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The effects of denervation and formoterol administration on facial growth

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Objective: To identify and demonstrate possible alterations of skeletal structures which might follow either unilateral surgical denervation of the masseter muscle, unilateral intramuscular injection of formoterol directly into the masseter muscle, or intramuscular formoterol injection after surgical denervation.

Materials and methods: Male Sprague Dawley rats (N = 16; four weeks of age) were prepared as four groups: 1. surgical sham + saline injection into the masseter muscle (*sham*); 2. surgical denervation of the masseter muscle only (*den.*); 3. surgical denervation of the masseter muscle plus intramuscular formoterol injection into the affected muscle (*den.+form.*); 4. intramuscular formoterol injection into the masseter muscle only (*form.*). The specimens were submitted for CT examination, the skulls and hemi-mandibles were photographed and measurements of craniofacial bones were made.

Results: In this relatively small sample, comparisons between non-experimental and experimental sides revealed differences, both *within* the groups and for the same measurements *between* groups, with the *den.* and *den.+form.* groups showing the most change. Relative increases in the gonial angle shown in these groups occurred bilaterally, with the change on the experimental side always greater in magnitude than the change on the contralateral side.

Conclusions: Surgical denervation of the masseter muscle leads to an alteration in the size and shape of the skeletal structures close to the zygoma and the mandible. The intramuscular injection of formoterol into denervated masseter muscle seems to limit this skeletal alteration after surgical denervation.

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Introduction

The muscles of mastication are responsible for performing the essential functions of chewing, swallowing, speaking and breathing. In conjunction with these functional roles, these muscles are also considered to be key regulators influencing the growth and development of the craniofacial skeleton.[1] As orthodontic interventions are often timed to alter or take advantage of facial growth, the influence of the muscles is likely to affect orthodontic treatment and its outcome. It has been postulated that, in the future, it may well be possible to purposely alter the functional characteristics of the mandibular muscles and, in turn, their associated effects on the adjacent bony structures, in an attempt to increase the efficiency of orthodontic treatment and the stability of the gained results.[1]

Many previous animal studies have involved an assessment of the effects of altering craniofacial muscle structure and function on hard tissue growth. These studies have revealed that normal craniofacial growth and function can indeed be affected by a reduction in, or an enhancement of, the function of the facial and masticatory muscles.[2-12]

β_2-adrenoceptor agonists (β_2-agonists) are commonly used for the treatment of asthma in humans, but some can also induce skeletal muscle hypertrophy or attenuate skeletal muscle atrophy concomitant with denervation, chronic glucocorticoid administration or

physical inactivity.[13-19] β_2-agonists are known to cause an increase in individual muscle fibre size and can even alter muscle fibre composition.[16,17,19,20]

Based on the current knowledge of the differential effects of surgical denervation and formoterol administration on skeletal muscle structure and function, this present study was designed to identify specific alterations of skeletal structures which might follow unilateral surgical denervation of the masseter muscle; to assess alterations in skeletal growth which might follow unilateral intramuscular injection of formoterol into the masseter muscle; and to determine the skeletal and dental effects of such intramuscular formoterol administration after surgical denervation. The gained information was expected to provide further evidence for the development of possible future protocols for formoterol administration before, during or after orthodontic treatment in humans.

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 four 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 assigned 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 N = 4 rats per 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 administered 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 arbitrarily chosen as the 'experimental' side in all animals. Blunt dissection was performed to expose the masseteric nerve in the sigmoid notch of the coronoid process of the mandible. In the *sham* group, the masseteric nerve was identified but not cut. In the *den.* and *den+form.* the masseteric nerve was cut and a 5 to 10 mm section excised. No surgery was performed on the *form.* group.

Formoterol administration

The *sham* group received an intramuscular injection of sterile saline (100 µL/0.9%) into the left side masseter muscle every third day for eight weeks. The *den.+form.* and *form.* groups received intramuscular injections of formoterol (100 µg in saline; Astra-Zeneca, 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 with an overdose of 6 mg/kg i.p. pentobarbital sodium (Nembutal, Rhone Merieux, Pinkenba, QLD, Australia).

