# The effect of botulinum toxin injected into masseter muscle on condyle and masseter muscle of young beagle dogs

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# The effect of botulinum toxin injected into masseter muscle on condyle and masseter muscle of young beagle dogs

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# 감사의 글

이 논문이 완성되기까지 세심한 배려와 아낌없는 격려로 저를 지도해 주시고, 부족한 제가 올바른 학문의 길로 갈수 있도록 인도해주셨던 황충주 지도 교수님께 진심으로 감사 드립니다. 연구의 시작에서부터 함께 고민해 주시고, 논문이 완성될 때까지 늘 정성으로 조언해주시고 지도해 주신 김성택 교수님께도 진심으로 감사 드립니다. 또한 논문을 위해 따뜻한 조언을 해주시고 힘이 되어 주신 유형석 교수님, 차정열 교수님, 조성원 교수님께 깊이 감사 드립니다. 교정학이라는 학문의 길에 들어설 수 있게 이끌어주시고, 지금도 늘 곁에서 보살펴주시는 박영철 교수님께 깊은 감사의 말씀을 전합니다. 언제나 따뜻하고도 세심한 조언으로 함께 해주시는 백형선 교수님, 김경호 교수님, 이기준 교수님, 정주령 교수님께도 진심으로 감사 드립니다. 치과대학 학생때의 생활뿐만 아니라 치과 의사로 살아가는데 있어서 늘 관심과 사랑으로 이끌어주시는 참스승이신 김종관 교수님께도 감사 드립니다. 그리고 멀리서 저를 늘 지켜봐 주시고 학문의 길잡이가 되어 주시는 교합학의 스승이신 김인권 교수님과 송영복 교수님께도 깊은 감사를 드립니다.

부족한 저를 응원해주시고 언제나 곁에서 행복을 위해 함께 해주시는 연세여우치과 동료 원장이신 김두형, 박준선, 이선복 선배님들께도 진심으로 감사 드립니다. 또한 한결 같은 마음으로 용기를 주시는 소성수 선배님께도 감사를 드립니다.

연구의 마무리에 도움을 준 최윤정 교수님, 실험실에서 함께했던 최성환, 김영훈, 이미림, 뭉해돌람, 이성일 선생께도 깊은 감사를 드립니다. 연구의 어려움을 격을 때 머나먼 미국에서까지 세심한 조언을 해준 권혁제 선생과 격조 있는 영문 교정에 도움을 준 안하영 선생께도 감사 드립니다.

그리고 무엇보다도 저에게 세상의 빛을 보게 해주시고 정성과 사랑으로 바르게 길러주셔서 올바른 한 사람으로 떳떳하게 살아가게 해주신, 세상에서 가장 존경하는 아버지 김덕영 옹과 사랑하는 어머니 김경숙 여사께 진심으로 깊은 감사를 드리고 영광을 받칩니다. 저를 늘 아들처럼 사랑해주시고 감싸 안아주시는 장모님 라분순 여사와 장인어른 김성환 옹께도 진심으로 감사 드립니다. 든든한 지원군 치과의사 동료이자 사랑하는 동생 김정주에게도 고마움을 전합니다.

마지막으로, 연구를 완성하기까지 묵묵히 늘 곁에서 힘이 되어 준 저의 아내와 자랑스런 세 아들에게 감사의 마음을 전합니다. 남편으로서 늘 부족한 저를 믿고, 언제나 손을 꼭 잡고 행복을 위해 함께 해주는 영원한 인생의 동반자인 사랑하는 아내 김지현과 늘 온화한 미소를 품은 듬직하고 지혜로운 똘똘이 큰아들 김성윤, 가족 생각을 누구보다 많이 하는 정많고 착하며 영리한 재주꾼 둘째 아들 김성우, 늘 가족들에게 웃음을 주는 밝고 애교 많은 똑똑한 귀염둥이 셋째 아들 김성민에게도 진심으로 고맙고 영원히 사랑한다는 말을 전합니다.

2015년 6월 저자 씀

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**Abstract**

## The effect of botulinum toxin injected into masseter muscle on condyle and masseter muscle of young beagle dogs

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This study was based on the previous studies that muscular function can affect craniofacial and mandibular growth during growth period. There had been numerous attempts to regulate mandibular growth by controlling mandiblar function. Botulinum toxin (BoNT) had been studied as a treatment medicine for muscle hypertrophy. The purpose of this study is to analysis the histological effect of BoNT injected into the masseter muscle on the condylar cartilage and the masseter muscle in young beagle dog. Samples were divided into three groups, such as the saline injection, 10U BoNT injection and 20U BoNT injection. BoNT injection was carried-out twice every 4 weeks. After 10 weeks from the first injection, the histological differences in the condylar cartilage and masseter muscle were evaluated.

In condylar cartilage the differences of the thickness in P zones were statistically significant between the two groups (group 10U & Control) and group 20U. H zones were significantly different among three groups. Especially in group 20U thinner P zone and H zone were shown.

And Histological differences of masseter muscle between experimental groups (group 20U and group 10U) and control group (group Control) were observed. The shape of masseter

muscle fibers in group 10U and group 20U was different from that of group Control. And endomysium was seen clearly with the separated cell connections in group 10U and 20U. The area of cross sectioned muscle fiber among three groups showed a significant difference statistically ($p$<0.05) each other. Each muscle fiber per unit area of group 20U was the smallest.

As a result of this, it was confirmed that BoNT injected into the masseter muscle could affect cell differentiation of condylar cartilage, which could ensure that consistent with the results of the previous studies made in lower animals, such as rat. If the further study that BoNT could affect the morphological changes in the mandible is carried out, this study could be a basis for the growth modification treatment of human mandible by using BoNT.

Key words : botulinum toxin, condylar cartilage, masseter muscle, mandibular growth, beagle

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## I. Introduction

Botulinum toxin (BoNT) temporarily inhibits the acetylcholine release at the neuromuscular junction and decreases muscular contractions. BoNT based on this mechanism was used in the treatment of strabismus (Scott et al., 1989), and it has been shown to be effective in treating disorders characterized by local muscle hyperactivity. Such use has emerged in the field of dentistry. In dentistry region BoNT is used to treat the masticatory and facial muscle spasms, dystonias, orofacial dyskinesias, facial tics as well as pain disorders without a clear-cut motor hyperactivity basis in the field of the orofacial region including dentistry (Clark et al., 2007).

