Effects of Clenbuterol, a β_2-Adrenergic Agonist, on Sizes of Masseter, Temporalis, Digastric, and Tongue muscles

Chieko Ishikawa[1], Takumi Ogawa[2], Tomoko Ikawa[2] and Akira Yamane[3]*

Departments of [1]Removable Prosthodontics, [2]Fixed Prosthetic Dentistry and [3]Biophyscis, Tsurumi University School of Dental Medicine, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230-8501, Japan

Abstract: We compared the hypertrophic effects of clenbuterol, a β_2-adrenergic agonist, on the masseter, digastric, and temporalis with those on the tongue, tibialis anterior, soleus, diaphragm, and heart. The weights of masseter, digastric and temporalis in the clenbuterol group were 36 ~ 56% greater than those in the control group, whereas those of the tibialis anterior, diaphragm, and heart weights in the clenbuterol group were 9 ~ 33% greater than those in the control group. No significant difference in the weights of the soleus and tongue was found between the control and clenbuterol groups. Taken together with our present and previously reported results, it is suggested that the hypertrophic effects of clenbuterol on the masseter, digastric, and temporalis are greater than those on the limb, trunk, and heart.

Keywords: Clenbuterol, hypertrophic effects, striated muscles, rat.

INTRODUCTION

Clenbuterol [4-amino-α (t-butyl-amino) methyl-3,5-dichlorobenzyl alcohol] is a β_2-adrenergic agonist and non-steroidal anabolic drug for sports doping. According to the recent World Anti-Doping Agency documents, the use of clenbuterol was the fifth most common case in the number of anabolic drugs–used contravention in 2006 (53 cases) [1]. Clenbuterol is known to induce hypertrophy of skeletal muscles such as the soleus, gastrocnemius, and extensor digitorum longus, as well as on the masseter and heart muscles [2-10]. The precise mechanism for the hypertrophy of skeletal and cardiac muscles induced by clenbuterol remains unknown. One leading hypothesis is that clenbuterol induces the hypertrophy of the skeletal muscles through the β_2-adrenergic receptor by up-regulating the expression of insulin-like growth factors (IGFs) [7, 11-13] which play essential roles in the development, growth, and regeneration of skeletal muscles [14-20].

Muscle satellite cells are mononucleated and quiescent stem cells that reside between the sarcolemma and basal lamina of adult myofibers [21,22]. In response to stimuli such as mechanical loading, unloading, denervation, and injury, the satellite cells are activated through several growth factors containing IGFs and this activation is thought to induce adaptive changes of skeletal muscle such as hypertrophy, the alteration of fiber type, and regeneration [16, 21-23]. Recently, we have reported that the pool size of satellite cells in the masseter muscle of the muscle dystrophy model mouse (mdx) is greater than those of other muscles such as the gastrocnemius, soleus, and diaphragm [24] and we hypothesized that the hypertrophic effect of clenbuterol on the craniofacial muscles containing the masseter muscle is greater than on other muscles.

In the present study, to test this hypothesis, we measured the wet weights of masseter, temporalis, digastric and tongue muscles, and compared them with those of tibialis anterior, soleus, diaphragm, and heart muscles after oral administration of clenbuterol for 3 weeks. Furthermore, to exclude the possibility that clenbuterol secondarily leads to the hypertrophy of the masseter, temporalis, and digastric muscles by directly inducing the hypertrophy of the mandible and maxilla, we measured the distance between the origin and insertion of the muscles by three-dimensionally reconstructing the images of micro-computed tomography (μCT).

