial.

Some researchers demonstrated that a soft diet, which results in reduced loading of the tissue, could lead to a narrower maxillary arch (12-14) and reduced maxillary intramolar width (15), while others reported no significant influences (16,17).

To our knowledge, no studies have investigated the effects of craniofacial and mandibular shapes and mandibular volume, and mandibular bone mineralization, and dental arch width in extremely decreased masticatory function using in vivo micro computer tomography (micro-CT) with which live photography is possible.

In this study, the authors used in vivo micro-CT to examine rats that had undergone extraction of all maxillary molars and had been reared on a powdered diet to minimize masticatory movement and force during childhood, and investigated the changes in craniofacial bone size, mandibular bone volume, bone mineralization, and jawbone and dental arch widths in adulthood.

Materials and methods

Animals

Four-week-old Wistar rats (10 males) were purchased from Sankyo Labo Service and divided into two groups of 5 rats each.

(a) Experimental group: All maxillary molars of the 5-week-old rats were extracted by expanding the socket with a probe and spoon excavator under general anesthesia with intraperitoneal pentobarbital (30 mg/kg). After molar extraction, rats were reared on powdered standard chow (MF; Oriental Yeast Co., Ltd., Tokyo) until 20 weeks of age.

(b) Control group: No molar was extracted, and the rats were reared on solid standard chow.

Micro-CT imaging

The heads of all rats were scanned at 20 weeks of age by *in vivo* micro-CT (R_mCT®; Rigaku, Tokyo, Japan) under general anesthesia with intraperitoneal pentobarbital (30 mg/kg).

Imaging conditions were as follows: tube voltage, 50 kV; tube current, 90 μA; magnification, 2×; measurement time, 17 s; slice thickness, 0.800 mm; and slice interval, 0.800 mm.

Morphological analysis

Micro-CT images were reconstructed in three dimensions and the cranial bones were observed. The maxillary complex and mandible were measured using TRI/3D-BON (Ratoc System Engineering Co., Ltd.

Bone volume and Bone mineral density

Using the 3D structural analysis software (TRI/3D-BON), the slice images obtained by micro-CT were reconstructed; the mandibular bone volumes and bone mineral density were analyzed.

Measurements of craniofacial bone

1. Craniofacial bone size (Fig.1)

(a) N-Oc length (distance between the nasion and the most distal point of the occipital bone)

N : Nasion

Oc : The most distal point of the occipital bone

(b) Interorbital width (distance between the most interior point on margin of left-right orbits)

Or : The most interior poit on margin of orbit

(c) Bizygomatic arch width (distance between the most exterior point of left-right zygomatic arches)

Zy : The most exterior point of zygomatic arch

(d) Vertical height of neurocranium (distance between the highest point of parietal bone and the lowest point of tympanic bulb)

Pa : The highest point of parietal bone

Tb : The lowest point of tympanic bulb

2. Mandibular size (Fig.2)

(a) Co- Am length (distance between the central point of the margin of condyle head and the highest point of mesial alveolar bone at the lower first molar)

Co : The central point of the margin of condylar head

Am : The highest point of mesial alveolar bone at the lower first molar

(b) Am-Me length (distance between the highest point of mesial alveolar bone at the lower molar and the lowest point on the lower border of the mandibular body)

Am : The highest point of mesial alveolar bone at the lower first molar

Me : The lowest point on the lower border of the mandibular body

(c) Co-Mm length (distance between the central point of the margin of the condylar head and the lowest margin of the mandibular angle)

Co : The central point of the margin of condylar head

Mm : The lowest point of the mandibular angle

(d) Co-Me length (distance between the central point of the margin of the condylar head and the lowest point on the lower border of the mandibular body)

Co : The central point of the margin of condylar head

Me : The lowest point on the lower border of the mandibular body

(e) Condylar head width

(f) Condylar process neck width

(g) Condylar thickness width

(h) Co-Co' length (distance between central points of the margin of left and right condylar heads)

Co : The central point of the margin of condylar head

(i) Go-Go' length (distance between the most posterior points at left and right mandibular angles)

Go : The most posterior point at the mandibular angle

(j) Mandibular angle (angle with Co-Go plane and Me-Mm plane)

(k) Mandibular volume

3. Lower dental arch width (Fig.3-1)

(a) Dental arch width at the lower first molars (distance between buccal cusps of left and right lower first molars)

(b) Dental arch width at the lower third molars (distance between buccal cusps of left and right lower third molars)

4. Femur length and volume (Fig.3-2)

(a) Femur length (distance between the most medial side and the most distal side of femur head)

(b) Femur volume

5. BMD of mandible and femur

(a) BMD of mandible

(b) BMD of femur

Statistical analysis

Data was denoted by mean value and standard deviations in each group.

Statistical analysis by Student's t-test for unpaired samples was used to test differences between the experimental group and control group.

Animal experiment approval

This study was approved by the ethical committee of the Nihon University School of Dentistry at Matsudo (Animal experiment approval number: No. AP09MD030).

Results

1. Body weight at 20 weeks of age

Fig 4 shows that there was no significant difference in body weight between the extraction and control groups at 20 weeks.

Craniofacial bone size

Na-Oc length (Fig.5-1), Interorbital width (Fig.5-2), Bizygomatic arch width (Fig.5-3) and Vertical height of neurocranium (Fig.5-4) were 44.36 mm±1.28 mm, 9.14 mm±0.44 mm, 22.02 mm±0.53 mm, 15.35 mm±0.16 mm, in the experimental group, and 45.24 mm±0.37 mm, 9.13 mm±0.22 mm, 22.11 mm±0.30 mm, 15.82 mm±0.46 mm in the control group, respectively. No significant difference in Na-Oc length, Interorbital width, Bizygomatic arch width and Vertical height of neurocranium were seen between experimental group and control group.

2. Mandibular size

Co-Am length (Fig.6-1) and Am-Me length (Fig.6-2) were 17.27 mm±0.43 mm, 7.33 mm±0.42 mm in the experiment group, and 17.70 mm±0.10 mm, 7.65 mm±0.15 mm in the control group, respectively. No significant difference in Co-Am length and Am-Me length were seen between experimental group and control group.

Co-Mm length (Fig.6-3) and Co-Me length (Fig.6-4) were 10.26 mm±0.25 mm, 20.80 mm±0.59 mm in the experimental group, and 11.49 mm±0.24 mm, 21.08 mm±0.50 mm in the control group, respectively which were significantly different.