It is also often used on patients with masseter muscle hypertrophy for esthetic improvement. The maximum bite force was significantly reduced after injection of BoNT for treating masseter muscle hypertrophy (Ahn and Kim., 2007). Other authors reported the effect of BoNT in reducing the masseter muscle, which was documented by ultrasonography and computed tomography (To et al., 2001). BoNT can reduce facial muscle thickness, and it can lead to changes in the facial contour (Kim et al., 2010). This therefore has been used for the esthetic effect in plastic surgery clinics. BoNT is also well known for relieving severe neurological disorders related bruxism.

Some BoNT study trying to figure out if it can be applied to the regulation of bone growth had been attempted, which was based on the functional matrix theory. 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. They have noted that muscle function is one of the most important epigenetic factors involved in guiding facial bone growth. Mandibular growth depends upon multiple factors and has therapeutic–clinical importance for so-called functional orthopedic treatment which aims at targeted modification of growth processes in the viscerocranium (Schumacher and Dokládal., 1968; Schumacher., 1968).

Mandibular growth is closely related to the occurrence and growth of temporomandibular joint. The secondary growth cartilage associated with temporomandibular joint development forms condylar cartilage. In some ways this is similar to that found in long bone epiphyseal cartilage during development stage. The condylar process of cartilage has proliferation layers of cells that can divide, which carries out role by progenitor cell for the cartilage growth. The cells become chondroblast and they form the extracellular matrix of cartilage by secreting type Ⅱ collagen and proteoglycan. After that they become chondorocytes. At the same time, the size of chondroblast is enlarged. After the cartilage formed, minerals are deposited on the cartilage, blood vessels come in the cartilage and the chondrocyte are destroyed. The differentiation of osteoblast takes place a series of processes such as formation of bone minerals deposited on the cartilage structure. The formation of bone through the differentiation of cartilage is subjected to these processes (Nanci., 2008).

Many studies tried to figure out that the control of mandibular function can engage in such cartilage differentiation. Previous animal studies of masticatory hypofunction demonstrated

less growth of the mandibular ramus in both vertical and anterior-posterior dimensions. The angular and condylar processes were also dimensionally smaller (Kiliaridis et al., 1985, 1988; Kiliaridis and Shyu., 1988). Morphological changes during craniofacial growth have been investigated in animals using approaches such as altering food consistency and performing a myoectomy, myotomy, or denervation (von Wowern and Stoltze., 1978; Behrents and Johnston., 1984; Bouvier and Hylander., 1984; Navarro et al., 1995; Ulgen et al., 1997).

Recent studies using BoNT (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 BoNT on skeletal development. They have shown that BoNT can have inhibitory effects on development rat mandible.

In a study of rat mandibles injected with BoNT in bilateral masseter muscles, mandibular dimensions were reduced compared with those of saline-injected rats (Kim et al., 2008). Furthermore, unilateral injection of BoNT was associated with reduced mandibular growth on the BoNT injected side compared with the non-injected side. Localized BoNT injection may induce craniofacial growth changes (Park et al., 2015).

The purpose of this study is to analysis the effect of BoNT injected into the masseter muscle on the condylar cartilage and the masseter muscle in young beagle dog. Whereas previous studies have made in the rat or rabbit, the experiment is significant that one step further made in the higher animals.

# II. Materials and Methods

## 1. Materials

Four male beagle dogs aged 15 weeks and weighing 10–11 kg were used for this study in accordance with protocols approved by the Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

Botox®(Allegan. Inc. USA) was supplied as BoNT injection.

## 2. Methods

Subjects were classified into three groups. They were divided into control group (group Control : 1 beagle) and two experimental groups (group 10U : 2 beagles, group 20U : 1 beagle).

***1) Animal preparation***

Prepared animals were observed for 1 week during adaptation period. After 1 week of observation period, the first injection protocol was carried-out. The animals were then initially anaesthetized by a subcutaneous injection of atropine (Daewon Pharmaceutical Co. Ltd., Seoul, Korea) with a dose of 0.05 mg/kg body weight, followed by an intravenous injection of Zoletil (Virbac Korea Co. Ltd., Seoul, Korea) at 5 mg/kg body weight and Rompun (Bayer Korea Ltd., Seoul, Korea) at 0.2 mg/kg body weight. General anesthesia was then preserved by 2% enflurane. After checking that the animal was sedated, the injection procedure was performed.

***2) Botulinum toxin injection***

Botulinum toxin mixture for injection was supplied as a freeze-dried powder. One vial (100U) of Botox® was mixed with 2 ml of saline. Injection points were marked at 5 mm, 7.5 mm height from mandible lower edge on both sides of beagle's masseter muscle (maximum prominent point).

Each beagle belonging to experimental groups (group 10U & 20U) was injected with BoNT and saline mixture at 4 injection points each accordingly (Figure 1). Control group (group control) was injected with saline.

group Control : 0.2ml of saline on each point

group 10U : 0.1ml (5U) of mixture on each point (10U per side (20U in total))

group 20U : 0.2ml (10U) of mixture on each point (20U per side (40U in total)

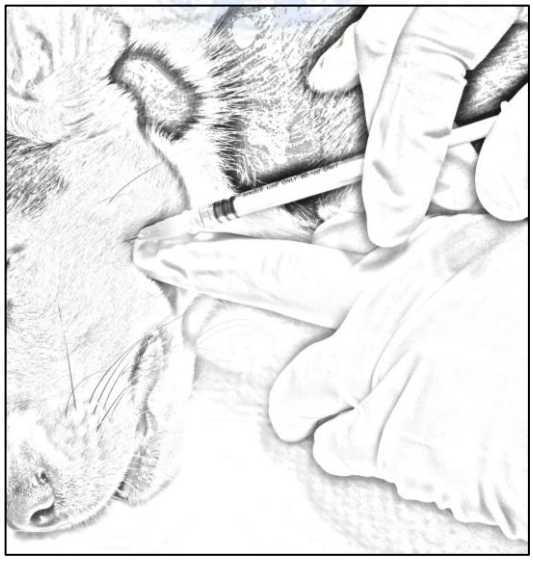

Figure 1. Botulinum toxin (BoNT) injection

After 4 weeks, the second injection was followed. The procedure was same with the first injection.

During the experimental period all the animals were provided with dog meal mixed with water for soft diet. It was in order to exclude the variable to strengthen the function of the muscles during the experimental period. Total experiment was performed for 11 weeks (Figure 2). After 6 weeks from the second injection, the animals were sacrificed. Cardiac anesthesia followed by KCl (potassium chloride) intravenous injection was conducted.