MATERIALS AND METHODS

Experimental Animals, Administration of Clenbuterol, and Weighing Muscles

Ten male Wistar rats were purchased from Clea Japan, Inc., (Tokyo, Japan) and fed a hard diet (CE-2; Clea Japan, Inc., Tokyo, Japan). They were divided into control and clenbuterol groups of five rats each at 8 weeks of age. We orally administered 30 μg/ml of clenbuterol (C5423; Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA) to the rats in the clenbuterol group via their drinking water for 3 weeks, while pure water was given to the rats in the control group. We daily measured the weight of each rat and consumption of pure water or water containing clenbuterol for each rat to estimate the daily dose of clenbuterol. The dose of clenbuterol was approximately 4 mg/kg of body weight/day. After 3 weeks, all the animals were killed by exsanguinations under ether anesthesia. The masseter, temporalis, digastric (anterior belly), tongue, tibialis anterior, soleus, diaphragm, and heart were immediately dissected and weighed. After removing the muscles, the whole heads were frozen and

*Address correspondence to this author at the Department of Biophysics, Tsurumi University School of Dental Medicine, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230-8501, Japan; Fax: +81-45-583-7828;
E-mail: yamane-a@tsurumi-u.ac.jp

1874-2106/09

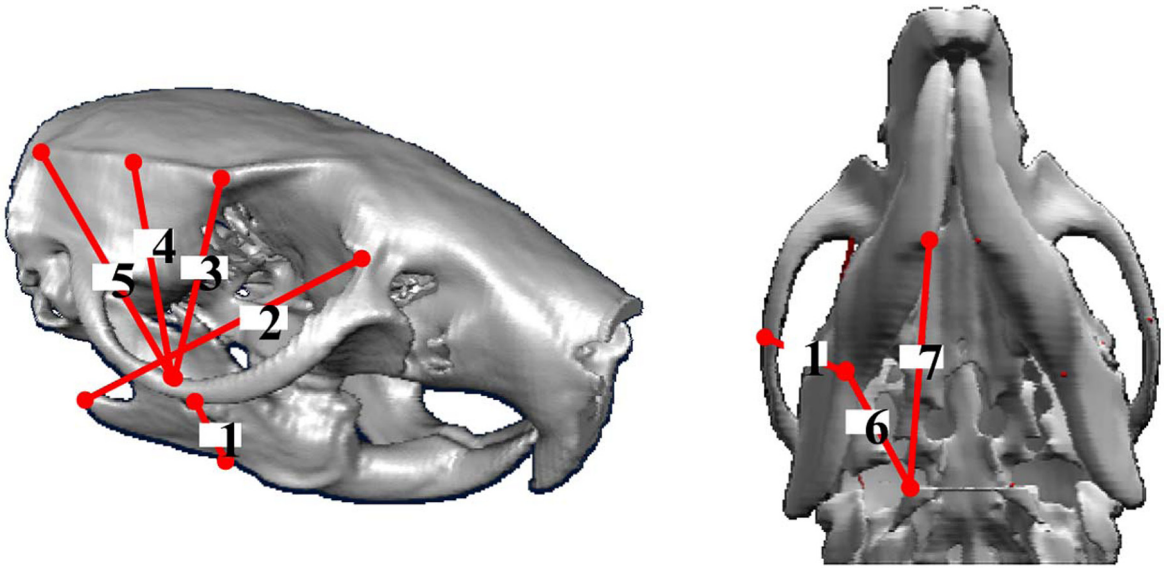

Fig. (1). Three-dimensionally reconstructed images of mandible and maxilla by μCT showing the measured points for the origins and insertions of masseter (1, 2), temporalis (3, 4, 5), and digastric (6, 7) muscles.