Condylar head width (Fig.6-5), condylar process neck width (Fig.6-6) and condylar thickness width (Fig.7-1) were 3.00 mm±0.13 mm, 2.65 mm±0.17 mm, 1.11 mm±0.09 mm in the experimental group, and 3.39 mm±0.15 mm, 3.38 mm±0.14 mm, 1.45 mm±0.06 mm in the control group, respectively which were significantly different.

Co-Co' length (Fig.7-2) and Go-Go' length (Fig.7-3) were 14.92 mm±0.19 mm and 18.23 mm±0.30 mm in the experimental group, and 15.19 mm±0.47 mm and 18.64 mm±0.29 mm in the control group, respectively which were not significantly different.

Mandibular angle (Fig.7-4) and Mandibular volume (Fig.7-5) were 89.60°±1.69° and 0.46 mm^3±0.03 mm^3 in the experimental group, and 81.80°±2.19° and 0.58 mm^3±0.02 mm^3 in the control group, respectively which were significantly different.

3. Lower dental arch width

Lower first molar width (Fig.8-1) and third molar width (Fig.8-2) were 7.42 mm±0.42 mm and 7.67 mm±0.3 mm in the experimental group and 7.89 mm±0.25 mm and 8.65 mm±0.11 mm in the control group, respectively.

There was significant difference in the third molar width; however, no significant difference was seen in the first molar width.

4. Femur length and volume

Femur length (Fig.9-1) and femur volume (Fig.9-2) were 36.17 mm±0.74 mm and 0.47 mm^3±0.04 mm^3 in the experimental group and 36.28 mm±0.60 mm and 0.48 mm^3±0.02 mm^3 in the control group, respectively. No significant differences were seen in femur size and volume.

5. BMD of mandible and femur

BMD of mandible (Fig.10-1) and BMD of femur (Fig.10-2) were 89.60 mg/cm^3±1.69 mg/cm^3 and 728.88 mg/cm^3±13.55 mg/cm^3 in the experimental group, and 81.80 mg/cm^3±2.19 mg/cm^3 and 742.08 mg/cm^3±10.45 mg/cm^3 in the control group. No significant difference was seen in mandibular BMD and femur BMD.

Discussion

The reason for the increasing occurrence of malocclusion in Japanese has received much attention these days. It is said that the children with malocclusion are increasing, which has been explained by a discrepancy in jaw and tooth sizes. There have been numerous studies on the effects of reduced masticatory function on jawbone growth, which have reported that the jawbones of mice fed soft food are smaller, and that extraction of the maxillary molars of rats reduces mandibular bone mass (17-19). Inbred mice and rats are suitable for genetic and environmental research since the genes are almost 100% homozygous and controlled.

Nonaka et al. (18) reported that the genetic component of variance significantly increased until the 80th day, the maternal component of variance showed a large value during the early stage of postnatal growth and gradually decreased thereafter to a very small amount by the 80th day in rats.

Murai (19) reported that mandibular growth increases rapidly till 40 days of age and then slows down after that. In order to examine the influence of reduced masticatory function in childhood on jawbone development in adulthood, the molars were extracted at 5 weeks of age and powdered diet was continued until 20 weeks of age, and the jawbone was analyzed at 20 weeks by which growth change was complete. Development is affected by health condition. In the rats used this study, body weight measurements tended to increase similarly in both groups with no significant differences between the two groups during the experimental period, indicating that there were no differences in nutritional status. Thus, the growth differences in jawbone between the experiment and control groups were not the result of nutritional status. Tanaka et al. (20) and Katsaros et al. (12) also reported that the changes in body weight of the soft diet group were similar to those in the hard diet group, showing no significant difference.

It is well known that growth of the craniofacial bone is regulated by genetic and environmental factors, however, which region of the bone is affected strongly by the interaction of these two factors is still not clear. Gene expression levels related to mandibular condylar cartilage growth were found to differ markedly before and after the initiation of mastication in mice (21). Okamoto et al. (22) reported that the inheritance pattern of horizontal and vertical dimensions in mandible differed.

Cranium and maxilla shape

He et al. (23) reported that the transverse dimensions of the neurocranium were found to be generally smaller in soft diet group ferrets fed at 5 weeks of age. The reduced contractile strength of

the masticatory muscles induced by lower functional demands in ferrets fed a soft diet, compared with the ferrets in the hard diet group, may have exerted less tension on the periosteal membrane of the cranial bones, resulting in less periosteal bone apposition in the inserting areas, and/or in the bone elements of the bones being pulled apart following reduced suture growth.

In this study, cranium showed no significant differences in N-Oc, Zy-Zy' and Pa-Tb sizes between the two groups, however, the tendency for the length (N-Oc), width (Zy-Zy') and height (Pa-Tb) in the experimental group was smaller than the control group.

Growth of maxillary complex depth depends on growth of the base of the skull, mainly by synchondrosis, which exhibits a nervous-system type pattern (24, 25), meaning it may be less susceptible to the effects of differences in masticatory function, which constitutes an acquired environmental factor. The effects of masticatory function on the growth of the neurocranium, i.e., the calvaria and the cranial base, have been investigated in a number of studies by means such as removal of the masticatory muscle (26), molar extraction (27) and variations in food hardness (28-30), and they were found to have no effect on the neurocranium. Moore (29) reported that in rats aged 1 month, the viscerocranium had grown to approximately 75% of its size in mature rats, and the size of the neurocranium had reached 93%. This means that neurocranial growth was already complete in 5-week-old rats at the start of our experiment. This may also explain why no significant differences in transverse dimensions of cranium were observed in the rats aged 5 weeks fed powdered diet and extracted upper molars in this study.

In the maxilla, the distance between upper left and right molars could not be measured, since all upper molars were extracted.

Mandibular shape and volume

The lower jaw consists of different functional units of body including alveolar part and ramus, : the alveolar part housing the continuously erupting incisors, the alveolar process of the molars and the angular, coronoid, and condylar process, which are all sites of attachment for the major masticatory muscles (31).

The changes in craniofacial structure of the masticatory muscles may be related to the decreased electro-myographic activity during soft food intake, which can produce a low training effect on the masticatory muscles and may explain the low biting forces in rats fed a soft diet (32).