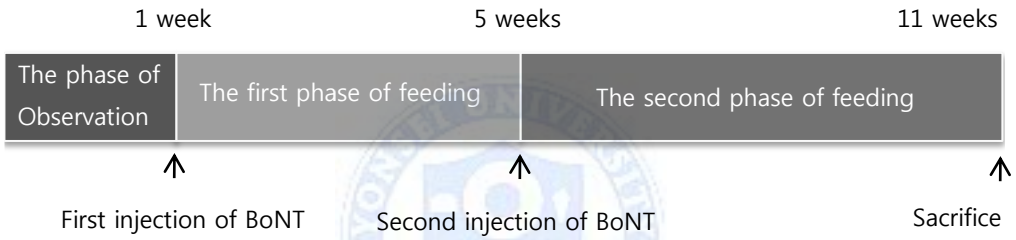


Figure 2. Time table of study protocol

### *3) Collection of tissue samples*

Both condyles of beagles were taken (Figure 3B). Specimens of masseter muscle were taken in the form of a 10 mm X 10 mm square column relative to most prominent point, based on the mandible lower edge (Figure 3C). The total of eight condyles and eight masseter muscle tissue specimens were collected.

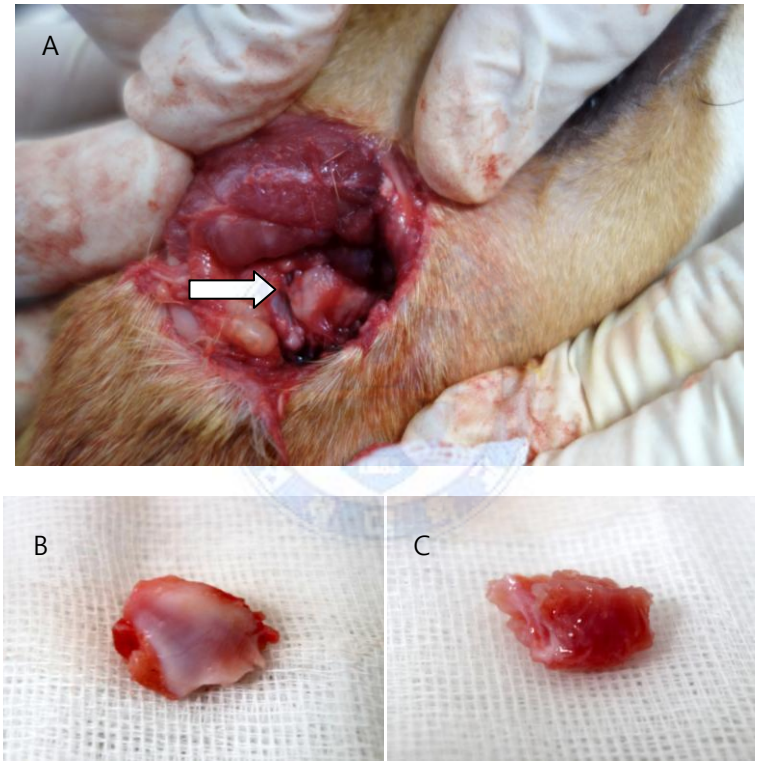


Figure 3. Collection of sample specimen

A. Condyle of beagle (the white arrow indicates condyle)

B. Condyle specimen

C. Masseter muscle specimen

***4) Histological procedure & analysis***

The block specimens were rinsed in sterile saline. Each acquired samples were fixed by perfusion with a 10% formalin solution. The specimens of condylar cartilage were decalcified with 5% Nitric acid. After then, all the specimens were dehydrated in an ascending graded ethanol series (70%, 80%, 90%, 95%, and 100%) and cleared in xylene. These were then embedded in paraffin. Specimens of condylar cartilage were cut in serial sagittal sections and specimens of masseter muscle were cut in cross-sections. All sections were stained with hematoxylin and eosin (H-E) as a conventional method. The stained sections were evaluated with aid of a light microscope.

The thickness of four zones such as F, R, P, H zones was measured at ten regions of condylar cartilage surface on each side (right and left side). Twenty measurements were obtained per a dog. All measurement sites were on point 2 mm away from the top of the condylar cartilage. And twenty five samples were extracted from the muscle fibers of each side randomly, and then the cross section area of the muscle fibers was measured. 'Image Pro' software (Media Cyberbetis Inc. USA) was utilized for the measurement of length and area.

***5) Statistical analysis***

Comparative analysis of acquired data was performed with SPSS version 20 statistical software (SPSS Inc., Chicago, IL, USA). Data were evaluated by paired t-test, Wilcoxon signed rank test and one-way ANOVA for significance of differences. Statistical significance level for all was adopted in the $p<0.05$ level.

# III. Results

The differences in the tissue among the three groups were compared with each other.

## 1. Condyle

The cell layers on condylar cartilage surface can be divided into the four following zones (Shen and Darendeliler., 2005). ; fibrous covering, reserve, proliferation and hypertrophic zones (Figure 4). Calcification zone and ossification zone are below the four zones (Figure 4).