Imaging

Computed tomography (CT) images of the intact, articulated specimens were made (110 kVp, 36 mA., 0.75 mm slice thickness, slice distance 0.5 mm. Somatom Emotion 16 Excell, Siemens, Germany) at the Faculty of Veterinary Science at The University of Melbourne. A ruler of known length was included in the scanned field. The specimens were then de-fleshed by bulk tissue removal, after which disarticulation of the hemi-mandibles was performed by hand, with the final cleaning performed by demestid larvae and beetles. Standardised photographs were taken of the superior aspects of all skulls, with the occlusal planes parallel to the film plane. Standardised photographs were also taken of the medial aspect of each hemi-mandible (Figures 1 and 2) (F40, 1/250 sec, ratio 1:28. Nikkon DX2, Nikkon 105 AF MicroNikkor. Nikkon. Tokyo, Japan).

Data collection

The CT images were calibrated using the standard ruler. All of the specimens were orientated with the maxillary occlusal plane horizontal and the coronal slice established perpendicular to the occlusal plane and 0.5 mm posterior to the distal surface of the maxillary third molar teeth. Vertical, transverse

Figure 1. Superior view of the rat skull displaying the anatomical points used for analysis. (see Table I for definitions of all points)

Figure 2. Medial aspect of the rat hemi-mandible, displaying the anatomical points used for analysis. (see Table I for definitions of all points)

and angular measurements were taken from the established coronal slice using an Onis Viewer 2.3 (Figure 3) (DigitalCore, Co. Ltd, Tokyo, Japan). A lateral cephalogram was also generated from these images (Figure 4).

The photographic images of the skulls and the medial surfaces of the hemi-mandibles were also calibrated using the same standard ruler. Anatomical points were identified and angular and linear measurements recorded using Photoshop CS3 software (Adobe Systems Incorporated. San Jose, CA, USA).

The definitions of all anatomical points and planes used in this study are shown in Tables I and II.

Statistical analysis

All statistical tests were performed using SPSS 17.0 statistical software (SPSS Inc., CHI, USA). Descriptive statistics were calculated for all data. Differences between groups were assessed using two-tailed paired *t*-tests with the level of significance set at

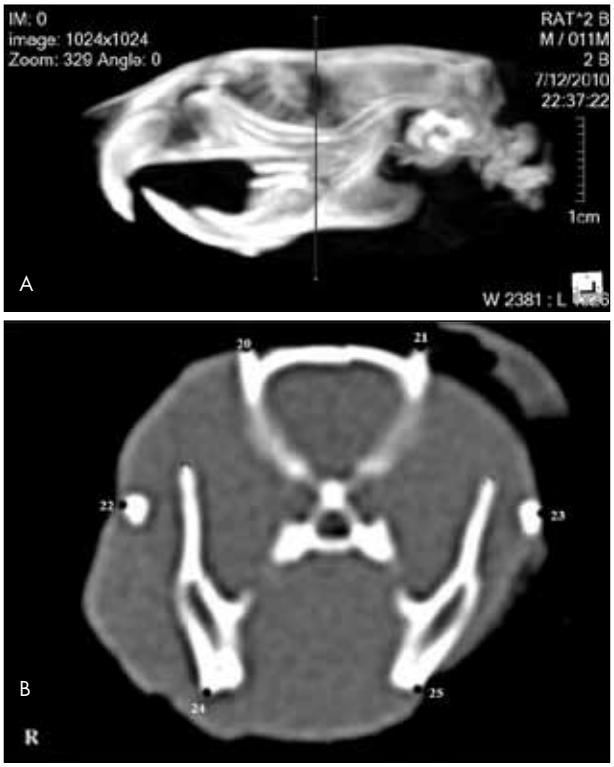

Figure 3. (A) The orientation of the coronal slice taken. (B) The coronal CT slice taken 0.5 mm distal to the third maxillary molar with the occlusal plane horizontal, displaying the anatomical points used for analysis. (see Table I for definitions of all points)

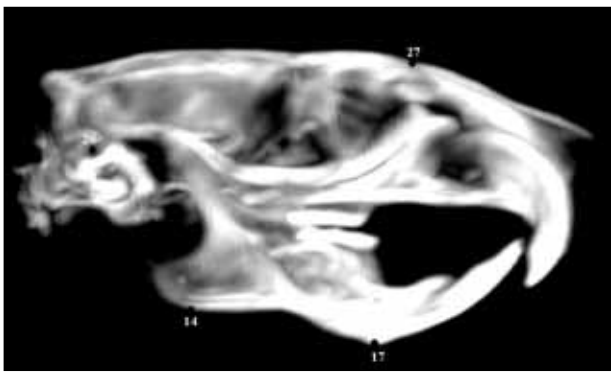

Figure 4. The lateral cephalogram generated from the CT images displaying the anatomical points used for analysis. (see Table I for definitions of all points)

$p < 0.05$. One way analysis of variance (ANOVA) tests were used to find differences between the four groups ($p < 0.05$) and a post-hoc Fisher's least significant difference test was performed when significance was detected.

