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Growth effects of botulinum toxin type A injected unilaterally into the masseter muscle of developing rats*

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Abstract: Objective: To evaluate the effects of botulinum toxin type A (BTX-A) on mandible skeletal development by inducing muscle hypofunction. Methods: Four-week-old Sprague-Dawley rats (*n*=60) were divided into three groups: Group 1 animals served as controls and were injected with saline; Group 2 animals were injected unilaterally with BTX-A (the contralateral side was injected with saline); and Group 3 animals were injected bilaterally with BTX-A. In Group 2, the saline-injected side was designated the control side (Group 2-1), whereas the BTX-A-injected side was designated the experimental side (Group 2-2). After four weeks, the animals were sacrificed, dry skulls were prepared, and mandibles were measured. Results: In the unilateral group, the experimental side (Group 2-2) had reduced dimensions for all mandible measurements compared with the control side (Group 2-1), suggesting a local effect of BTX-A on mandible growth, likely due to muscle reduction. Conclusions: Localized BTX-A injection induced a change in craniofacial growth, and the skeletal effect was unilateral despite both sides of the mandible functioning as one unit.

Key words: Botulinum toxin, Craniofacial growth, Mandible
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1 Introduction

Botulinum toxin type A (BTX-A) reduces muscular contractions by temporarily inhibiting acetylcholine release at the neuromuscular junction. Since its initial use in the treatment of strabismus (Scott *et al.*, 1989), BTX-A has been shown to be effective in treating disorders characterized by local muscle hyperactivity. In past decades, the use of BTX-A as a therapeutic agent has expanded widely. It has been used to treat adult patients with severe primary axillary hyperhidrosis, cervical dystonia, strabismus, blepharospasm, and other conditions. Among pediatric patients, even though all uses are off-label, BTX-A is used in cerebral palsy to reduce spasticity, leading to slight improvements in gait patterns (da Fonseca and Casamassimo, 2011).

The use of BTX-A in the orofacial region has emerged in the field of dentistry. It is used to treat primary and secondary masticatory and facial muscle spasms, facial tics, orofacial dyskinesias, dystonias, as well as pain disorders without a clear-cut motor hyperactivity basis (Clark *et al.*, 2007). It is also often used in patients with masseter muscle hypertrophy (Ahn and Kim, 2007). BTX-A reduces muscle thickness, leading to changes in the facial contour (Kim *et al.*, 2010). It is well known that a change in muscle activity not only has an esthetic effect, but also may relieve severe bruxism-related neurological disorders.

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This study aimed to investigate the effects of BTX-A beyond the treatment options described above. We focused on the effects of reducing volume and altering the function of muscle hypertrophy and skeletal development. According to the functional matrix theory (Moss and Rankow, 1968), craniofacial growth and development are not intrinsically regulated by bone or cartilage, but by the surrounding muscle. Thus, the induction of muscle hypofunction may influence facial growth. Recent studies using BTX-A (Kim *et al.*, 2008; Tsai *et al.*, 2010) have supported the theory. These studies included animal experiments designed to investigate changes in masticatory muscle function due to the effects of BTX-A on skeletal development. They have shown that BTX-A can have inhibitory effects in the developing rat mandible.

In a study of rat mandibles injected with BTX-A in bilateral masseter muscles, mandibular dimensions were reduced compared with those of saline-injected rats (Kim *et al.*, 2008). However, a limitation of that study was that the comparison was made between different individuals. To overcome this limitation, our study was designed to evaluate the effects of BTX-A in the same individual—one side of the mandible was injected with BTX-A and compared to the other side, which served as a control, injected with saline.

Therefore, this study investigated the effects of unilateral injection of BTX-A into the masseter muscle in growing rat mandibles.

