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Denervation and β_2-adrenoceptor-agonist administration on craniofacial bone density

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Objective: β_2-agonist medications are thought to have adverse effects on bone density. Surgical denervation and intramuscular β_2-agonist injections appear to have opposing effects on skeletal muscles. The present study has been designed to assess the effects of denervation of the masseter, intramuscular injection of a β_2-agonist and the combination of both procedures, on bone density in the craniofacial skeleton in rats.

Materials and methods: Sprague-Dawley rats were prepared as four groups: 1. surgical sham + saline injection into the masseter (*sham*); 2. surgical denervation of the masseter (*den.*); 3. surgical denervation of the masseter + intramuscular formoterol injection into the affected muscle (*den.+form.*); 4. intramuscular formoterol injection into the masseter (*form.*). All specimens were submitted for CT examination and volumetric calculations of the mineralised bone tissue were performed.

Results: The sham and form. groups had a greater volume of mineralised bone in the zygoma on the experimental side compared with the control side. The maxilla on the experimental side had a higher volume of mineralised bone in the *den.+form.* and *form.* groups compared with the *sham* and *den.* groups. The control side of the maxilla had a higher volume of mineralised bone in the *den.+form.* and *form.* groups compared with the *den.* group only.

Conclusion: Intramuscular administration of formoterol appears to induce a bilateral increase in bone mineral density in the maxilla and the zygoma, likely explained as a secondary effect of the well-described increase in muscle mass and strength associated with β_2-agonist administration.

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Introduction

The muscles of mastication are considered to play an important role in craniofacial growth and development.[1] To comply with Wolff's law, the forces that these muscles exert on the craniofacial skeleton result in the modelling of the bones to achieve a structure that can best withstand applied mechanical loads.[2] Previous studies in animals have assessed the impact that alteration in masticatory muscle structure and function might have on hard tissue growth.[3-8]

β_2-adrenoceptor agonists (β_2-agonists) are pharmacological agents commonly used to relieve bronchospasm in asthmatics.[9] Systemic administration of β_2-agonists has been shown to decrease bone mineral content, reduce bone mineral density, alter bone micro-architecture and increase the risk of hip and femur fractures.[10-12] When β_2-agonists are used in both healthy and atrophy states, increases in individual muscle fibre size are possible, as well as a potential shift from slow to fast muscle fibre types.[13,14] Formoterol is a β_2-agonist with increased selectivity for β_2-adrenoceptors compared with other drugs of the same family.[15]

In healthy individuals, the maintenance of bone density relies on sufficient physiological loading. When either the metabolic health of the individual or physiological loading of the bones is altered, bone reacts by remodelling and altering its mineral content.[16-23] Knowing the opposing effects that

surgical denervation and intramuscular formoterol administration have on skeletal muscle size and activity, the present study was designed to assess the likely effects of various combinations of surgical denervation of the masseter muscle and intramuscular formoterol injection on volumetric bone tissue density in the craniofacial skeleton.

Materials and methods

All experiments were approved by the Animal Ethics Committee of The University of Melbourne (AECC number 0704146).

Animals

Sixteen male Sprague-Dawley rats aged 4 weeks (~110 g body mass) were housed in standard cages in the Biological Research Facility at The University of Melbourne under a 12:12 hour light-dark cycle with *ad libitum* access to food and water. The rats were randomly allocated to one of four groups: 1. surgical sham + saline injection (*sham*); 2. surgical denervation only (*den.*); 3. surgical denervation + formoterol injection (*den.+form.*); or 4. formoterol injection only (*form.*), with four rats assigned to each group.

Surgical procedure

The rats were anaesthetised with an intraperitoneal (i.p.) injection of ketamine (225 mg/kg) and xylazine (30 mg/kg) with supplemental doses administrated as required to maintain the appropriate depth of anaesthesia.

In the *sham*, *den.* and *den.+form.* groups, an incision was made below the left eye and directly parallel to the zygomatic arch. The left side of the rat was chosen as the 'experimental' side in all animals. Blunt dissection was performed to expose the masseteric nerve in the sigmoid notch of the mandibular coronoid process. The masseteric nerve was identified but not cut in the *sham* group. In the *den.* and *den+form.* groups, the masseteric nerve was cut and a 5 to 10 mm section removed. Surgery was not performed on the *form.* group.

Formoterol administration

The *sham* group received an intramuscular injection of sterile saline (100 µL/0.9%) every third day for eight weeks into the left side masseter muscle. The *den.+form.* and *form.* groups received intramuscular injections of formoterol (100 µg in saline; Astra-Zenica, Molndal, Sweden) in the left side masseter muscle for the same period. For all of these intramuscular injections, the rats were anaesthetised with 5% isoflurane (3 ml; CENVET, Australia).