Poorer masseter muscle development in mice fed a soft diet compared with those fed a hard diet (33), as well as the influence of the masseter muscle on the angle of the mandible (34), have been reported. This study observed little change in the angle of mandible at 20 weeks of age in the experimental group. The decreased functional demands in animals fed a soft diet caused changes in the size and distribution of the muscle fibers (32). Yosida et al.(35) reported that the weight of the superficial and deep parts of the masseter and temporal muscles was significantly smaller, while there was no influence on the anterior belly of the digastric muscle in the liquid diet group. The author concluded that feeding liquid diet affected the differentiation and development of muscle fibers of masticatory muscles in mice. According to Yamada et al. (36), rats fed a solid diet bite

their food 2-3 times with the incisors and then chew it with the molars for several seconds, whereas those fed a powdered diet only chew it with the molars for a few seconds, with a clear difference in the masticatory muscle activity. In this study, all maxillary molars were extracted and rats were also fed a powdered diet; thus, muscle activity would have been even lower than that reported by Yamada et al. (36). It reported that although there were no differences in mandibular size between developing rats reared on a solid or powdered diet, mandibular ramus length was significantly smaller in rats fed a powdered diet after 30 days and mandibular thickness was significantly smaller after 120 days when compared with those of rats fed a solid diet, consistent with the results of the present study (36).

The authors determined the mandibular ramus width and intra-arch width at lower left and right molars, since this study were analyzed by in vivo micro CT. There were no significant differences in the distances between the left and right condyles and gonions. The distance between lower left and right third molars was significantly narrower in the experimental group, whereas no significant difference was seen in the distance of the first molars. These results were completely in agreement with that of Katsaros et al. (12), who stated that this may be because the first and second molars had been in occlusion at the beginning of the experimental period, while the third molars were not. We thought that the third molar region would be strongly affected than the first molar region by masseter muscular force.

There are only a few reports involving 3D measurements of mandibular bone volume in which the presence of regions with significantly lower bone volumes in rats fed a soft diet were compared

with those fed a hard diet in two-dimensional cross-sectional images (36). In this study, the mandibular bone volumes in rats with extracted upper molars and fed soft diet were also less than the controls. Mastication of a hard diet results in exertion of a significantly greater mechanical force on the temporomandibular joint than mastication of a soft diet (37).

No reports are available about mandibular width in live mice since in vivo micro-CT was not performed. It has been impossible to measure the width because the midline of the menton in mice is a fibrous ossification, and it can be easily separated. Since the present study used in vivo micro-CT, the mandibular width could be measured. The results of the distance between left- right condyle, and left- right gonion in the experimental group and control group were not significantly different, but the experiment group tended to be narrower. A smaller condyle and thinner condylar cartilage were seen in the experiment group compared with control group, and these findings were in agreement with Bouvier et al. (16). It is clear that the condylar size was also affected by masticatory muscle activity. The difference in vertical length of mandible could be due to the attachment of the muscles. Low masseter muscle activity in the experiment group cause the alveolar process narrow followed by the distance between left and right lower third molar significantly.

Mandibular bone mineral density (BMD)

The internal structure of bone is constantly adapting to its functional environment through processes that remove existing bone and deposit new bone. The muscle provides an important mechanical stimulus for bone formation. In animal limb models, a reduction in habitual mechanical

loading leads to clear decrease in apparent bone density (38). In this study, no significant differences in size and BMD of mice femur between the two groups were seen, which suggested that the decreased masticatory muscle had no effect on the femur.

Mavropoulos et al. (39) classified six geometrically defined areas in mandible and reported that the reduction of the forces exerted during mastication (soft diet) resulted in reduction of bone density in all regions after 6 weeks of soft diet, and the BMD was significantly lower in the coronoid and the angular processes of the mandible in a powder-diet group. Tanaka et al. (20) also observed that high degree of mineralization of mandible was shown in the hard diet group than in the soft diet group since the change to a soft diet linked with reduced forces applied to the mandible during mastication is assumed to result in a reduction of new bone formation with normal bone resorption.

Bresin et al. (40) reported that reduced masticatory function in the soft diet group produced reduced bone density. As the degree of mineralization depends on the remodeling rate of bone, the degree of mineralization can give information on bone formation/resorption rate (41, 42). It seemed that sufficient masticatory function results in new bone formation and resorption as in active bone metabolism in which there is high bone mineralization. Mavropoulos et al. (39) found a significant reduction of the bone density in the mandibular alveolar bone after 6 weeks of soft diet. Therefore, the 20 weeks of soft diet after molar extraction in the present study can be considered sufficient to discriminate between the experimental and control groups.

The authors have no explanation for the higher degree of mineralization in the control group than in the powdered diet and extracted molar group. Possibly, it could be due to the fact that the mandibles of the rats were still developing. In mature rats of 20 weeks of age where a normal diet would not be considered as excessive loading, the soft diet may result in reduced mineralization.

No significant difference was observed in the bone mineralization at the femur in both groups, which means the decreased masticatory muscle activity did not affect the femur.

Based on the present study, although the rat mandible differs significantly both morphologically and functionally from the human mandible, it could be assumed that low masticatory function produces changes in the morphology, size, volume and bone mineralization density, causing malocclusion.

Acknowledgement

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 23792450) .

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Figure legends

Fig.1 Craniofacial bone size

a: N-Oc length, b: Interorbital width, c: Bizygomatic arch width and d: Vertical height of neurocranium are shown.

Fig.2 Mandibular size

a: Co-Am length, b: Am-Me length, c: Co-Mm length, d: Co-Me length, e: Condylar head width, f: Condylar process neck width, g: Condylar thickness width, h: Co-Co' length, i: Go-Go' length, j: Mandibular angle and k: Mandibular volume are shown.

Fig.3-1 Lower dental arch width

Lower dental arch width at first molar and third molar in the experimental group and the control group is shown.

Fig.3-2 Femur size

Femur length in the experimental group and the control group is shown.

Fig.4-1 N-Oc length

N-Oc length in the experimental group and the control group is shown.

Fig.4-2 Interorbital width

Interorbital width in the experimental group and the control group is shown.

Fig.4-3 Bizygomatic arch width

Bizygomatic arch width in the experimental group and the control group is shown.

Fig.4-4 Vertical height of neurocranium

Vertical height of neurocranium in the experimental group and the control group is shown.

Fig.5-1 Co-Am length

Co-Am length in the experimental group and the control group is shown.

Fig.5-2 Am-Me length

Am-Me length in the experimental group and the control group is shown.

Fig.5-3 Co-Mm length

Co-Mm length in the experimental group and the control group is shown.

Fig.5-4 Co-Me length

Co-Me length in the experimental group and the control group is shown.

Fig.5-5 Condylar head width

Condylar head width in the experimental group and the control group is shown.