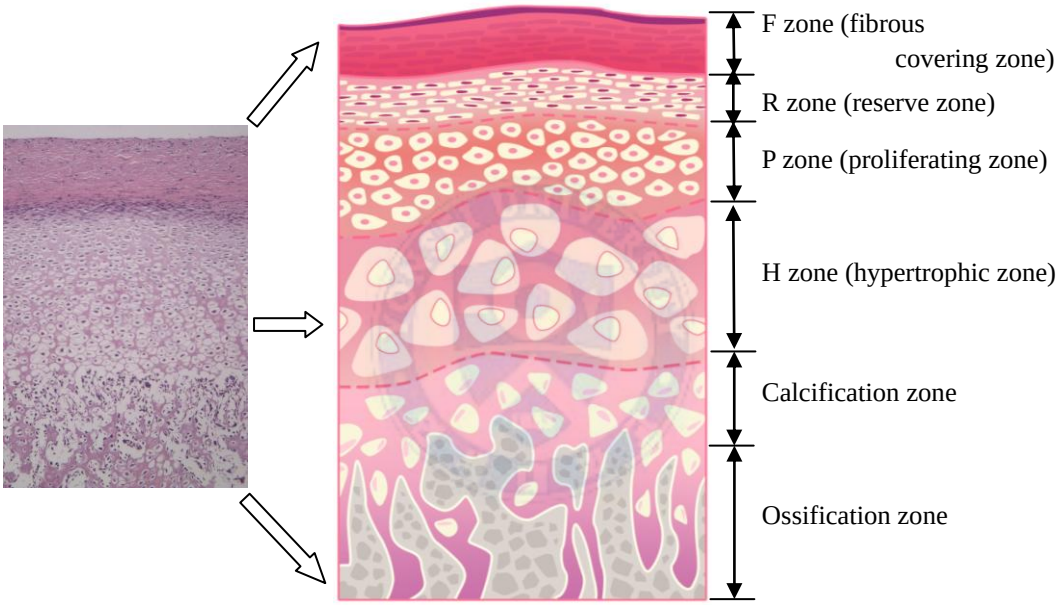


Figure 4. The schematic image of condylar cartilage by layers

F zone : The most superficial zone covering the articular surface is the articular fibrous layer, in which there are densely packed collagen fibers with fibroblasts.

R zone : Beneath the F zone is the condylar cartilage. The superficial layer of the cartilage is a zone of reserve cartilage cells. The cartilage cells in this zone are small, and the amount of chondroid matrix is less, relative to the deeper layer.

P zone : This deeper layer consists of mature cartilage with abundant intercellular cartilaginous matrix. The individual cartilage cells are relatively larger than in the overlying layer.

H zone : The chondrocytes in this zone become highly mature. It should be noted that hypertrophic chondrocytes do not lose proliferative activity, at least during the embryonic period.

Each tissue section was observed for these four zones mainly of condylar cartilage.

The sagittal sections of condylar cartilage of subjects are shown in Figure 5.

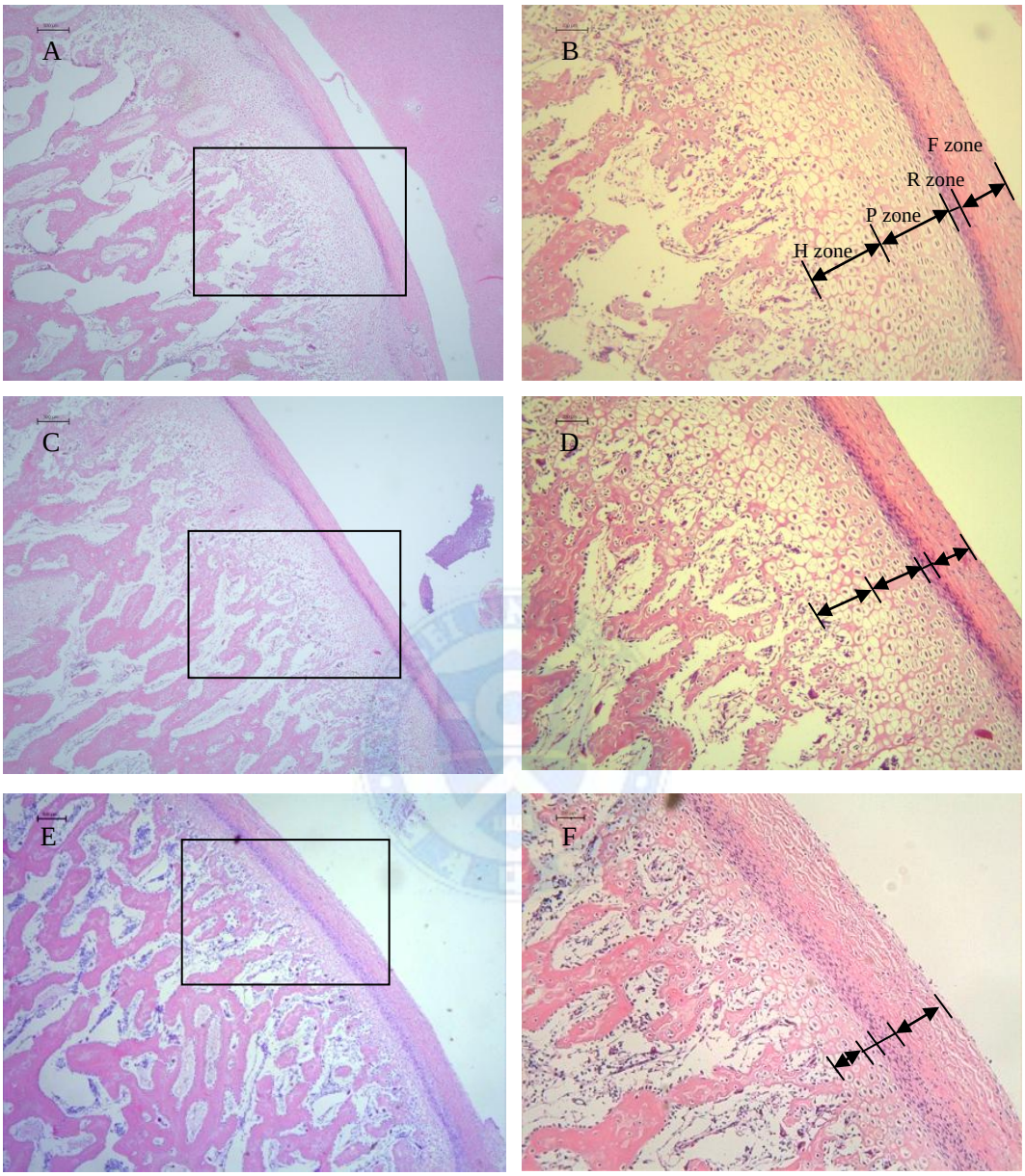


Figure 5. Histological comparison image of condylar cartilage of group Control (A, B), group 10 U (C, D), and group 20 U (E, F).

A, B Group Control shows normal condylar surface with multiple layers in beagle in growth period.
: fibrous covering zone, reserve zone, proliferation zone, hypertrophic zone, calcification zone, and ossification zone

C, D Group 10U shows no significant difference compared to group Control.

E, F Group 20U shows markedly reduced proliferation and hypertrophic zone compared to group Control and group 10U.

( magnification. A, C, E : x40 ; B, D, F : x100 / scale bar. A, C, E : 500μm ; B, D, F : 200μm )

Histological differences among three groups may describe as follows (Table 1, Figure 6).