2 Materials and methods

2.1 Subjects and study design

A total of 63 four-week-old (T_0) male Sprague-Dawley rats were randomly divided into three groups, with 21 subjects in each group: Group 1, control group; Group 2, unilateral BTX-A group; and, Group 3, bilateral BTX-A group. The body weight of each rat was measured. The BTX-A used in this study was Botox® (Allergan Inc., Irvine, CA, USA). One vial of Botox® (100 U) was diluted with saline to make 3 U of Botox® in a 0.05-ml solution.

The masseter muscle of each rat was injected with either saline or BTX-A solution, depending on the group to which it was assigned. (1) Control group (Group 1): 0.05 ml of saline was administered to both sides of the superficial portion of the masseter muscle. (2) Unilateral BTX-A group (Group 2): one masseter muscle of each rat was injected with 0.05 ml saline, whereas the masseter muscle on the other side was injected with 3 U (0.05 ml) of BTX-A solution. The saline side was designated as the control side (Group 2-1), and the BTX-A side was designated as the experimental side (Group 2-2). Rats were randomly assigned to groups. Half were injected with BTX-A on the left side and half were injected with BTX-A on the right side. (3) Bilateral BTX-A group (Group 3): 3 U (0.05 ml) of BTX-A solution was administered to bilateral masseter muscles.

Experimental animals were fed ground rat pellets along with standard pellets to compensate for the possible adverse effect of a reduced incisive function in BTX-A-injected animals. Pellets that were fed to each subject were weighed weekly. All subjects were also weighed weekly (from T_0 to T_1, T_2, T_3, and T_4) until sacrifice at four weeks (T_4).

This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Samsung Biomedical Research Institute (SBRI), Korea.

2.2 Dry mandibles

The mandibles were dissected from sacrificed animals and sectioned into two halves. Photographs of dry mandibles were taken using a digital camera, which was held at a constant distance from the mandible. Photos were transferred to computer images. The mandibular measurements used in this study (Fig. 1) were described by Asano (1986). All measurements were performed by three investigators.

2.3 Statistical analysis

The overall body weights at T_0 were evaluated for inter-group variability using the Kruskal-Wallis test with Bonferroni's correction. Differences between specific groups were assessed using multiple comparison tests. The rate of weight increase was estimated using weights from T_0 to T_4 and was evaluated between groups using the Kruskal-Wallis test and multiple comparison tests. Mandibular measurements are described by means and standard deviations (SDs). The repeated-measures analysis of variance (ANOVA) was used for the evaluation of each mandibular measurement for all groups. A P value of

<0.05 was considered to be statistically significant. The reliability of the three investigators was evaluated using the intraclass correlation test.

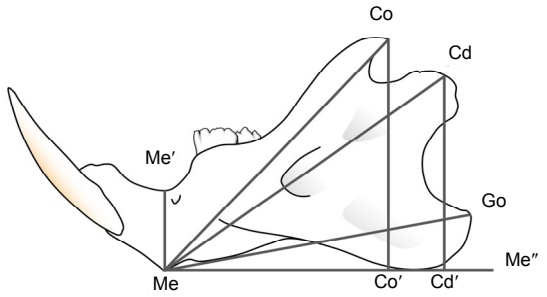

Fig. 1 Landmarks and measurements of the mandible used in this study

Me, the most inferior point of mental protuberance; Me′, the most inferior point of anterior alveolar bone; Me-Me″, the tangent to the bottom of the angular process through Me; Go, the most posterior tip of the angular process; Cd, the central point of the condyle; Cd′, the crossing point on Me-Me″ perpendicular to Me-Me″ from Cd; Co, the tip of the coronoid process; Co′, the crossing point on Me-Me″ perpendicular to Me-Me″ from Co; Me-Go, mandibular body length; Me-Cd, condylar length; Me-Co, coronoid process length; Me-Me′, anterior region height; Co-Co′, coronoid process height; Cd-Cd′, condylar height. The figure has been adopted from the study of Asano (1986)

3 Results

Sixty of 63 animals completed the study protocol. Some mandibles were excluded from the measurement due to fractures during processing. The intraclass correlation test for reliability gave a mean coefficient of 0.6165, demonstrating good reliability between investigators.