Fig.5-6 Condylar process neck width

Condylar process neck width in the experimental group and the control group is shown.

Fig.6-1 Condylar thickness width

Condylar thickness width in the experimental group and the control group is shown.

Fig.6-2 Co-Co' distance

Co-Co' length in the experimental group and the control group is shown.

Fig.6-3 Go-Go' distance

Go-Go' length in the experimental group and the control group is shown.

Fig.6-4 Mandibular angle

Mandibular angle in the experimental group and the control group is shown.

Fig.6-5 Mandibular volume

Mandibular volume in the experimental group and the control group is shown.

Fig.7-1 M1-M1' distance in mandible

Inter lower first molars distance in the experimental group and the control group is shown.

Fig.7-2 Lower third molar width

Lower third molar width in the experimental group and the control group is shown.

Fig.8-1 Femur length

Femur length in the experimental group and the control group is shown.

Fig.8-2 Femur volume

Femur volume in the experimental group and the control group is shown.

Fig.9-1 Femur volume

Femur volume in the experimental group and the control group is shown.

Fig.9-2 BMD of femur

BMD of femur in the experimental group and the control group is shown.

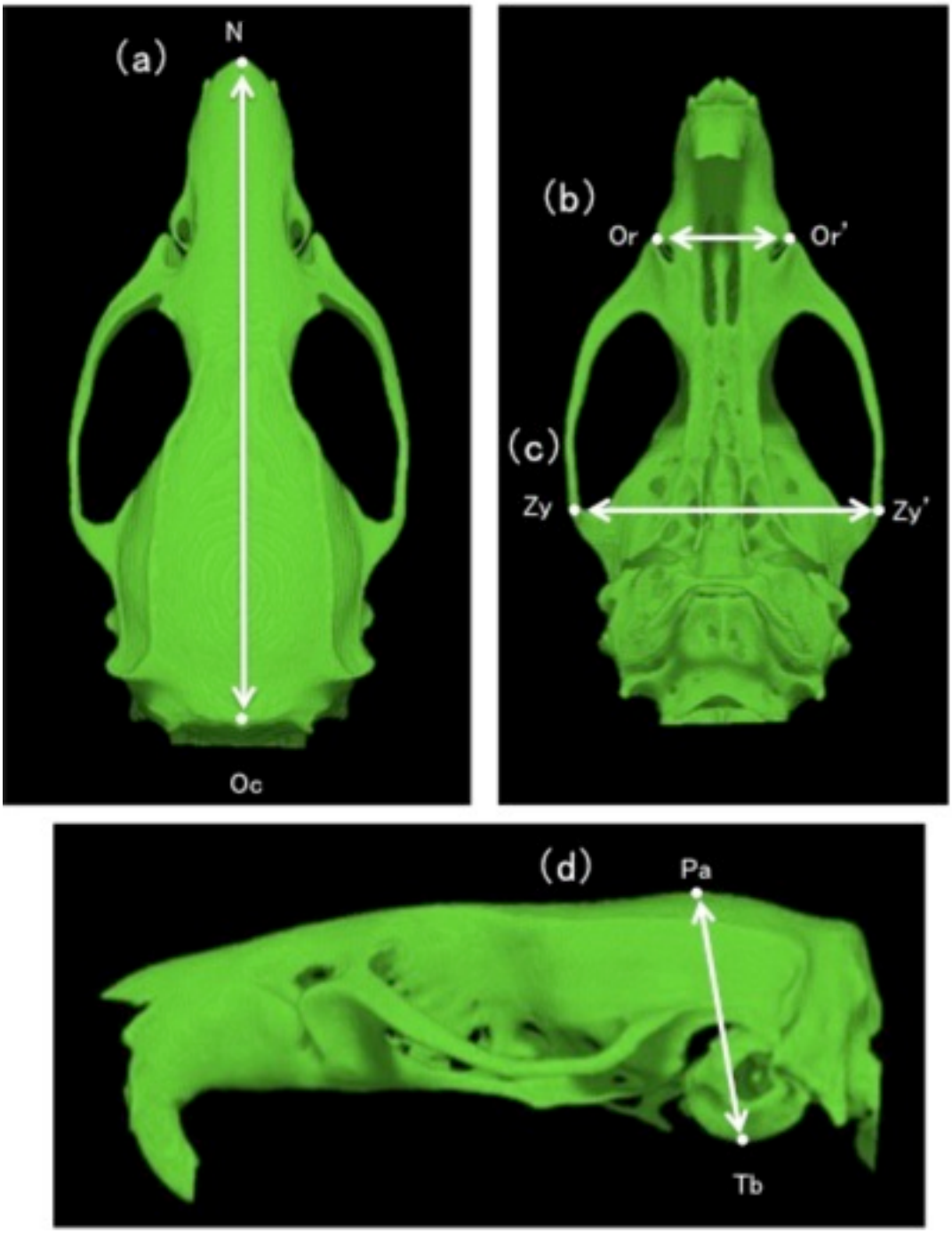