Error measurement

Repeat measures were taken for the *sham* group one month after the initial measurements were recorded. Intra-class correlations were performed to assess

Table I. Definitions of the points used for the craniofacial analysis.

Anatomical structure	Point number (right, left)	Definition
TFa	1, 2	The most anterior point of the temporal fossa
TFm	3, 4	The most medial point of the temporal fossa
Zi	5, 6	Widest point of the temporal fossa on the internal aspect of the zygomatic arch
ZMm	7, 8	The medial point of the middle of the suture between the zygoma and maxilla
ZMl	9, 10	The lateral point of the middle of the suture between the zygoma and maxilla
TFp	11, 12	The most posterior point of the temporal fossa
Co	13	The most superior posterior point on the mandibular condyle
Gn	14	The most inferior point below the ramus of the mandible
Ra	15	The most posterior point on the posterior bony projection from the ramus of the mandible
I	16	The point on the lingual surface of the mandibular incisor where it meets the most superior part of the alveolar bone
Me	17	The most inferior point on the anterior part of the body of the mandible
MandM1	18	The cusp tip of the most anterior cusp of the most mesial mandibular molar tooth
MandM3	19	The cusp tip of the most distal buccal cusp of the most distal mandibular molar tooth
TCct	20, 21	The most superior point of the temporal crest on the CT image
Zct	22, 23	The most lateral point of the zygoma on the CT image
MRct	24, 25	The most inferior point of the mandibular ridge on the CT image
Po	26	The centre of the shadow of the external auditory meatus on the lateral cephalogram
NNFF	27	The midline junction of the sutures between the nasal bones and frontal bones

Table II. Definitions of the anatomical planes used for analysis.

Anatomical planes	Definition
Frankfort plane	A plane intersecting points NNFF and Po as seen on the lateral cephalogram
Mandibular plane	A plane intersecting points Gn and Me
MdVert	A plane intersecting points Co and Gn
MdBody	A plane intersecting Ra and I

the reliability of the measurements. All intra-class correlation coefficients ranged from 0.894 – 1 and no significant differences were found ($p < 0.05$) between initial and repeated measurements.

Results

Changes between right (non-experimental) and left (experimental) sides (Table III)

Group 1 (Sham)

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

Group 2 (Den.)

A significant difference was found for the measurement between TCct and Zct (experimental side reduced, mean change of 2.4%).

Group 3 (Den.+form.)

Significant differences were found between measurements of the transverse thickness of the zygomatic bone, ZMm to ZMl (experimental side reduced, mean change 23%), mandibular length Ra to I (experimental side reduced, mean change 1.9%), Ra to MandM1 (experimental side reduced, mean change 2.2%), Ra to MandM3 (experimental side reduced, mean change 3.8%), and TCct to Zct

Table III. Statistically significant changes ($p < 0.05$) between control and experimental sides.

Significant measurements	Control (right)		Experimental (left)	
Definition	Mean	SD	Mean	SD
Sham group				
Nil				
Den. group				
TCct to Zct	12.98	0.18	12.68	0.20
Den.+form. group				
ZMm to ZMl	1.59	0.18	1.21	0.05
TCct to Zct	13.13*	0.25	12.60*	0.33
Ra to I	29.77	0.17	29.21	0.30
Ra to MandM1	21.63	0.30	21.16	0.33
Ra to MandM3	15.75	0.25	15.25	0.30
Form. group				
Nil				

All values are expressed in millimetres
SD = standard deviation
All changes significant ($p < 0.05$) (2-tailed)
* Highly significant ($p < 0.01$) (2-tailed)

(experimental side reduced, mean change 4.4%).

Group 4 (form.)