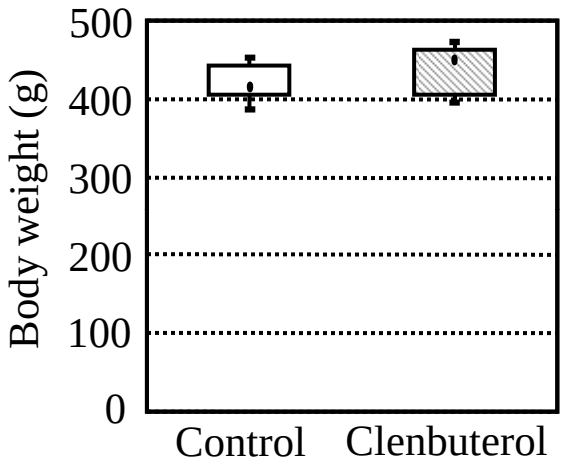

Fig. (2). Box and whisker plot of body weights in the control and clenbuterol groups after oral administration of clenbuterol for 3 weeks. There were five rats in each group. No significant difference in the body weight was found between the control and clenbuterol groups.

stored in -30°C until subsequent μCT analysis. Experimental protocols concerning animal handling were reviewed and approved by the Institutional Animal Care Committee of Tsurumi University School of Dental Medicine.

Morphometric Analysis of Rat Head by Micro-Computed Tomography (μCT)

The distance between the origin and insertion of masseter, digastric and temporalis muscles are analyzed by three-dimensionally reconstructing the images of μCT. The whole head of the rat was transversely scanned at 750 μm intervals with a μCT system (LCT-100, ALOKA CO., LTD., Tokyo, Japan). The images at a resolution of 480 X 480 pixels were reconstructed in three directions using image analysis software (Amira 3.1, Mercury Computer Systems, San Diego, CA) and the distances between the origin and insertion of masseter, digastric and temporalis muscles on the mandible and maxilla were measured on the three-dimensional images. Fig. (**1**) shows the measured points for the origins and insertions of masseter, temporalis and digastric muscles on the mandible and maxilla.

Statistical Analysis

A Mann-Whitney U-test was used to compare the median values between the clenbuterol and control groups.

RESULTS

Fig. (**2**) shows box and whisker plots of the body weight in the control and clenbuterol groups at 21 days of clenbuterol administration. In the plot, the dots represent the median value, the boxes represent the interquartile range, and the whiskers represent the full range of data. The median values of the body weight in the clenbuterol and control groups were 414 g and 449 g, respectively, with no statistically significant difference between the two groups.

Fig. (**3**) shows box and whisker plots of the masseter (A), digastric (B), temporalis (C), and tongue (D) weights in the control and clenbuterol groups at 21 days of clenbuterol administration. The median values of the masseter, digastric, and temporalis weights in the clenbuterol group were 1.588, 0.147, and 0.762 g, which were 46, 36, and 56% greater than those in the control group (1.091, 0.108, and 0.490 g; $p<0.01$), respectively. The median values of the tongue weight in the clenbuterol and control groups were 0.375 and 0.353 g, respectively, with no statistically significant difference between the two groups.

Fig. (**4**) shows box and whisker plots of the tibialis anterior (A), soleus (B), diaphragm (C), and heart (D) weights in the control and clenbuterol groups at 21 days of clenbuterol administration. The median values of the tibialis anterior, diaphragm, and heart weights in the clenbuterol group were 1.111, 1.099, and 0.923 g, which were 33, 17, and 9% greater than those in the control group (0.837, 0.937, and 0.845 g; $p<0.05 \sim 0.01$), respectively. The median values of the soleus weight in the clenbuterol and control groups were