Fig.1 Craniofacial bone size

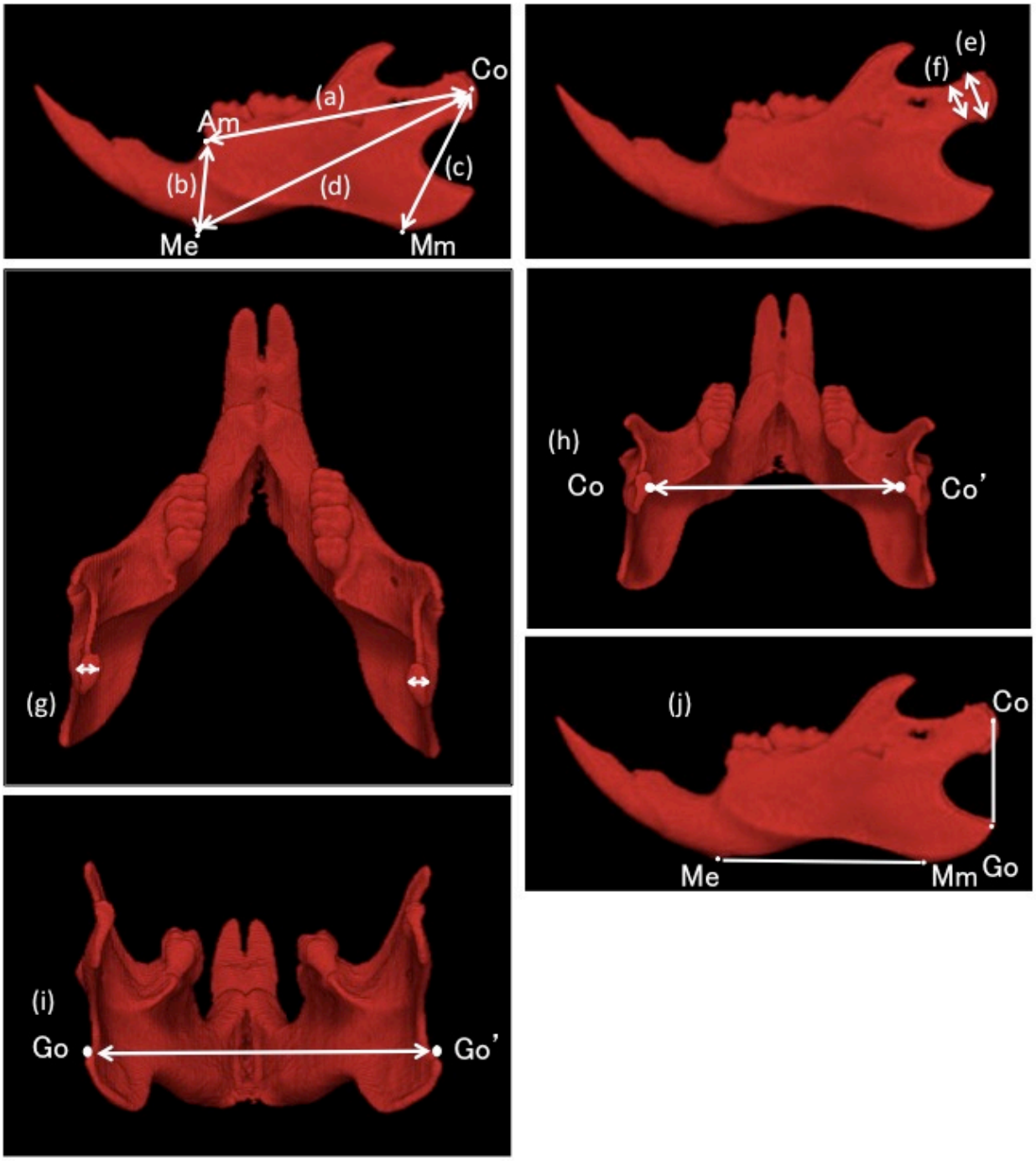

Fig.2 Mandibular size

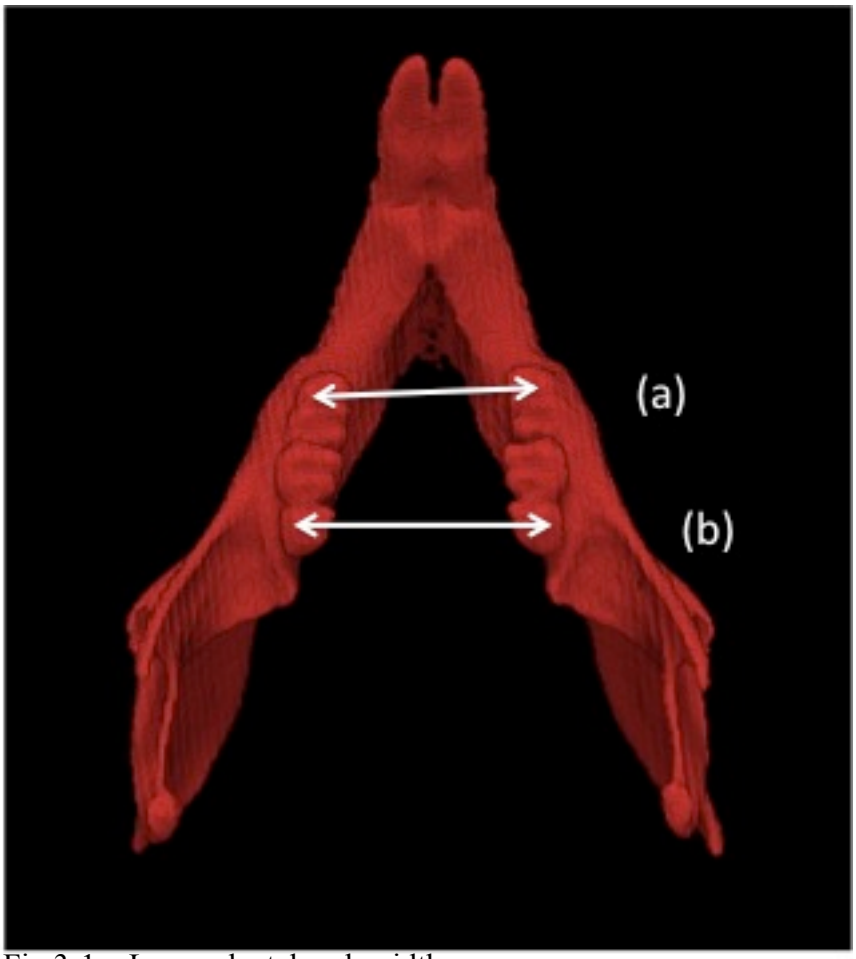

Fig.3-1 Lower dental arch width

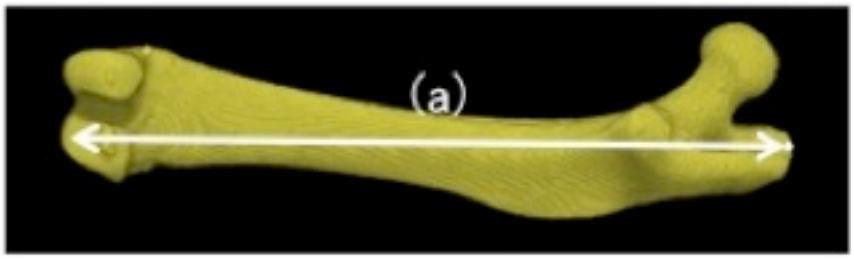

Fig.3-2 Femur size

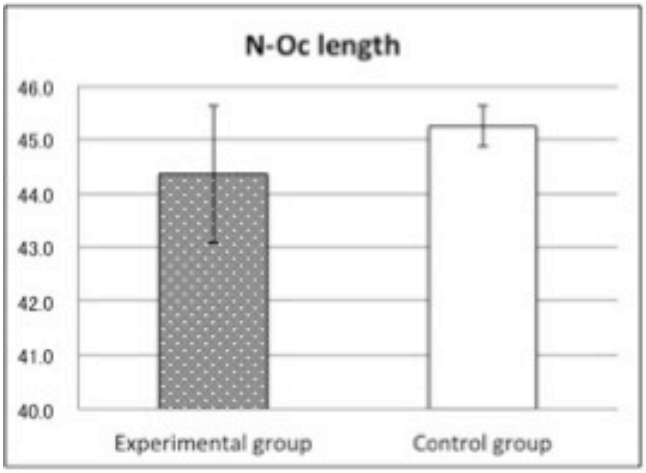

Fig.4-1

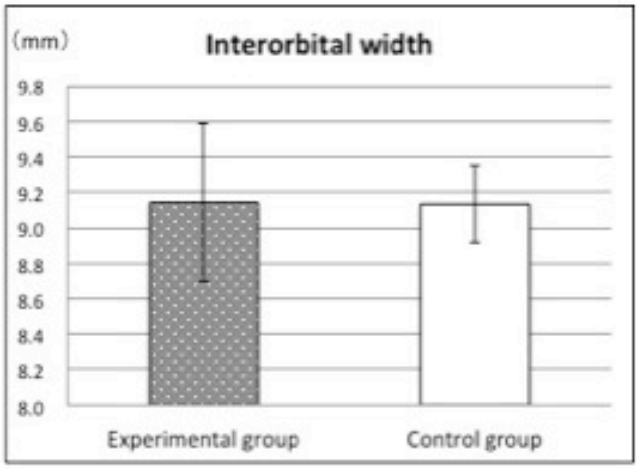

Fig.4-2

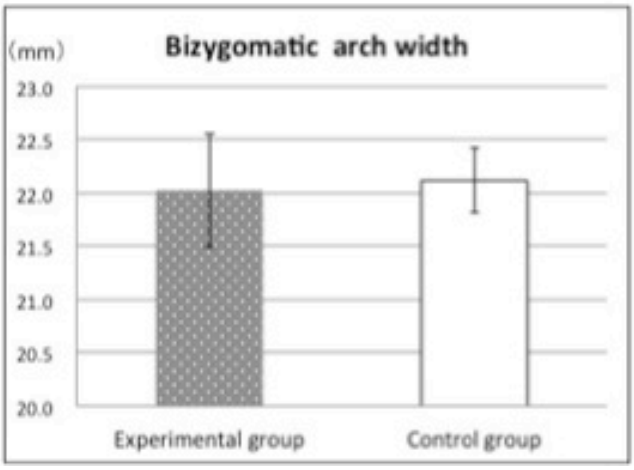

Fig.4-3

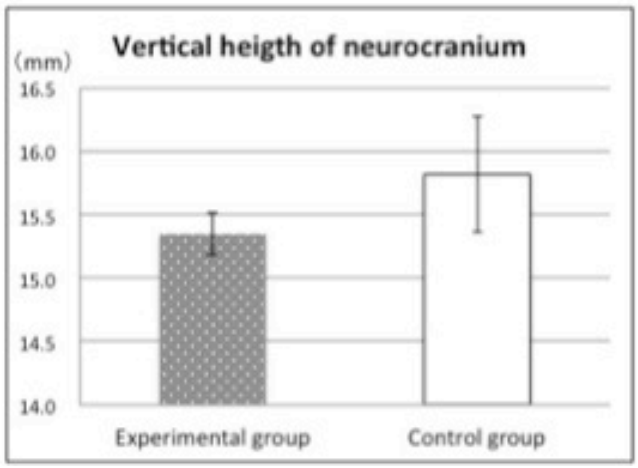

Fig.4-4

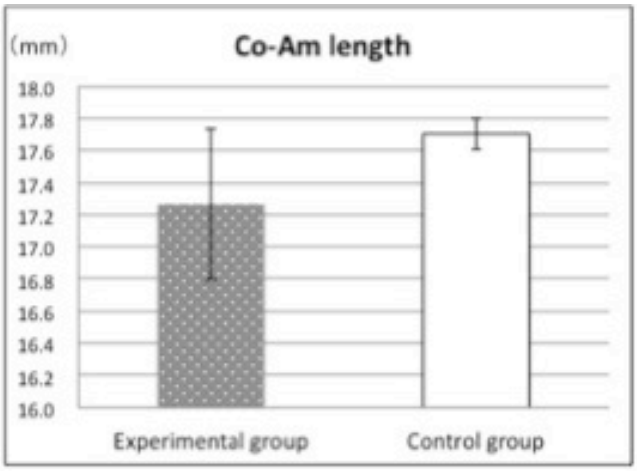

Fig.5-1

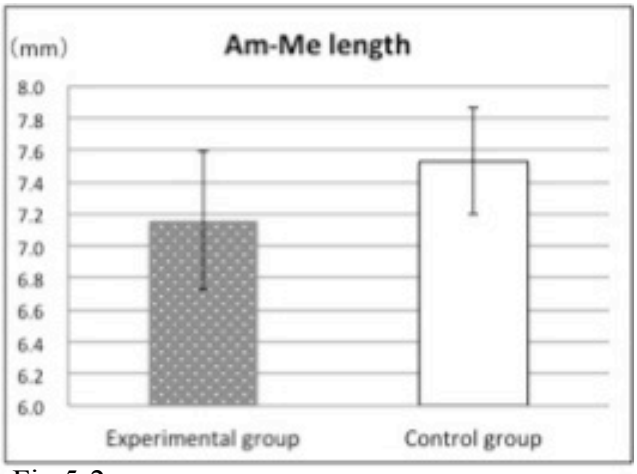

Fig.5-2

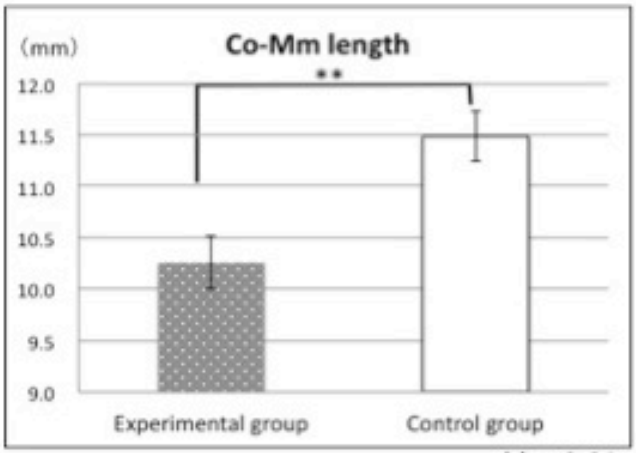

Fig.5-3 **p<0.01

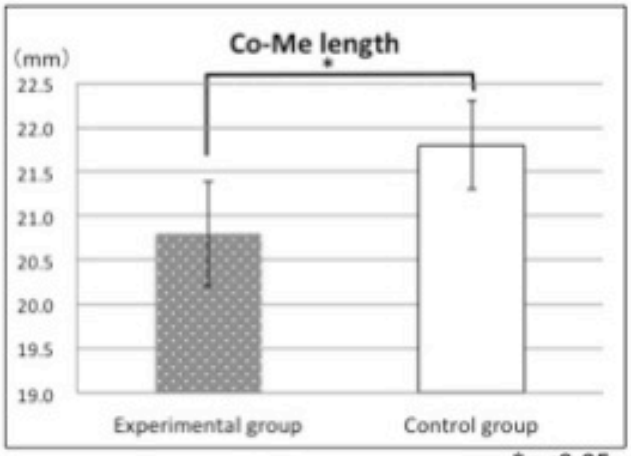