Table 1. Mean thickness of four zones of the condylar cartilage for each groups (unit : μm)

| | Group | Right | Left | Mean |
|---|---|---|---|---|
| **Fibrous covering zone** | Control | 168.1±21.4 | 170.6±23.2 | 169.6±21.6 |
| | 10U | 175.2±10.6 | 177.7±12.2 | 176.4± 11.6 |
| | 20U | 173.6±19.9 | 176.2±19.8 | 173.6± 21.7 |
| **Reserve zone** | Control | 81.6±16.0 | 83.6±12.8 | 82.8± 14.0 |
| | 10U | 83.2±8.4 | 82.4±6.4 | 82.9± 7.2 |
| | 20U | 88.0±9.6 | 89.2±9.2 | 88.8± 8.4 |
| **Proliferation zone** | Control | 305.2±26.4 | 312.4±23.2 | 308.8±24.4 |
| | 10U | 241.2±21.2 | 246.0±23.2 | 243.6± 21.6 |
| | 20U | 88.8±21.6 | 87.6±22.0 | 87.2± 20.0 |
| **Hypertrophic zone** | Control | 346.4±36.8 | 354.0±35.6 | 350.4± 36.0 |
| | 10U | 379.6±35.6 | 390.4±36.4 | 385.6± 36.1 |
| | 20U | 88.4±11.6 | 87.6±7.6 | 88.0± 9.6 |

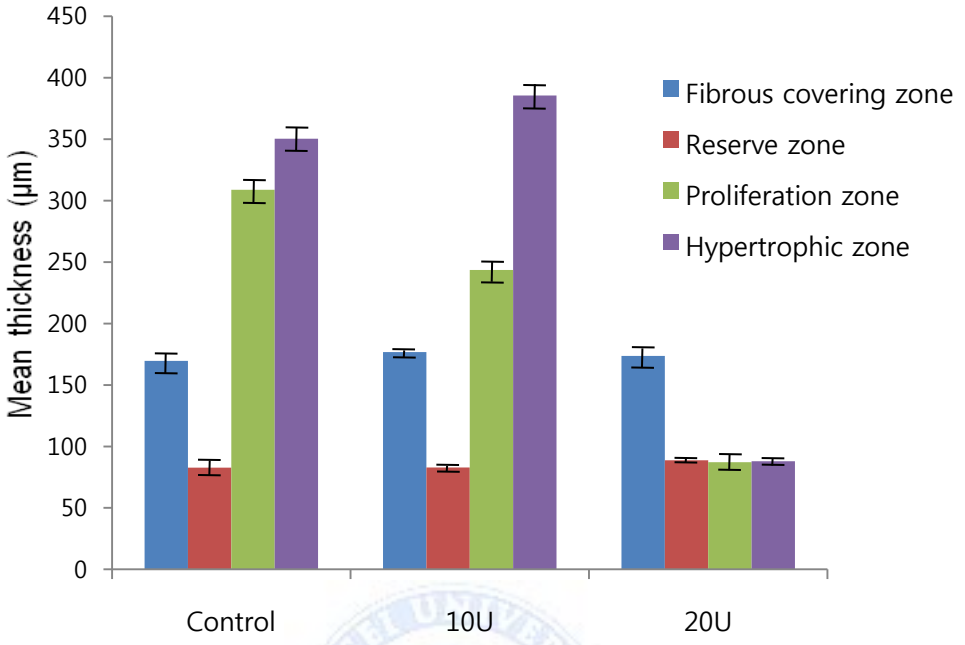


Figure 6. Comparison of thickness in four zones among three groups

In F zone and R zone of the condylar cartilage showed no significant difference among the three groups. However, in P zone and H zone the differences were able to observe between the groups. In P zones differences were not observed compared to the group 10U and Control. But differences of the thickness in P zones were statistically significant between the two groups (group 10U & Control) and group 20U ($p<0.05$). Table 2 and figure 6 show the differences. In group 20U thinner P and H zones were observed, and especially in H zone the difference was clear. And H zones were significantly different among three groups. There were no significant differences of the thickness in four zones between right and left in all groups. There were no differences in calcification zone and ossification zone among three groups. Bone trabecular patterns were irregular and severe bony defects were not detected in all groups. Areas occupied by ossifying bone per unit area were not statistically different among three groups.

## 2. Masseter muscle

Masseter muscle samples of all groups were cut in cross-sections. Histological analysis of the masseter muscle in three groups is shown Figure 7.