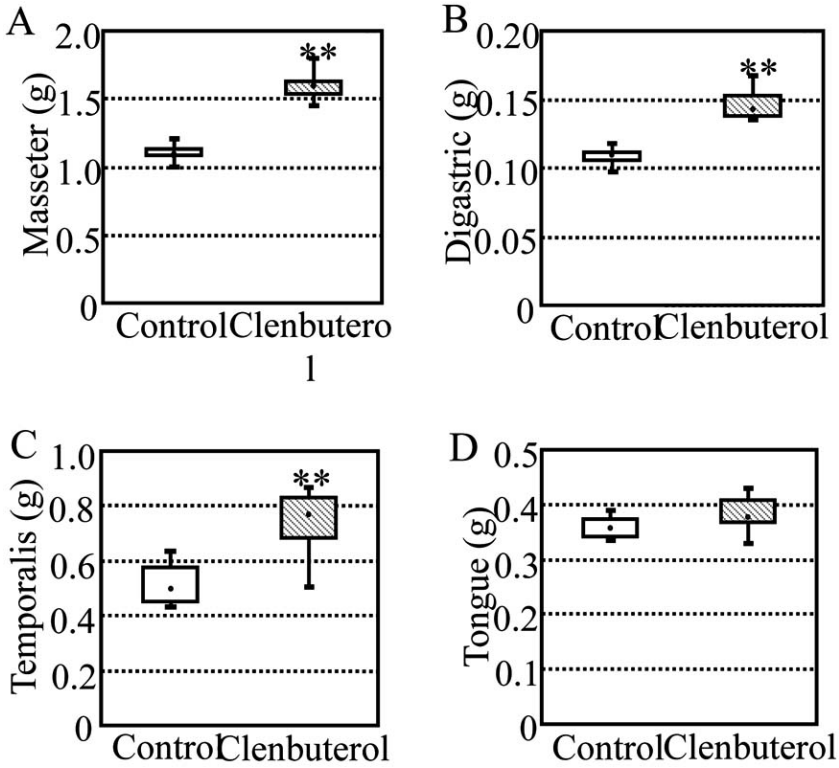

Fig. (3). Box and whisker plots of weights of masseter (**A**), digastric (**B**), temporalis (**C**), and tongue (**D**) in the control and clenbuterol groups after oral administration of clenbuterol for 3 weeks. There were five rats in each group. Significant difference between the control and clenbuterol groups, **p<0.01. The median values of the masseter, digastric, and temporalis weights in the clenbuterol group were 46, 36, and 56% greater than those in the control group, respectively, but no significant difference in the tongue weight was found between the control and clenbuterol groups.