At the end of the eight-week experimental period the rats were sacrificed by an overdose of 6 mg/kg i.p. pentobarbital sodium (Nembutal, Rhone Merieux, Pinkenba, QLD, Australia).

Data collection

Computed tomography (CT) was used to obtain images of the intact heads of the specimens (110 kVp, 36 mA, 0.75 mm slice thickness, slice distance 0.5 mm, Somatom Emotion 16 Excell; Siemens, Germany) at the Department of Radiology, Faculty of Veterinary Science of The University of Melbourne. The images were analysed using Syngo CT 2009E software (Siemens, Germany).

The CT images were orientated with the maxillary occlusal plane horizontal and the coronal slice used for measurements was established perpendicular to the occlusal plane and 0.5 mm posterior to the distal surface of the maxillary third molar teeth. The measurements were recorded in Hounsfield units using the circular region of interest (ROI) tool. The ROI tool calculated the mean attenuation value for voxels within a 0.38 mm^2 area. The radio-density values were recorded bilaterally for the following bony structures:

1. The crest defining the superior attachment of the temporalis muscle (temporal crest).
2. The zygomatic bone.
3. The maxillary tuberosity.
4. The crest defining the inferior attachment of the masseter muscle (masseteric ridge).

(Figures 1, 2)

Quantitative CT calibration

To ensure standardisation of the density measurements, the CT images were taken with a calibration phantom containing plastic materials of known densities (Mindways Software, Inc. Austin, TX, USA. Part No. 04499). The calibration phantom was used

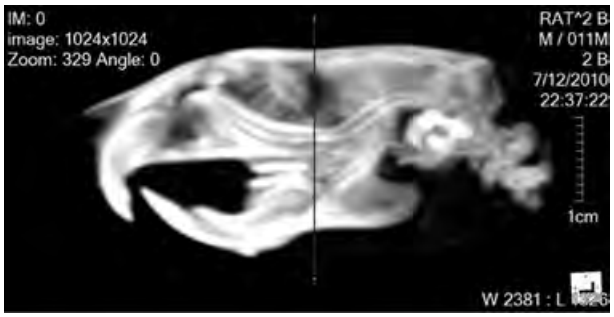

Figure 1. Lateral image of subject 2B indicating the position and orientation of the image slice to be analysed.

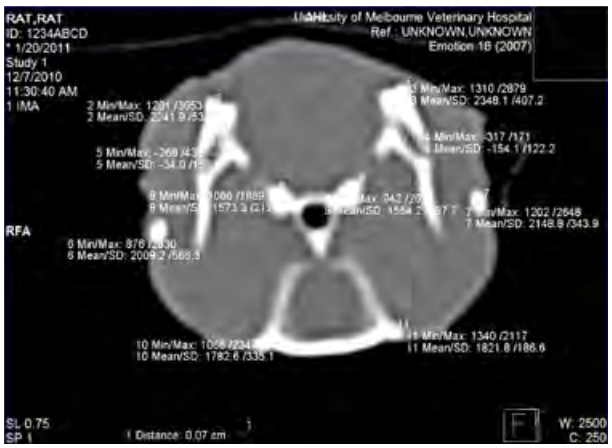

Figure 2. Coronal slice showing positions and attenuation values for the regions of interest for the craniofacial bones surveyed.

to convert the x-ray attenuation measurements (in Hounsfield units) to a bone mineral equivalent density measurement expressed in mg/cm^3 equivalent aqueous K_2HPO_4, the standard unit for Quantitative CT (QCT).

Statistical analysis

All statistical tests were performed using SPSS 17.0 statistical software (SPSS Inc. Chicago, IL, USA). Descriptive statistics were calculated for all data. Differences between groups were assessed using 2-tailed paired *t*-tests with the level of significance set at $p < 0.05$. One-way analysis of variance (ANOVA) tests were used to find differences between the four groups ($p < 0.05$) and post-hoc Fisher's least significant difference (LSD) tests were performed when significance was detected (Table I).

Error measurement

Repeat measures were taken for the *sham* group one month after the initial measurements were recorded. Intraclass correlations were performed to assess the reliability of the measurements. All intraclass correlation coefficients ranged from 0.894-1.0 and no significant differences were found ($p < 0.05$) between initial and repeat measures (Table II).

Results

All results are displayed in Table I.

Changes between right (control) and left (experimental) sides

Group 1 (sham)

Significant differences were found between the measurements of the left and right zygomatic bones (left side increased by 157.9 mg/cm^3 equivalent aqueous K_2HPO_4, $p = 0.008$).

Group 2 (den.)

No significant differences were found between right and left measurements in this group.

Group 3 (den.+form.)

No significant differences were found between right and left measurements in this group.

Group 4 (form.)