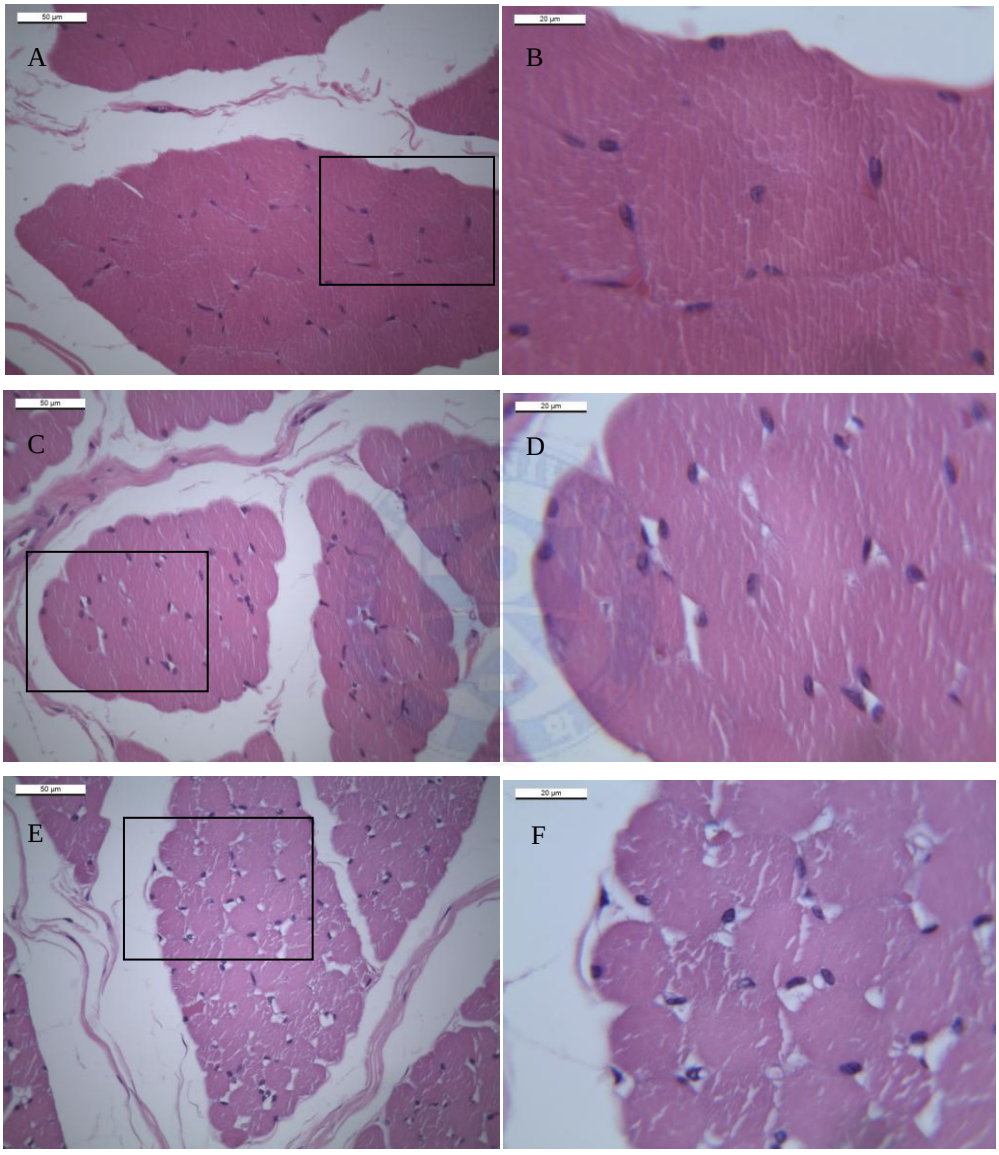


Figure 7. Histological analysis of the masseter muscles in group Control (A, B), group 10U (C, D), and group 20U (E, F).

A, B Muscle fibers of group Control have polygonal form.

C, D Group 10 U shows reduced size of muscle fiber compared to group Control.

E, F Group 20U shows reduced size of muscle fiber compared to group Control and group 10U.

( magnification. A, C, E : x400 ; B, D, F : x1000 / scale bar. A, C, E : 50㎛ ; B, D, F : 20㎛)

In the group Control, endomysium is unclear, and the adjacent muscle fibers are connected tightly. However, in the group 10U and 20U, endomysium was seen clearly with the separated cell connections. In group Control, muscle fibers had normal polygonal shape without necrosis or inflammation. The shape of muscle fibers of groups 10U and 20U was more round and diameter of the fiber decreased. Histological differences on area of cross sectioned muscle fiber among three groups may describe as follows (Table 2, Figure 8).

Table 2. Mean area of cross sectioned muscle fiber in groups (unit : $\mu m^2$)

| Group | Right | Left | Mean |
|---|---|---|---|
| **Control** | 1176.6±37.9 | 1180.9±44.4 | 1178.8±41.0 |
| **10U** | 839.8±49.5 | 842.3±48.5 | 841.1±48.5 |
| **20U** | 647.4±25.1 | 650.2±26.9 | 648.8±25.8 |

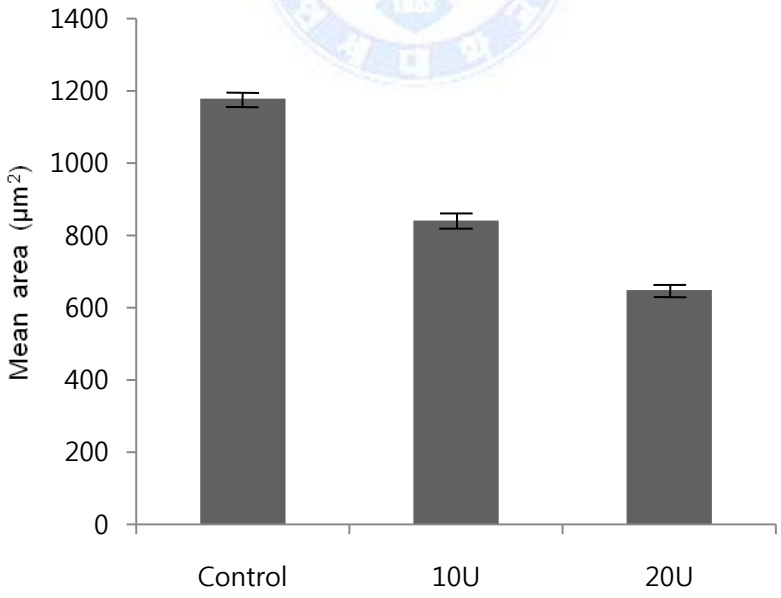


Figure 8. Comparison of muscle fiber cross sectioned area in groups

Area occupied by each muscle fiber per unit area of group 10U and group 20U were smaller than that of group Control. Area measurement using 'Image Pro' program shows area of each muscle fiber (Table 2 and Figure 8). There were no significant differences of the muscle fiber areas between right and left in all groups. The area of cross sectioned muscle fiber among three groups showed a significant difference statistically ($p<0.05$) each other. Each muscle fiber per unit area of group 20U was the smallest.

# IV. Discussion

The functional matrix theory (Moss and Rankow., 1968) states that craniofacial growth and development are by the surrounding muscle. Many studies have investigated the influence of muscle on craniofacial growth and development by changing function of masseter muscle to prove the functional matrix theory.

Other studies attempted to control skeletal growth with the aim of restoring normal function and facial appearance by changing masticatory muscle function. They used invasive method to reduce function and force of masseter muscle. For example, there are myectomy, denervation procedure of the masseter muscle (Horowitz et al., 1955; Moore et al., 1973; Sato et al., 1986). But invasive method such as myectomy and surgical denervation may leave scar on tissue or damage other structures that can change growth patterns (Gardner et al., 1980). And other studies researched local mechanical regulations affecting the condyle including intermaxillary fixation, removal of incisors, alteration of dietary consistency, muscle resection, and application of orthodontic appliances (Bouvier and Hylander., 1984).

Recent studies have used non-invasive methods to control the masticatory muscle. The most active research is the study by using the botulinum toxin (BoNT). BoNT is the paralytic neurotoxin from produced clostridim botulinum. It attaches at the cholinergic motor end plate, and cause paralysis of motor neuron (Borodic et al., 1994; Kim et al., 2003; Kwon et al., 2007). It is life-threatening toxin known to bind to receptors at the neuromuscular junctions of target nerves where they cleave soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which are responsible for the process of vesicle-plasma membrane fusion (Aoki et al., 2002; Rosales et al., 2006).