3.1 Amount of pellets

The total amounts of pellets fed to the animals did not differ significantly between groups. However, when the amounts of ground pellets and standard pellets were analyzed separately, significant differences were observed. At T_1, T_2, and T_3, rats in Group 3 ingested fewer standard pellets than those in Group 1. In contrast, when comparing amounts of ground pellets, Group 3 rats ingested more than Group 1 rats at T_1 and T_3.

3.2 Weight measurements

Although the experimental animals were randomly divided into three groups, there was a statistical difference in body weight among the three groups at the start of the trial. Average body weight was highest in the control group ((91.19±5.72) g), with the lowest weight in the unilateral BTX-A group ((83.19±1.65) g), as shown in Table 1. The rate of weight increase was estimated in each group ((W_4−W_0)/W_0×100%, where W_4 and W_0 are the weights at T_4 and T_0, respectively) and statistically analyzed. The rate was significantly higher in Group 1 ((273.48±22.14)%) and Group 2 ((270.26±21.67)%) than in Group 3 ((249.97±24.37)%), but there was no significant difference between Groups 1 and 2.

3.3 Mandibular measurements

Mandibular measurements for all groups are shown in Table 2, and comparisons between groups are shown in Table 3 (P<0.05). For all six mandibular measurements in Group 2, the experimental side (Group 2-2) had significantly smaller dimensions than the control side (Group 2-1). Differences in measurements between the control group (Group 1) and the control side of Group 2 (Group 2-1) were not statistically significant, except for condylar height. In contrast, most mandibular measurements of the experimental side of Group 2 (Group 2-2) had smaller dimensions than those of the control group (Group 1), with the exception of condylar length, which was not significantly different between these two groups. Comparison between the control group and the bilateral BTX-A group (Group 3) showed shorter lengths in Group 3, with the exception of the condylar and coronoid process lengths. Differences between the experimental side of Group 2 (Group 2-2) and Group 3 were not statistically significant.

4 Discussion

It is essential to understand the process of craniofacial growth and development, especially in the field of pediatric dentistry. Growth deficiencies or overgrowth of the mandible may result in malocclusion, which can influence masticatory function as well as esthetics among growing children. Many studies have attempted to control skeletal growth with the aim of restoring normal function and facial appearance by altering masticatory muscle function.

Table 1 Mean body weight of rats in the three groups measured weekly during the experimental period

Group	Body weight (g)					Weight increase rate (%)
	T_0	T_1	T_2	T_3	T_4	
1	91.19±5.72	154.19±9.09	220.82±12.74	281.89±15.75	339.71±18.44	273.48±22.14
2	83.19±1.65	135.09±5.85	195.24±9.98	254.74±13.88	308.12±20.46	270.26±21.67
3	88.14±1.28	131.57±9.95	191.13±13.89	252.19±19.06	308.60±23.63	249.97±24.37

Group 1: control group (n=20); Group 2: unilateral BTX-A injection group (n=19); Group 3: bilateral BTX-A injection group (n=21). The weight increase rate was calculated using weights at T_0 (4 weeks of age, W_0) and T_4 (8 weeks of age, W_4): $(W_4-W_0)/W_0\times100\%$. T_1, T_2, and T_3: one, two, and three weeks after T_0. Data are expressed as mean±SD

Table 2 Mean mandibular measurements at the time of sacrifice (T_4, 8 weeks of age) for each group

Group	Mandibular body length (mm)	Condylar length (mm)	Coronoid process length (mm)	Anterior region height (mm)	Condylar height (mm)	Coronoid process height (mm)
1	20.43±0.39	21.89±0.44	19.58±0.43	4.80±0.14	10.81±0.41	13.06±0.34
2-1	20.07±0.44	21.91±0.43	19.43±0.44	4.75±0.12	10.43±0.60	12.95±0.36
2-2	19.74±0.36	21.61±0.51	18.97±0.38	4.50±0.17	9.95±0.36	12.32±0.37
3	19.78±0.59	21.52±0.51	19.24±0.52	4.48±0.15	10.08±0.45	12.48±0.39