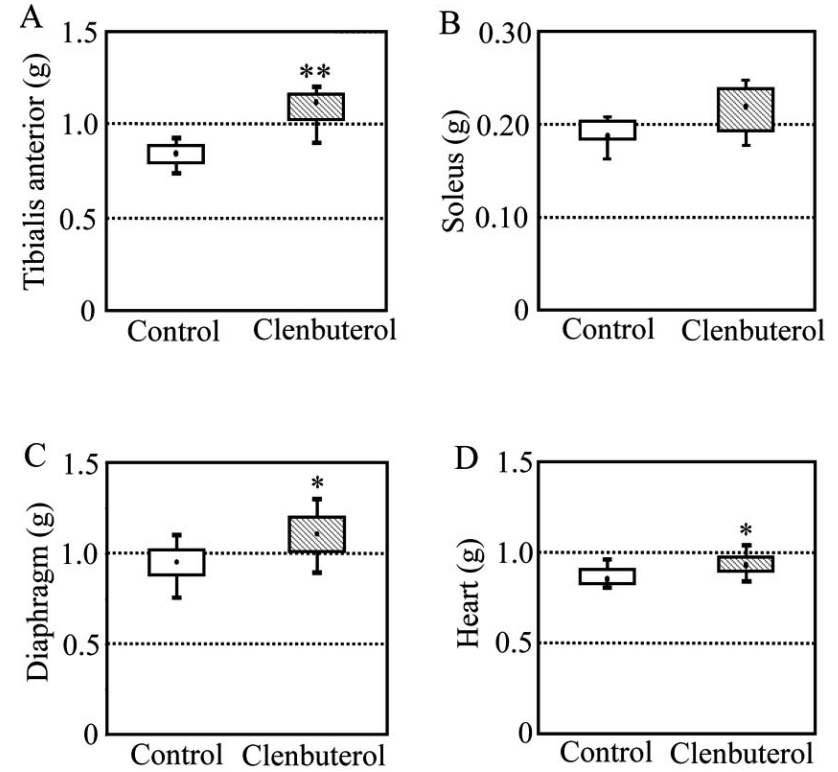

Fig. (4). Box and whisker plots of weights of tibialis anterior (**A**), soleus (**B**), diaphragm (**C**), and heart (**D**) in the control and clenbuterol groups after oral administration of clenbuterol for 3 weeks. There were five rats in each group. Significant differences between the control and clenbuterol groups: *p<0.05, **p<0.01. The median values of the tibialis anterior, diaphragm, and heart weights in the clenbuterol group were 33, 17, and 9% greater than those in the control group, respectively, but no significant difference in the soleus weight was found between the control and clenbuterol groups.

Table 1. The Distances (mm) Between the Origin and Insertion of Masseter, Digastric, and Temporalis Muscles Analyzed by μCT

	Control	Clenbuterol	Significance
Masseter			
1	9.87 ± 0.67	9.47 ± 0.74	NS
2	19.90 ± 1.22	19.35 ± 0.39	NS
Temporalis			
3	10.10 ± 0.63	10.49 ± 0.50	NS
4	11.12 ± 0.66	12.07 ± 0.70	NS
5	14.20 ± 1.09	15.44 ± 0.82	NS
Digastric			
6	13.52 ± 1.22	13.18 ± 0.48	NS
7	6.62 ± 1.05	7.05 ± 0.60	NS

The distance was expressed as the mean ± standard deviation for five rats.

Table 2. Reported Hypertrophic Rate of Murine Limb, Trunk, and Heart Muscles Induced by Clenbuterol

Dose (mg/kg/day)	Duration (days)	Method	Hypertrophic rate (%)						Heart	No. of Ref.
			Sol	Gas	PI	EDL	TA	DP		
0.01	14	M.P.	12	--	18	--	11	--	12	[25]
0.25	7	S.C.	18	22	22	--	--	--	0	[26]
0.25	56	S.C.	0	--	--	20	--	--	--	[6]
1.0	14	S.C.	14	17	17	19	15	--	10	[27]
1.0	21	S.C.	--	5	--	--	--	--	--	[28]
1.0	42	S.C.	17	--	--	--	--	--	--	[29]
2.0	16	S.C.	--	13	--	--	--	--	0	[2]
2.0	14	S.C.	6	18	15	--	--	--	--	[30]
2.0	14	S.C.	--	19	--	--	--	--	--	[3]
2.0	105	Oral	17	--	--	0	--	--	--	[31]
3.0	9	M.P.	--	12	--	--	11	--	--	[14]
#	14	Oral	8	24	--	19	--	--	--	[4]
#	28	Oral	27	--	--	--	--	--	--	[5]
4.0	21	Oral	0	--	--	--	33	17	9	*

The percentage of the wet weight of the soleus (Sol), plantaris (PI), extensor digitorum longus (EDL), tibialis anterior (TA), diaphragm (DP), and heart are expressed relative to the control. Dashes (--) indicate that the muscle mass was not measured. S.C., M.P., and Oral denote subcutaneous injection, continuous injection by subcutaneously implanted osmotic mini-pump, and oral administration, respectively. *Data from the present study is included for comparison. #In these two studies, 30 μg/ml of clenbuterol was orally administered to animals via their drinking water and the exact dose of clenbuterol was not determined.

0.186 and 0.218 g, respectively, with no statistically significant difference between the two groups.

To exclude the possibility that clenbuterol secondarily leads to the hypertrophy of the masseter, temporalis, and digastric muscles by directly inducing the hypertrophy of the mandible and maxilla, we measured the distance between the origin and insertion of the muscles by μCT (Table **1**) and found no statistically significant differences in these distances. This result indicates that the hypertrophic effect of clenbuterol on masseter, temporalis, and digastric muscles is not a secondary effect.