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

Changes between groups

Linear measurements

Significant differences between experimental groups were found for the maximum transverse measurement of the experimental side temporal fossa, TFm to Zi (*form.* > *sham*, mean change 9.1%); maximum length of the experimental side temporal fossa, TFa to TFp (*den.* > *sham*, mean change 8.3%. *den.+form.* > *sham*, mean change 7.0%); experimental side TCct to Zct bone (*form.* > *sham*, mean change 4.5%. *form.* > *den.*, mean change 3.5%. *form.* > *den.+form.*, mean change 4.1%); and experimental side TCct to MRct (*den.* > *sham*, mean change 6.2%. *den.* > *den.+form.*, mean change 7.4%) (Table IV).

Angular measurements

Significant angular differences were found for measurements of the control side gonial angle, the angle between the MdVert and MdBody planes (*den.+form.* > *sham*, mean change 3.83°, *den.+form.* > *form.*, mean change 5.55°, *den.* > *form.*, mean change 4.88°); the experimental side gonial angle (*den.* > *sham*, mean change 7.05°, *den.* > *den.+form.*, mean change 5.23°, *den.* > *form.*, mean change 8.03°, *den.+form.* > *form.*, mean change 2.8°); and the cephalometric mandibular plane angle, between the Frankfort and Mandibular planes (*den.* > *sham*, mean change 5.33°, *den.* > *den.+form.*, mean change 5.50°, *den.* > *form.*, mean change 5.67°) (Table V) .

Discussion

Changes associated with the zygoma

Since the zygoma comprises the cranial attachment for the masseter muscle, changes in the dimensions associated with the zygoma have often been seen following alterations in masseter muscle function. For example, decreases in external zygomatic length[7] and volume[8] have been reported in rabbits when botulinum neurotoxin A was used to paralyse the masseter muscle. A similar result was found in the present study when the masseter muscle was denervated.

An assessment of possible vertical changes in the zygoma associated with masseter muscle function has not been previously reported. In the present study, a consistent increase in the vertical distance from the zygoma to the temporal crest was found on the experimental side following the intramuscular administration of formoterol, while a reduction occurred on the experimental side of the *den.* group when compared with the control side. The vertical height from the temporal crest to the mandibular ridge was found to increase in the *den.* group on the experimental side, compared with the *sham* and *den.+form.* groups. It is

Table IV. Statistically significant linear changes ($p < 0.05$) between groups.

	Group 1 (*sham*)		Group 2 (*den.*)		Group 3 (*den.+form.*)		Group 4 (*form.*)	
Measurement definition	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control side (right) – linear								
Nil								
Experimental side (left) – linear								
TFm to Zi	6.08[a]	0.27	6.33	0.20	6.34	0.10	6.69[a]	0.39
TFa to TFp	14.46[a,b]	0.70	15.76[a]	0.19	15.55[b]	0.32	14.91	0.81
TCct to Zct	12.55[a]	0.26	12.68[b]	0.20	12.60[c]	0.33	13.13[a,b,c]	0.13
MRct to TCct	21.10[a]	0.26	22.20[a,b]	0.71	20.73[b]	0.61	21.53	0.82

All values are expressed in millimetres
SD = standard deviation
All shown measurements have significant differences between groups ($p < 0.05$) (one-way ANOVA)
[a,b,c] denote significant differences between groups for the same measurement

Table V. Statistically significant angular changes ($p < 0.05$) between groups.

	Group 1 (*sham*)		Group 2 (*den.*)		Group 3 (*den.+form*)		Group 4 (*form.*)	
Measurement definition	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control side (right) – angular								
Gonial angle (angle between Co-Gn and Ra-I)	95.18[a]	1.56	98.33[c]	2.98	99.00[a,b]	2.95	93.45[b,c]	1.74
Experimental side (left) – angular								
Gonial angle (angle between Co-Gn and Ra-I)	95.60[a]	0.32	102.65[a,b,c]	2.63	97.43[b,d]	0.53	94.63[c,d]	1.24
Midline								
Mandibular plane angle (relative to Frankfort Plane)	22.81[a]	1.11	28.14[a,b,c]	0.48	22.64[b]	2.08	22.48[c]	1.29

All values are expressed in degrees
SD = standard deviation
All shown measurements have significant differences between groups ($p < 0.05$) (one-way ANOVA)
[a,b,c,d] denote group differences

interesting to note that Kwon et al.[7] observed a decrease in the length from the anterior zygomatic arch to the anterior masseteric attachment on the lower border of the mandible following masseteric denervation. This may, however, be at least partly explained by the reduction in zygomatic length also reported in their sample, rather than simply being directly related to vertical change. Whatever the case, the resultant vertical position of the zygoma, subsequent to various alterations in masseter muscle function, does require further investigation.