Significant differences were found between the measurements of the left and right zygomatic bones (left side increased by 155.9 mg/cm^3 equivalent aqueous K_2HPO_4, $p = 0.001$).

Changes between groups

Significant differences between groups were found on both sides of the maxilla. On the left side, the *den.+form.* group showed a significantly greater mean density of the measured region relative to the *sham* and *den.* group of 290.8 and 279.8 mg/cm^3 equivalent aqueous K_2HPO_4, respectively ($p < 0.001$). The *form.* group also showed a significant increase in mean density relative to the *sham* and *den.* groups ($p < 0.001$) for the region in the left side of the maxilla. The mean differences between these groups were of 275.9 for the *sham* group and 265.0 mg/cm^3 equivalent aqueous K_2HPO_4 for the *den.* group. The region in the right side of the maxilla in the *den.* group, had a significantly lower mean density compared with the *den.+form.* group (mean difference 199.0 mg/cm^3 equivalent aqueous K_2HPO_4) and *form.* groups (mean difference 254.3 mg/cm^3 equivalent aqueous K_2HPO_4) ($p = 0.034$).

Table I. Bone mineral density assessment within and between groups.*

	Group 1 (*sham*)		Group 2 (*den.*)		Group 3 (*den.+form.*)		Group 4 (*form.*)	
Measurement	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Temporal crest – experimental side (Left)	362.5	247.5	470.3	152.2	552.2	111.9	535.0	147.9
Temporal crest – control side (Right)	358.1	219.1	505.5	55.8	532.8	61.8	610.3	53.8
Zygoma – experimental side (Left)	892.4#	223.4	856.7	167.5	880.0	94.0	904.2#	195.0
Zygoma - control side (Right)	734.5#	251.9	736.3	128.3	754.6	151.7	748.2#	194.0
Maxilla - experimental side (Left)	122.7[a,b]	78.1	133.7[c,d]	102.5	413.5[a,c]	79.5	398.7[b,d]	96.9
Maxilla - control side (Right)	248.6	128.5	119.2[a,b]	153.4	318.2[a]	82.5	373.5[b]	28.9
Masseteric ridge - experimental side (Left)	860.4	148.6	992.1	93.7	956.1	166.3	946.7	134.6
Masseteric ridge - control side (Right)	907.0	237.2	1047.6	54.0	944.8	165.4	770.0	103.9

* All measurements are in mg/cm^3 equivalent aqueous K_2HPO_4
\# Statistically significant left/right differences ($p < 0.05$) within groups (2-tailed)
[a,b,c,d] represent statistically significant differences ($p < 0.05$) between similarly labelled groups in the same row (one-way ANOVA)

Table II. Intraclass correlation of repeat measurements for Group 1 (*sham*).

			95% Confidence interval	
Measurement		Intraclass correlation	Lower bound	Upper bound
Temporal crest - experimental side (Left)	Single measures	0.996	0.944	1.000
	Average measures	0.998	0.971	1.000
Temporal crest - control side (Right)	Single measures	0.999	0.980	1.000
	Average measures	0.999	0.990	1.000
Zygoma - experimental side (Left)	Single measures	0.967	0.593	0.998
	Average measures	0.983	0.745	0.999
Zygoma - control side (Right)	Single measures	0.979	0.718	0.999
	Average measures	0.989	0.836	0.999
Maxilla - experimental side (Left)	Single measures	0.855	-0.092	0.990
	Average measures	0.922	-0.203	0.995
Maxilla - control side (Right)	Single measures	0.865	-0.055	0.991
	Average measures	0.928	-0.116	0.995
Masseteric ridge - experimental side (Left)	Single measures	0.959	0.509	0.997
	Average measures	0.979	0.675	0.999
Masseteric ridge - control side (Right)	Single measures	0.983	0.765	0.999
	Average measures	0.991	0.867	0.999

Discussion

Drug safety

For formoterol to be considered for therapeutic application to alter the masticatory muscle function, its side effects from acute and chronic administration must be considered. As β_2-adrenergic receptors are so widely distributed throughout the body, potential off-target side effects may occur. To date, side effects of β_2-agonists have been assessed for inhaled systemic doses for asthmatics. The heart and blood vessels contain large numbers of β_2-adrenoceptors and the most serious side effects of β_2-agonists are those related to the cardiovascular system. These include increased risk of ischaemia, congestive heart failure, arrhythmia and sudden death, with cardiac hypertrophy being the most well established cardiovascular change after chronic use of β_2-agonists in high doses.[24-27]

As functional β_2-adrenoceptors are also found on osteoblasts and osteoclasts, it would also be expected that β_2-agonists would affect bone.[28] Stimulation of β_2-adrenoceptors on bone cells by β_2-agonists might be expected to increase osteoclast formation and multi-cellularity, but no changes in osteoblast function have been reported.[29] Animal studies, and epidemiological evidence in humans have directly and indirectly linked systemic administration of β_2-agonists to a decrease in bone mineral content, which may consequently reduce its flexural strength and increase fracture risk.[10-12,16,29] The present study appears to be the first to investigate changes in bone mineral density associated with intramuscular injection of the β_2-agonist, formoterol.