DISCUSSION

In the present study, the hypertrophic rate associated with clenbuterol in the craniofacial muscles, including the masseter, temporalis, and digastric muscles, ranged from 36 to

56%, whereas those of the tibialis anterior, diaphragm, and heart ranged from 9 to 33%. Comparative data from previous studies of murine limb, trunk, and heart muscles are presented in tabular form in Table **2** [25]. The maximum value of the hypertrophic rate in the previous studies is 27%, which was induced in the soleus following 27 days of oral administration of 30 μg/ml of clenbuterol; the hypertrophic rates ranges from 0 to 27%. Since the dose, duration, and method of administration of clenbuterol varied among these studies, they are very difficult to compare with the present study. However, the hypertrophic rates shown in Table **2** are less than those of the craniofacial muscles in the present study.

Muscle satellite cells are activated and induce adaptive changes of skeletal muscle such as hypertrophy, the alteration of fiber type, and regeneration by external stimuli such as mechanical loading, unloading, denervation, and injury through growth factors such as IGFs, myostatin and IL-6 [16,21-23,32,33]. We have reported that the pool size of satellite cells in the masseter muscle of a muscle dystrophy model mouse (mdx) is greater than those of other muscles such as the gastrocnemius, soleus, and diaphragm [24]. It also has been also reported that clenbuterol stimulates the activation, proliferation and differentiation of satellite cells [34-36] and, in the mouse disrupting β_2 adrenergic receptor gene, clenbuterol is not able to induce the hypertrophy of skeletal muscles [14]. These reports suggest a direct relationship among the satellite cells and hypertrophy of skeletal muscle induced by clenbuterol. In the present study, the clenbuterol-induced hypertrophic rates of craniofacial muscles were greater than those of the tibialis anterior, soleus, and diaphragm muscles, and greater than those seen in previous studies. This result supports our hypothesis that the hypertrophic effect of clenbuterol on the craniofacial muscles containing the masseter muscle is greater than on other muscles

We found no significant difference induced by clenbuterol in the weight of the tongue between the control and clenbuterol groups. Although the tongue muscles constitute a subset of the craniofacial muscles, the developmental origins of tongue muscles involves the hypaxial somites 2 ~5, and not the somitomeres, which are involved in the developmental origin of the craniofacial muscles [37-39]. Further, the program governing tongue myogenesis is similar to that for limb myogenesis and distinct from that for craniofacial myogenesis [40]. These differences may be responsible for the observed differences in the hypertrophic effect on tongue muscles by clenbuterol relative to the other craniofacial muscles such as the masseter, digastric, and temporalis.

ACKNOWLEDGEMENTS

This study was supported in part by grants-in-aid for funding scientific research (No. 19592266 to C.I. and No. 20592190 to A.Y.), for funding the Bio-ventures and High-Technology Research Center from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan, and by grant-in-aid for funding scientific research form the Society for Tsurumi University School of Dental Medicine.

REFERENCES

[1] Sato S, Nomura S, Kawano F, Tanihata J, Tachiyashiki K, Imaizumi K. Effects of the β_2-agonist clenbuterol on β_1- and β_2-adrenoceptor mRNA expressions of rat skeletal and left ventricle muscles. J Pharmacol Sci 2008; 107: 393-400.

[2] Emery PW, Rothwell NJ, Stock MJ, Winter PD. Chronic effects of β_2-adrenergic agonists on body composition and protein synthesis in the rat. Biosci Reports 1984; 4: 83-91.

[3] Benson DW, Foley-Nelson T, Chance WT, Zhang FS, James JH, Fischer JE. Decreased myofibrillar protein breakdown following treatment with clenbuterol. J Surg Res 1991; 50: 1-5.

[4] Stevens L, Firinga C, Gohlsch B, Bastide B, Mounier Y, Pette D. Effects of unweighting and clenbuterol on myosin light and heavy chains in fast and slow muscles of rat. Am J Physiol Cell Physiol 2000; 279: C1558-63.