Since a study verified that BoNT temporarily inhibits acetylcholine release at the neuromuscular junction and reduces muscular contractions, BoNT has been used as an effective method for reducing muscle activity and function. Because BoNT injection is a simple procedure, there is little room for experiment mistake. However, the accurate position during the injection of BoNT is important.

There are many studies that examine the effect of BoNT injection for mandible growth control in rats. BoNT influences on inhibitory action of the developing mandible due to apoptosis at the proliferation stage of the reserve zone of the condylar cartilage in developing rat mandible (Kim et al., 2010). When the rats were injected with BoNT on masseter muscle during growth period, the rats after growing showed a decrease in mandibular height, but the mandibular length and intergonial distance were not affected (Huang JJ et al., 2010). Other study showed a facial morphology typical of a dolichofacial profile: short upper face accompanied by a long lower face with an extended mandibular length and ramus height and constricted bicoronoidal and bigonial widths which suggest that induction of localized masticatory muscle atrophy with BoNT alters craniofacial growth and development (Tsai CY. et al., 2010).

In another study, BoNT injection into the temporalis and masseter muscles of growing rats induced reduction cortical bone thickness and BMD (Bone marrow density) of the skull and mandibular bone structure. The volumes of the temporalis and masseter muscles injected with BoNT were smaller (Tsai CY. et al., 2011). Apart from this, there were several studies on whether BoNT injection on masseter muscle induces mandibular asymmetry. Out of these studies, in most studies that were conducted on rat, morphological analysis indicates that facial asymmetry can be induced due to paralysis of masseter muscle. The muscles injected with BoNT were smaller than the sham or control muscles and anthropometric measurements

of the bony structures attached to the masseter muscle showed a significant treatment effect (Tsai CY et al., 2009). Unilaterally localized BoNT injection induced a change in craniofacial growth, and the skeletal effect was unilateral despite both sides of the mandible functioning as one unit (Park et al., 2015).

In comparison to such results, in studies carried out on medium sized mammals such as rabbit, showed that although there could be subtle difference between structures (mandibular ramus length, zygomatic length, masseteric length, etc.) related to masseter muscle on two sides, it does not indicate that unilateral injection of BoNT caused significant facial asymmetry (Kwon et al., 2007). This suggests that, for more evolved mid-sized mammals with more complex mandible, activity of one side of normally functioning masseter muscle can influence the activity of masseter muscle on the other side.

Furthermore, due variation for time and period for growth depending on type of animal, there can be difference in period for effect of BoNT to be maintained. Therefore, it is possible for results of experiments done on one side to be different to the other.

Considering above previous results, in this study design, BoNT was injected to masseter muscles on both sides of beagle dog, a higher animal than rabbit, to eliminate any factors that could affect the results. We designed to evaluate the influence of BoNT on mandibular growth by comparing histological difference between BoNT injection (10U per one side, 20U per one side) groups and saline injection group. Previous studies with rats proposed the dose of BoNT injection. Those were about 0.02~0.05 U per gram for rat (Babuccu et al., 2009; Brodic et al., 1994; Kim et.al., 2003).

We determined the dose for our experiment by reference to these studies. But because each animal might have different doses, we established two experimental groups (10U per one side, 20U per one side). Beagles' weights were from 10 to 11Kg.

Histological examination of condylar cartilage and masseter muscle of beagle was carried out after 11 weeks of growth period and injection of BoNT twice. Masseter muscle specimens of all groups were cut in cross-sections. And the section of condyle specimen was sagittal section.

Histological comparison of the condylar cartilage in the control, 10U injection, and 20U injection groups are shown in Figure 5. Points more than 2.0 mm away from the top of condylar cartilage were the distinction of the four zones which showed an irregular pattern. Around the top portion of the condyle, the differentiation of cells occurred actively and significant statistically differences were observed among groups. In P zones differences were not observed compared to the group 10U and Control. But differences of the thickness in P zones were statistically significant between the two groups (group 10U & Control) and group 20U ($p<0.05$). And H zones were significantly different among three groups. Especially in group 20U, where 20U was injected on each side, thinner proliferation zone and hypertrophic zone were shown. The thickness of the two layers of group 20U showed a significant difference statistically ($p<0.05$) compared to that of group 10U and Control. The average thickness of H zone in group 20U was less than one-third of that of group Control. But in F zone and R zone of the condylar cartilage showed no significant difference among the three groups. This result can be explained in two ways. This is either due to decrease in cell activity in proliferation zone or decrease in number of cells which induce proliferation. Study with rats showed that cell apoptosis in the proliferation zone of the BoNT group was increased compared with that of the control group. The cell death was detected in the proliferation zone adjacent to the reserve zone by Tunnel staining process. Cell death at the proliferation zone adjacent to the reserve zone is relative to the growth of the mandible and condyle in developing rat mandible (Kim et al., 2010).

The reserve zone has a growth potential (Kierszenbaum., 2002). It has also been known to play an important role in the proliferation of the cells. On our study we could not find significant difference of the reserve zone among groups.

And there were no differences in calcification zone and ossification zone among three zones. Bony trabecular patterns were same and severe bony defects were not detected in all groups. Areas occupied by ossifying bone per unit area were not statistically different among three groups.

Histological comparison of the masseter muscle in the control, 10U injection, and 20U injection groups are shown in Figure 7. The groups 10U and 20U showed the separated cell connections and cell degenerations with decrease in size of muscle fiber. The muscle fibers of the BoNT groups were more round and diameter of the fiber decreased. The area of cross sectioned muscle fiber among three groups showed a significant difference statistically ($p$<0.05) each other. Each muscle fiber per unit area of group 20U was the smallest and that of group Control was the largest. BoNT injection inhibits acetylcholine release at the neuromuscular junction and reduces muscular contractions. The formation of condensed muscle fibers in the BoNT injection group might be an expression of its degenerative atrophy (Akagawa et al., 1983). The histological assessment of muscle fiber implicate for reflection of muscle denervation after BoNT injection (Vilmann et al., 1990; Borodic and Pearce, 1994). The muscles injected with BoNT were smaller than control (Tsai CY et al., 2008). It has been studied that after injection of BoNT, the size of muscle fiber became smaller than the control group (Korfage et al., 2012). There was a study on the distribution of changes in fiber type in the muscle injected with BoNT. The investigation carried out on pigs showed that paralysed masseters displayed atrophic changes and the typical distributions of type IIa and IIb fiber

types in masticatory muscles were increased in the masseter muscles due to BoNT application (Gedrange., 2013).