Group 1: control group (n=20); Group 2-1: control side of unilateral BTX-A injection group (n=19); Group 2-2: experimental side of unilateral BTX-A injection group (n=19); Group 3: bilateral BTX-A injection group (n=21). Differences among groups were statistically significant (P<0.05). Data are expressed as mean±SD

Table 3 Significant differences in mandibular measurements between groups when compared pairwise

Group	Mandibular body length	Condylar length	Coronoid process length	Anterior region height	Condylar height	Coronoid process height
1 vs. 2-1	NS	NS	NS	NS	*	NS
1 vs. 2-2	*	NS	*	*	*	*
1 vs. 3	*	NS	NS	*	*	*
2-1 vs. 2-2	*	*	*	*	*	*
2-1 vs. 3	NS	*	NS	*	*	*
2-2 vs. 3	NS	NS	NS	NS	NS	NS

Group 1: control group; Group 2-1: control side of unilateral BTX-A injection group; Group 2-2: experimental side of unilateral BTX-A injection group; Group 3: bilateral BTX-A injection group. NS: not significant; *: a significant difference (P<0.05) between the groups

Horowitz and Shapiro (1955) and Moore (1973) altered masseter muscles by myectomy, resulting in vertically-directed growth patterns of the mandible. In another study, denervation of the masseter muscle resulted in decreased mandibular height and length (Sato *et al.*, 1986).

In this study, we used a non-invasive method involving BTX-A injection to alter masseter muscle function. Surgical denervation and myectomy may cause scar tissue or damage adjacent structures that can further alter growth patterns (Gardner *et al.*, 1980). This study was designed to evaluate the influence of BTX-A on mandibular development by comparing changes in dimensions between BTX-A-injected and saline-injected subjects. Since both sides of the mandible function as one unit, it is possible to experience a unilateral BTX-A injection effect leading to decreased bilateral movement of mandible. For this reason, growth of the control side of the unilateral group (Group 2-1) was compared to that of the control group (Group 1), and growth of the experimental side of the unilateral group (Group 2-2) was compared to that of the bilateral BTX-A-injected group (Group 3) to see whether BTX-A was effective, even though the mandible functions as a single unit.

4.1 Changes in mandibular measurements

Mandibular measurements were statistically smaller in Group 3 than in Group 1, with the exception of the condylar and coronoid process lengths. The lack of statistical differences for these two measurements was presumably due to observational errors stemming from the difficulty in locating the condylar point and the tip of the coronoid process. Our finding of statistically smaller dimensions in Group 3 coincides with that of a previous study (Kim *et al*., 2008). In previous studies, histological findings revealed an inhibitory action of BTX-A due to induced apoptosis at the proliferation stage of the reserve zone of condylar cartilage in developing mandibles. The

current study included unilateral injection because it enabled the measurement of differences in dimensions between treatments in a single rat, thereby minimizing subject-dependent variability.

When control and experimental sides of the unilateral BTX-A group were compared, all measurements showed statistically significant differences. Similar results were reported in another experiment using rabbits (Kwon *et al.*, 2007). They found that BTX-A induces site-specific muscular hypofunction and influences morphology at a local skeletal site. In the current study, BTX-A also induced localized craniofacial growth changes with unilateral effects.

There were some limitations to this study. Despite the fact that 63 rats were randomly divided into three groups, the average weight of the control group (Group 1) was significantly higher than those of the other two groups. Therefore, the statistical difference in mandibular measurements between Groups 1 and 3 may have been influenced by original weight differences at T_0, as well as by the presence of BTX-A. However, BTX-A injection seemed to have an effect on skeletal development, as comparisons between Group 2-1 and Group 3, and between Group 2-2 and Group 3, showed no significant weight differences.