[5] Oishi Y, Imoto K, Ogata T, Taniguchi K, Matsumoto H, Roy RR. Clenbuterol induces expression of multiple myosin heavy chain isoforms in rat soleus fibres. Acta Physiol Scand 2002; 176: 311-18.

[6] Rajab P, Fox J, Riaz S, Tomlinson D, Ball D, Greenhaff PL. Skeletal muscle myosin heavy chain isoforms and energy metabolism after clenbuterol treatment in the rat. Am J Physiol Regul Integr Comp Physiol 2000; 279: R1076-R81.

[7] Wakana N, Akutsu S, Yamane A. Effects of clenbuterol, a β_2-adrenergic agonist, on the myofiber diameter, fiber type, and expressions of insulin-like growth factors in the adult mouse masseter muscle. Jpn J Oral Biol 2003; 45: 418-27.

[8] Soppa GK, Smolenski RT, Latif N, *et al.* Effects of chronic administration of clenbuterol on function and metabolism of adult rat cardiac muscle. Am J Physiol Heart Circ Physiol 2005; 288: H1468-76.

[9] Wong K, Boheler KR, Bishop J, Petrou M, Yacoub MH. Clenbuterol induces cardiac hypertrophy with normal functional, morphological and molecular features. Cardiovasc Res 1998; 37: 115-22.

[10] Akutsu S, Shimada A, Yamane A. Transforming growth factor βs are upregulated in the rat masseter muscle hypertrophied by clenbuterol, a β_2 adrenergic agonist. Br J Pharmacol 2006; 147: 412-21.

[11] Awede BL, Thissen JP, Lebacq J. Role of IGF-I and IGFBPs in the changes of mass and phenotype induced in rat soleus muscle by clenbuterol. Am J Physiol Endocrinol Metab 2002; 282: E31-E7.

[12] Sneddon AA, Delday MI, Steven J, Maltin CA. Elevated IGF-II mRNA and phosphorylation of 4E-BP1 and p70S6k in muscle showing clenbuterol-induced anabolism. Am J Physiol Endocrinol Metab 2001; 281: E676-82.

[13] Matsumoto T, Akutsu S, Wakana N, Morito M, Shimada A, Yamane A. The expressions of insulin-like growth factors, their receptors, and binding proteins are related to the mechanism regulating masseter muscle mass in the rat. Arch Oral Biol 2006; 51: 603-11.

[14] Hinkle RT, Hodge KMB, Cody DB, Sheldon RJ, Kobilka BK, Isfort RJ. Skeletal muscle hypertrophy and anti-atrophy effects of clenbuterol are mediated by the β2-adrenergic receptor. Muscle Nerve 2002; 25: 729-34.

[15] Adams GR, Cheng DC, Haddad F, Baldwin KM. Skeletal muscle hypertrophy in response to isometric, lengthening, and shortening training bouts of equivalent duration. J Appl Physiol 2004; 96: 1613-8.

[16] Adams GR. Role of insulin-like growth factor-I in the regulation of skeletal muscle adaptation to increased loading. Exerc Sport Sci Rev 1998; 26: 31-60.

[17] Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: Biological actions. Endocr Rev 1995; 16: 3-34.

[18] Florini JR, Ewton DZ, Coolican SA. Growth hormone and the insulin-like growth factor system in myogenesis. Endocr Rev 1996; 17: 481-517.

[19] Yamane A, Urushiyama T, Diekwisch TGH. Roles of insulin-like growth factors and their binding proteins in the differentiation of mouse tongue myoblasts. Int J Dev Biol 2002; 46: 807-16.

[20] Yamane A, Amano O, Slavkin HC. Insulin-like growth factors, hepatocyte growth factor and transforming growth factor-α in mouse tongue myogenesis. Dev Growth Differ 2003; 45: 1-6.

[21] Charge SB, Rudnicki MA. Cellular and molecular regulation of muscle regeneration. Physiol Rev 2004; 84: 209-38.