Fig.5-4 *p<0.05

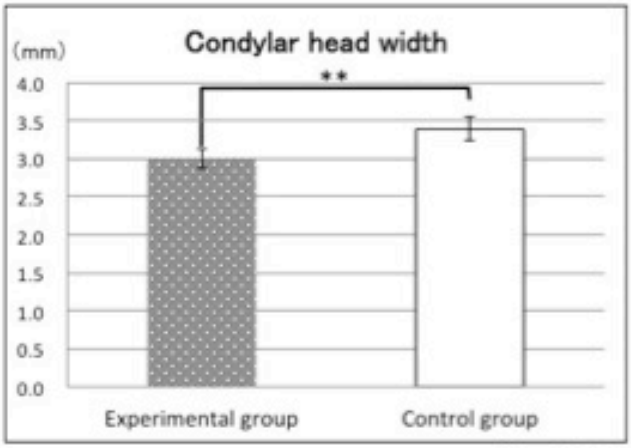

Fig.5-5 **p<0.01

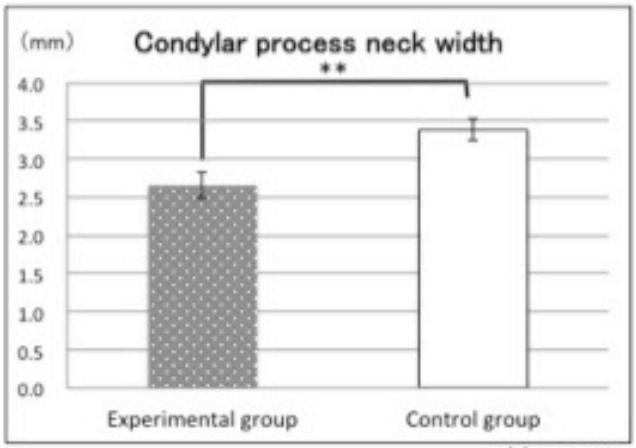

Fig.5-6 **p<0.01

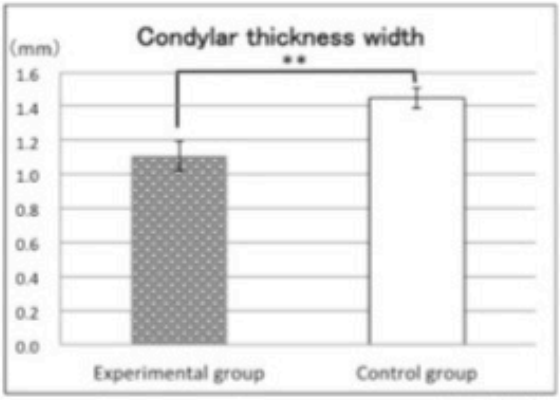

Fig.6-1

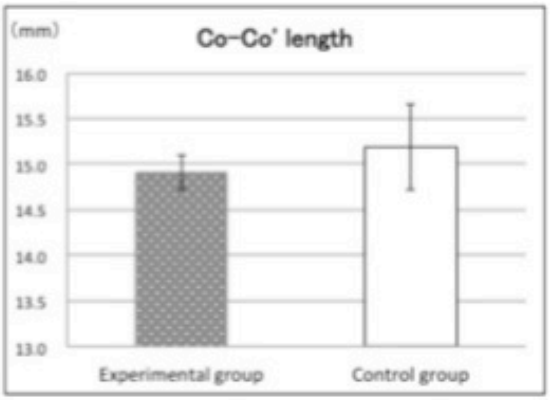

Fig.6-2

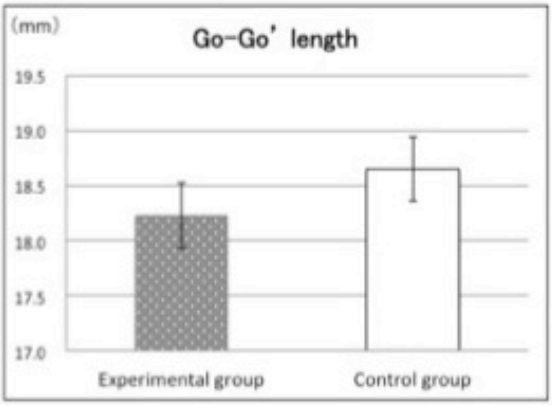

Fig.6-3

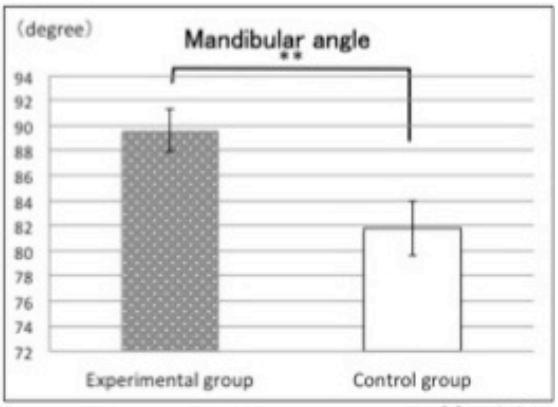

Fig.6-4

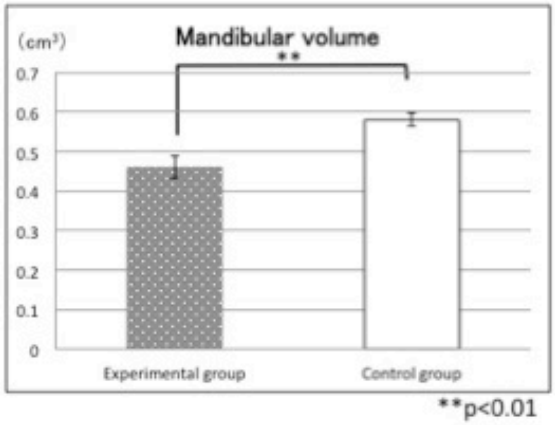

Fig.6-5

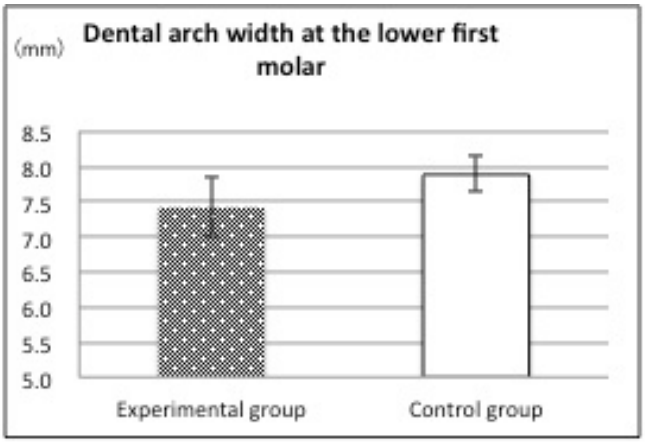
(mm)
Dental arch width at the lower first molar
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
Experimental group
Control group

Fig.7-1

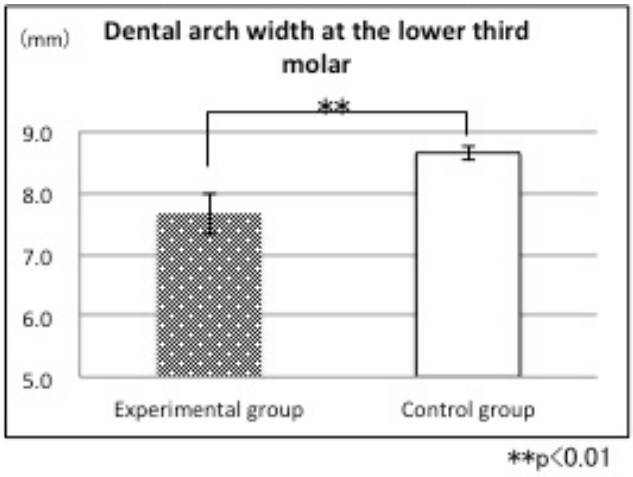
(mm)
Dental arch width at the lower third molar