4.2 Overall body weights and amounts of pellets consumed

When the weight increase in each group was compared, there were no statistically significant differences between Groups 1 and 2, even though Group 1 started out with a significantly higher average weight. These findings suggest that unilateral BTX-A injection did not have systemic effects, but rather localized effects on mandibular development at the injected site. This result coincides with those of other studies where the overall growth of rabbits and rats was not altered by local injections of BTX-A into the masticatory muscles (Matic *et al.*, 2007b; Tsai *et al.*, 2011). Reduced masticatory function due to the toxin did not alter the systemic functions of the rats.

However, weight gain was significantly higher in Groups 1 and 2 compared with Group 3. This finding coincides with that of our previous study (Kim *et al.*, 2008), in which we concluded that weight differences may be the result of reduced muscle tonicity and mass induced by bilateral BTX-A injection.

The results pertaining to weight increase also coincided with our findings regarding pellet weight measurements. Group 3 rats ate more ground pellets and fewer standard pellets. Even though the overall pellet weight was not significantly different between groups, Group 3 rats ingested increased amounts of softer ground pellets than standard pellets. This finding was related to decreased masticatory function in Group 3 compared with other groups, in keeping with the findings of Kiliaridis *et al.* (1985). They found that consuming ground pellets may alter masticatory function in rats.

4.3 Limitations of this study

Measuring muscle mass and monitoring electromyography (EMG) to improve our understanding of muscle activity should be attempted in future studies, to improve results and statistical analysis. Research on masticatory muscles other than the masseter muscle, including the temporal muscle and digastric muscles, will help to improve our knowledge of craniofacial morphology and the specific influence of masticatory muscles (Ueda *et al.*, 1998).

This study demonstrated that BTX-A could be used to inhibit masticatory muscle contraction and to influence mandible growth. Even though many other studies have also shown that BTX-A can affect masticatory muscles and influence skeletal development, the specific actions of BTX-A remain unknown (Matic *et al.*, 2007a). Therefore, further studies investigating the influence of BTX-A on muscle change and skeletal development must be performed. Clinical applications of results from these studies may offer increased possibilities for treating mandibular problems via non-invasive, non-surgical methods. However, it is important to remember that BTX-A is not widely used in the pediatric population, and safe dosages have yet to be established. Unusual systemic effects have been reported with repeated injections (Howell *et al.*, 2007). Therefore, additional studies are needed to determine the safety profile of BTX-A.

5 Conclusions

This study demonstrated that unilateral injection of BTX-A was associated with reduced mandibular growth on the BTX-A-injected side compared with the non-injected side. Localized BTX-A injection may induce craniofacial growth changes. Although both sides of the mandible function as one unit, the skeletal effects might be unilateral.

Compliance with ethics guidelines

Chanyoung PARK, Kitae PARK, and Jiyeon KIM declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：咬肌单侧注射 A 型肉毒毒素对大鼠下颌骨生长发育的影响

目　的：通过诱导肌肉功能衰退，评估 A 型肉毒毒素（BTX-A）对下颌骨生长发育的影响。

创新点：通过对同一个体的对照侧（组 2-1）和实验侧（组 2-2）下颌骨尺寸的测量分析，证明 BTX-A 对下颌骨生长具有局部效应。

方　法：四周龄的 Sprague-Dawley 大鼠（60 只）随机分为三组：第 1 组动物注射生理盐水作为对照组；第 2 组动物单侧注射 BTX-A（对侧注射生理盐水）；第 3 组动物双侧注射 BTX-A。在第 2 组中，注射生理盐水的一侧作为对照侧（组 2-1），而 BTX-A 注射的一侧作为实验侧（组 2-2）。四周后，处死动物，制备干头骨，测量下颌骨。

结　论：尽管下颌骨的两侧作为一个功能单元，但是局部注射 BTX-A 诱导产生的颅面生长改变以及对骨骼的影响却是单侧的。

关键词：肉毒毒素；颅面生长；下颌骨