If the proliferation and differentiation of the cells occurs in condyle, these results lead to the formation of bone in accordance with endochondral bone formation. The gene expression patterns of chondrocytes during condylar growth have been categorized into two phases: maturation and mineralization (Inoue et al., 1995). The chondrocyte maturation is initiated with mesenchyme differentiation into pre-chondroblasts and terminated with highly matured hypertrophic chondrocytes. This process is well-manifested by cellular and phenotypic responses of chondrocytes, resulting in a unique zone-like packing of condylar cartilage (Shen., 2000). As a result, cell proliferation can be explained that led to bone formation and balance.

Changes in the production of bone in growth may be a factor that influences the amount of growth can be confirmed in this previous study (Tsai CY et al., 2010; Park et al., 2015; Kim et al., 2010; Huang JJ et al., 2010). Thus the change in cell differentiation of the condylar cartilage due to the injection of BoNT can be a factor that can affect mandibular growth. As a result, the reduction in cell differentiation and proliferation caused by decrease of muscle function could make to reduce the bone formation of the condyle. According to these results, it is thought that it could reduce the growth of the mandible.

In the past BoNT had been used for treatment purposes only confined to the muscles. But currently, various studies suggested possibility of influence of the bone growth by reducing the function of the muscles and the usage range of BoNT as a therapeutic agent is widened. Whilst most studies were limited to rat study, this experiment applies far higher animals such as dog to support the results of previous research. Based on the results of this experiment, further research should be carried out in the future to analyze morphologically how

histological changes on condylar surfaces have influence on mandibular growth in long-term. Moreover, further studies should be applied on monkeys, resembling human rather than dogs.

Other treatments to control mandibular growth, especially which inhibit the overgrowth of mandible still have many issues in orthodontics. But we believe that BoNT could be applied to treatment for growth modification. Our experiment coupled with other studies so far can help to develop better treatment.

# V. Conclusion

1. In condylar cartilage the differences of the thickness in P zones were statistically significant between the two groups (group 10U & Control) and group 20U ($p<0.05$). And H zones were significantly different among three groups ($p<0.05$). Especially in group 20U thinner P zone and H zone were shown.
2. The shape of masseter muscle fibers in group 10U and group 20U was different from that of group Control. And endomysium was seen clearly with the separated cell connections in group 10U and 20U.
3. The area of cross sectioned muscle fiber among three groups showed a significant difference statistically ($p<0.05$) each other. Each muscle fiber per unit area of group 20U was the smallest.

As a result of this, it was confirmed that BoNT injected into the masseter muscle could affect cell differentiation of condylar cartilage, which could ensure that consistent with the results of the previous studies made in lower animals, such as rat.

If the further study that BoNT could affect the morphological changes in the mandible is carried out, this study could be a basis for the growth modification treatment of human mandible by using BoNT.

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## 국문요약

# 어린 비글견의 교근에 주입된 보툴리눔 독소가 하악과두 및 교근에 미치는 효과


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하악의 기능을 통제함으로써 하악 성장을 조절하려는 많은 시도들이 있었다. 보툴리눔 독소의 주입은 근육의 기능을 저하시킴으로써 하악의 기능을 통제할 수 있는 방법으로 사용될 수 있음이 이전의 연구들을 통하여 밝혀졌다. 본 연구는 근육의 기능이 성장 중에 있는 두개안면 및 하악의 성장에 영향을 줄 수 있음을 밝힌 선학들의 연구에 기초하고 있다. 본 연구는 성장기 비글견의 교근에 보툴리눔 독소를 주입하여 하악과두 및 교근의 조직학적 변화를 살펴봄으로써 보툴리눔의 작용에 따른 교근의 기능저하가 하악 과두 부위의 세포의 분화와 교근의 근섬유에 어떠한 영향을 주는지를 알아보고자 하였다.

총 4 마리의 비글견을 연구의 대상으로 하였으며, 실험군(3 마리), 대조군(1 마리)으로 나누어 실험을 진행하였다. 보툴리눔 독소는 Allergan 사의 Botox ® 를 사용하였으며, 실험군 중 2 마리는 편측당 10U(group 10U), 1 마리는 20U(group 20U)의 보툴리눔 독소를, 대조군은 saline 을 4 주 간격으로 양측 교근에 총 2 회 주입하였다. 총 11 주간의 사육기간 후 희생하여, 개체마다 양측 하악과두 및 교근의 조직시편을 채취하고 HE 염색을 통한 조직 표본을 분석하였다. 교근 및 하악과두의 연골 부위에서의 조직학적 비교분석 결과, 다음과 같은 결과를 얻었다.

1. 하악과두 연골에서, group 20U의 P zone의 두께는 group 10U와 group Control의 P zone 두께에 비해 얇았으며, 이러한 두께의 차이는 통계학적으로 유의한 차이($p$<0.05)를 보였다. 또한 H zone의 경우에는 모든 group간에 유의한 차이($p$<0.05)를 보였다. 특히 group 20U에서 더욱 얇은 P zone과 H zone을 관찰할 수 있었다.

2. group 10U와 group 20U의 교근 근섬유의 횡단면은 group Control의 근섬유의 횡단면과는 다른 형태를 가지고 있었으며, 근섬유 세포를 분리하는 분명한 근내막을 관찰할 수 있었다.

3. 세 군간에 통계학적으로 유의한 근섬유의 횡단면 면적 크기의 차이($p$<0.05)를 보였으며, 근섬유의 횡단면 면적은 group 20U에서 가장 작았다.

본 연구 결과, 교근으로의 보툴리눔 독소 주입이 하악과두 연골의 세포분화에 영향을 준다는 확인할 수 있었으며, 이는 백서 등의 하등동물에서 이루어진 이전의 연구들의 결과에 부합되는 것이었다.

연구 결과를 토대로, 향후 보툴리눔 독소가 하악 구조의 형태학적인 변화에 영향을 끼칠 수 있음을 더욱 많은 개체 수에서 증명한다면, 보툴리눔 독소를 이용한 사람의 하악골 성장조절 치료를 위한 중요한 근거가 될 수 있을 것으로 생각된다.

핵심되는 말 : 보툴리눔 독소, 하악과두 연골, 교근, 하악 성장, 비글견