Mandibular changes

Changes in mandibular growth related to masticatory muscle function have been examined extensively, but no previous study has assessed the potential for normalisation of growth after muscle function has already been reduced. The significant reduction found in the present study in the antero-posterior length of the experimental hemi-mandible, relative to the other side in the *den.+form.* group, is consistent with previous reports of formoterol administration following direct reduction in masseter function by surgical removal of the masseter muscle, surgical denervation or pharmacological paralysis.[5,21,22] The measurements in the posterior portion of the mandible indicated larger proportional changes in the length of the entire mandible, in turn reflecting the fact that the area posterior to the teeth is the area of greatest change. This is consistent with the changes in length occurring posterior to the first molar tooth, previously reported by Carter and Harkness.[5]

It has been suggested that the considerable increase in muscle size and function in myostatin-null mice leads to an increase in mandibular length.[11,12] Although formoterol is accepted as an anabolic agent likely to increase muscle mass and function, it is noteworthy

that no change in mandibular length was found in the *form.* group in the present study. It should be recognised, however, that the muscle hypertrophy exhibited in the myostatin-null mice would generally be considered to be at the uppermost limit, whereas the observed muscle hypertrophy occurring after β_2-agonist administration would be considerably less.

The gonial angle seems to react to changes in masseter muscle function in a consistent manner, with surgical and pharmacological reductions in muscle function generally resulting in an increase in the gonial angle.[4,23,24] A reduction in dietary hardness alone has been shown to produce a backward growth rotation of the mandible.[9,25-27] Interestingly then, the results of the present study did not show significant differences in the gonial angles between experimental and non-experimental sides when the entire sample was evaluated. Comparisons between groups revealed the resultant experimental side gonial angle was significantly greater in the *den.* group when compared with the other groups. As a likely representation of unilateral muscle alteration affecting contralateral structures, these significant changes in the gonial angle also occurred on the opposite side, although not to the same extent. Consistent with the gonial angle changes, the mandibular plane angle increased in the *den.* group relative to the other groups, a finding which also matched previous reports.[21,22] Although care must be taken when extrapolating the results of animal studies to likely effects in humans, the changes found in both the mandibular plane and gonial angles reflect the previously reported similar inverse relationship which exists between the size and strength of human masseter muscles and changes in the adjacent bony angles.[28-33]

β_2-agonists as muscle therapeutics

An aim of the present study was to assess how intramuscular administration of β_2-agonists might influence growth of the craniofacial skeleton. While the administration of formoterol, alone, tended to alter the skeleton in ways consistent with the effects of naturally larger masticatory muscles, the effect was only mild. Intramuscular injections of formoterol after denervation did, however, reduce the magnitude of the expected changes in both mandibular plane and gonial angle measurements, which largely offset the expected effects of denervation, alone.

Individual variation in orthodontic patients is not only present in their dental malocclusions, but also in the neuromuscular and skeletal environments in which those malocclusions develop. The findings of this study and similar previously reported studies suggest that, by altering the craniofacial musculature, a likely direct effect upon the underlying skeletal structures is produced. Further studies may lead to ways in which the mandibular muscles may be selectively altered, and therefore, in turn, affect dentofacial growth and development. Subsequently, it might be possible to influence individual muscular responses to orthodontic and orthognathic treatments in individual patients, and to somehow control long-term muscular behaviour following active treatment. For these reasons, studies of this type should be of interest to all clinicians working to control or alter the development of various dentofacial structures and their interrelationships.

Conclusions

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

- Surgical denervation of the masseter muscle is likely to lead to an alteration in the size and shape of skeletal structures close to its area of insertion, on both the zygomatic arch and the mandible, with limited effects on more distant structures.
- Intramuscular injection of formoterol into the masseter muscle is likely to have little effect on the skeletal structures in the craniofacial region.
- Intramuscular injection of formoterol into previously denervated masseter muscles is likely to limit the magnitude of any skeletal alterations following the surgical denervation.

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