[22] Hawke TJ, Garry DJ. Myogenic satellite cells: physiology to molecular biology. J Appl Physiol 2001; 91: 534-51.

[23] Sabourin LA, Rudnicki MA. The molecular regulation of myogenesis. Clin Genet 2000; 57: 16-25.
[24] Yamane A, Akutsu S, Diekwisch TG, Matsuda R. Satellite cells and utrophin are not directly correlated with the degree of skeletal muscle damage in mdx mice. Am J Physiol Cell Physiol 2005; 289: C42-8.
[25] Burniston JG, Clark WA, Tan LB, Goldspink DF. Dose-dependent separation of the hypertrophic and myotoxic effects of the β_2-adrenergic receptor agonist clenbuterol in rat striated muscles. Muscle Nerve 2006; 33: 655-63.
[26] MacLennan PA, Edwards RH. Effects of clenbuterol and propranolol on muscle mass: Evidence that clenbuterol stimulates muscle β-adrenoceptors to induce hypertrophy. Biochem J 1989; 264: 573-9.
[27] von Deutsch DA, Abukhalaf IK, Wineski LE, *et al.* β-agonist-induced alterations in organ weights and protein content: comparison of racemic clenbuterol and its enantiomers. Chirality 2000; 12: 637-48.
[28] Rothwell NJ, Stock MJ. Effect of a selective β_2-adrenergic agonist (clenbuterol) on energy balance and body composition in normal and protein deficient rats. Biosci Rep 1987; 7: 933-40.
[29] Criswell DS, Powers SK, Herb RA. Clenbuterol-induced fiber type transition in the soleus of adult rats. Eur J Appl Physiol Occup Physiol 1996; 74: 391-6.
[30] Dodd SL, Powers SK, Vrabas IS, Criswell D, Stetson S, Hussain R. Effects of clenbuterol on contractile and biochemical properties of skeletal muscle. Med Sci Sports Exerc 1996; 28: 669-76.
[31] Lynch GS, Hayes A, Campbell SP, Williams DA. Effects of β_2-agonist administration and exercise on contractile activation of skeletal muscle fibers. J Appl Physiol 1996; 81: 1610-8.
[32] Gilson H, Schakman O, Kalista S, Lause P, Tsuchida K, Thissen JP. Follistatin induces muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin and activin. Am J Physiol Endocrinol Metab 2009; 297: E157-64.
[33] Serrano AL, Baeza-Raja B, Perdiguero E, Jardi M, Munoz-Canoves P. Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. Cell Metab 2008; 7: 33-44.
[34] McMillan DN, Noble BS, Maltin CA. The effect of the β-adrenergic agonist clenbuterol on growth and protein metabolism in rat muscle cell cultures. J Anim Sci 1992; 70: 3014-23.
[35] Roberts P, McGeachie JK. The effects of clenbuterol on satellite cell activation and the regeneration of skeletal muscle: an autoradiographic and morphometric study of whole muscle transplants in mice. J Anat 1992; 180(Pt 1): 57-65.
[36] Maltin CA, Delday MI. Satellite cells in innervated and denervated muscles treated with clenbuterol. Muscle Nerve 1992; 15: 919-25.
[37] Noden DM. The embryonic origins of avian cephalic and cervical muscles and associated connective tissues. Am J Anat 1983; 168: 257-76.
[38] Christ B, Ordahl CP. Early stages of chick somite development. Anat Embryol 1995; 191: 381-96.
[39] Huang R, Zhi Q, Izpisua-Belmonte JC, Christ B, Patel K. Origin and development of the avian tongue muscles. Anat Embryol (Berl) 1999; 200: 137-52.
[40] Yamane A. Embryonic and postnatal development of masticatory and tongue muscles. Cell Tissue Res 2005; 322: 183-89.

Received: June 15, 2009 Revised: July 02, 2009 Accepted: July 31, 2009