Changes on the experimental side (left side)

The present study found no difference in the volume of mineralised bone tissue in measurements for the *den.* and *sham* groups. This observation differs from the significant decrease in bone density reported in a previous animal study following denervation of the limb muscles.[16] The maintenance of bone density in the present study may relate to the other normally functioning masticatory muscles providing sufficient loading to preserve bone density. The alternative muscles appear to exert sufficient stresses on the bone to maintain equilibrium of bone turnover, in accordance with Frost's 'mechanostat' theory.[30]

Current results showed the *form.* group had significantly higher levels of maxillary bone density relative to the *sham* and *den.* groups. The changes in bone density between the four groups on the experimental side of the maxilla appear related to the changes in skeletal muscle. Increasing masticatory loading by increasing diet hardness seems to increase the degree of bone mineralisation of the palate; increase bone mineral density of the mandible; increase the volume and thickness of trabeculae in mandibular alveolar bone[21,23] and increase mandibular bone mineralisation related to the rate of bone turnover.[20,22] Myostatin-deficient mice, which have twice the skeletal muscle mass of wild type controls,[18] exhibit increases in bone mineral density in the regions of the mandibular cortical bone in the lateral corpus and the articular area of the mandibular symphysis.[19]

The changes found in the maxilla in the present study were not, however, evident in the area of the masseteric mandibular insertions. The measured masseteric ridge region had a much greater density than the highest density recorded for the maxilla, which likely reflected the high proportion of cortical bone in this area. In previous studies, which assessed the density of the jawbones, changes in the thickness of the cortical bone of the palate[23] and bone mineral density of mandibular alveolar bone[21] may alter with different loading patterns. Due to a much higher bone mineral density of cortical bone, changes associated with loading may, in fact, be expressed as changes in dimension, rather than density. Due to the large voxel size of the CT scans in the present study, a measurement of cortical bone thickness was not performed and future work will be required to assess dimensional changes within the cortical bone of this region.

Changes on the control side (right side)

The stress distribution throughout the maxilla is complex, with areas of tension and compression seen at sites proximal and distal to the loaded region.[31,32] Finite element modelling has shown that unilateral loading of the maxilla is likely to induce stress, not only on the loaded side, but also on the contralateral side.[33] This concept was supported by the density observations in the present study, since the unilateral increases and decreases in bite force appear to be sufficient to have induced contralateral changes in bone density within the maxilla. Unilateral denervation may also have a deleterious effect on the neuronal reflexes of the opposing masseter muscle; due to contralateral muscle spindle degeneration following denervation.[34]

This could possibly exaggerate the difference between the *form.* and *den.+form.* groups relative to the *den.* group found in the present study.

Experimental side changes within groups

The assumed increase in loading on the experimental side in the *form.* group appeared to cause a higher bone density of the zygomatic bone on the ipsilateral side. The change appeared to be more modest than those between groups but was statistically significant and supported the concept that increased muscle loading affects bone density of surrounding local structures. However, no other significant differences were observed between left and right sides for other structures. A previous assessment of the distribution of masticatory force throughout the facial skeleton showed regions of high stress in the anterior root of the zygoma and the anterior portion of the zygomatic arch, with a steep strain gradient descending anteriorly to posteriorly along the arch.[35] Therefore, the change in zygomatic bone density found in the present study would not be entirely unexpected, although the lack of change in other bony structures is surprising.

While the results of previous studies might suggest that the use of a β_2-agonist adversely affects skeletal micro-architecture, bone mineral density and strength,[10,12] the results of the present study suggest that local muscular factors are more important determinants of bone tissue turnover than the drug itself.

Conclusions

Within the limits of the present rat study, the following conclusions may be drawn:

- Intramuscular formoterol injection into denervated or unaltered masseter muscles is unlikely to reduce bone mineral density, either at close or distant sites within the craniofacial skeleton.
- Intramuscular injection of formoterol into either unaltered or denervated masseter muscles is likely to increase bone mineral density of the maxilla on the *ipsilateral* side when compared with unaltered control or denervation groups.
- Relative to denervation alone, unilateral intramuscular injection of formoterol, regardless of the presence or absence of unilateral denervation, is likely to increase bone mineral density of the maxilla on the *contralateral* side.
- Unilateral intramuscular injection of formoterol into unaltered masseter muscles is likely to increase bone mineral density of the zygoma on the *ipsilateral* side.

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