**
9.0
8.0
7.0
6.0
5.0
Experimental group
Control group
**p<0.01

Fig.7-2

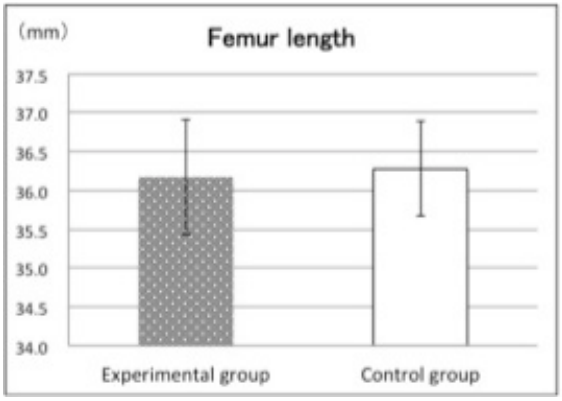
(mm)
Femur length
37.5
37.0
36.5
36.0
35.5
35.0
34.5
34.0
Experimental group
Control group

Fig.8-1

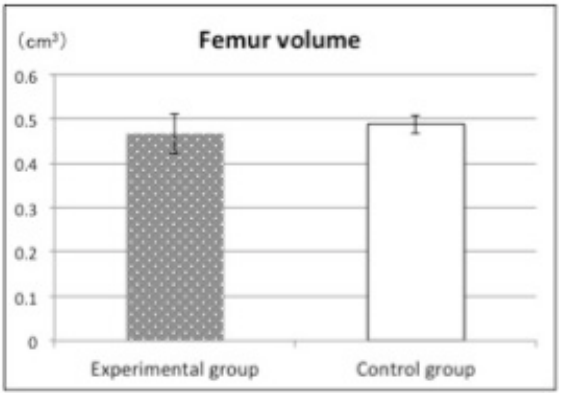
(cm³)
Femur volume
0.6
0.5
0.4
0.3
0.2
0.1
0
Experimental group
Control group

Fig.8-2

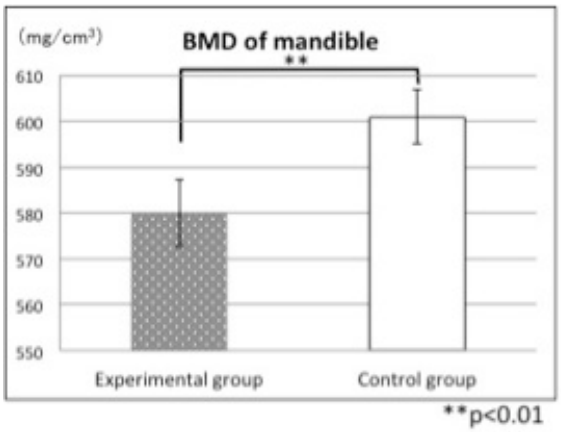
(mg/cm³)
BMD of mandible
**
610
600
590
580
570
560
550
Experimental group
Control group
**p<0.01

Fig.9-1

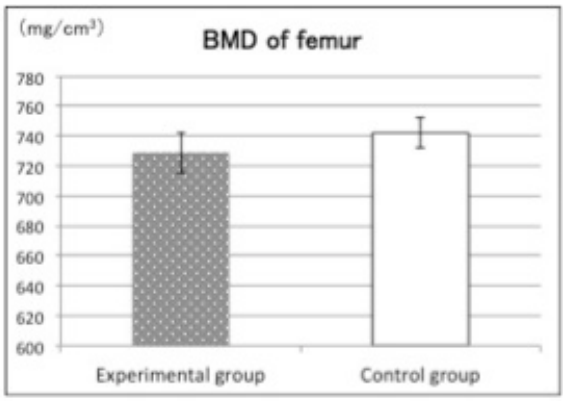
(mg/cm³)
BMD of femur
780
760
740
720
700
680
660
640
620
600
Experimental group
Control group

Fig.9-2