Effects of Dietary Protein and Energy on Growth Performance and Muscle Composition in Broilers Treated with Clenbuterol

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ABSTRACT : The present study was conducted to examine the effects of dietary protein (20, 22, 24%) with a constant protein-to-energy ratio on clenbuterol-induced performance in broilers. The protein-to-energy ratio was based on adequate level (22% protein, 3,100 kcal of energy). Female broiler chickens were used for a 3 × 2 factorial arrangement and fed diets with or without 1 ppm clenbuterol from 14- to 32-days of age. Feed efficiency improved with increasing dietary protein level, regardless of clenbuterol treatment. The dietary clenbuterol increased weights of breast and leg muscles (*gastrocnemius* and *peroneus longus*), and clenbuterol markedly reduced protein content of leg muscles in chickens fed the 20% protein diet, but did not in chickens fed the 22 and 24% protein diets. Feeding the 24% protein diet with clenbuterol improved the protien accretion (*peroneus longus*) by 8.4%. Clenbuterol decreased DNA content and increased the protein/DNA ratio in breast muscle regardless of dietary protein intake. Clenbuterol had no effect on RNA content in both breast and leg muscles. The present results demonstrated that various protein levels which retain the same protein-to-energy ratio in the diet markedly alter the protein accretion induced by β-agonist in broilers.

(**Key Words:** Clenbuterol, Protein and Energy Levels, Growth Performance, Muscle Composition, Protein Deposition)

INTRODUCTION

Dietary supplementation with β-adrenergic agonists (β-agonist) has been shown to increase skeletal muscle mass and protein accumulation and to decrease fat deposition in growing animals (Yang and McElligott, 1989; Wellenreiter, 1991). Moreover, the effect on animal growth is associated with nutritional condition, especially dietary protein level (Reeds and Mersmann, 1991; Dunshea et al., 1993; Mitchell et al., 1991). Feeding a low protein diet resulted in reducing growth performance of β-agonist-treated rats (Perez-Llamas et al., 1991), pigs (Bracher-Jakob and Blum, 1990) and broilers (Hamano et al., 1994). Other workers showed that β-agonist-fed pigs required increased amounts of dietary protein to maintain maximal growth performance (Mitchell et al., 1991; Dunshea et al., 1993; Oksbjerg et al., 1994). A previous study also indicated that increasing dietary protein intake of broilers resulted in an improved weight-gain in response to clenbuterol (Hamano et al., 1998).

Although dietary protein would be a limiting factor for animal growth, energy status also appears to affect the growth performance or metabolic response of β-agonist-fed animals. Kim et al. (1991) reported that feeding a diet containing 19% crude protein (CP) and 3,200 kcal/kg of metabolizable energy (ME) brought about the highest body weight gain and feed efficiency in broilers treated with a β-agonist cimaterol, during the period of 4 to 7 weeks of age. In addition, Reeds and Mersmann (1991) described the maximum response to β-agonist as declining when the protein-to-energy ratio was high. A high protein-to-energy ratio (30% CP) or a high energy level (3,500 kcal ME/kg) resulted in an absence of growth promotion on broiler (Hamano, 1996). Thus, a combined change in protein and energy content rather than alteration in protein level alone may affect the ability of β-agonist to deposit body protein. Likewise, differential involvement of dietary protein levels on muscle growth in β-agonist-fed chickens may be clarified by the dietary condition of a fixed protein-to-energy ratio.

Since the response of female chickens to clenbuterol has been shown to be more sensitive than males (Dalrymple et al., 1986), the present study used female broilers. Objective of this study was to define the effects of dietary protein levels on the growth response of

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broilers to clenbuterol under the dietary condition of a constant protein-to-energy ratio.

MATERIALS AND METHODS

Thirty female broiler chicks were selected and divided into 6 groups of 5 birds each at 14 days of age. The birds were individually housed in wire cages and used for a 3×2 factorial experiment design. The birds were fed diets containing three different levels of protein of 20, 22 and 24% CP. To maintain an equal ratio of protein-to-energy (%/kcal), dietary energy was added to each diet. The protein-to-energy ratio was adjusted to an adequate protein of 22% CP and energy of 3,100 kcal ME/kg. The experimental diets also had an approximately equivalent value of lysine-to-energy ratio (table 1). Thus, these diets comprised of different levels of protein and energy were represented as LPM (20%, CP; 2,826 kcal ME/kg), APM (22% CP; 3,154 kcal ME/kg) and HPM (24% CP; 3,380 kcal ME/kg) diets, with or without 1 ppm clenbuterol hydrochloride (Sigma Chemical, MO, USA). Each group was provided experimental diets of compositions shown in table 1 for 18 days.

Table 1. Composition of diet (g/kg)

Crude protein	Low (LPM)	Adequate (APM)	High (HPM)
Isolated soybean protein	162	176	205
Ground yellow corn	688	774	720
Vitamin and mineral mixture[1)]	37.3	37.3	37.3
DL-methionine	2.26	2.47	2.85
L-lysine · HCl	0.99	1.59	1.01
Glycine	1.48	1.15	2.20
L-threonine	0.21	0.23	0.32
Arginine	0.31	0.40	0.29
Cellulose	107.1	6.51	–
Analytical composition			
CP (%)	20.3	22.5	24.4
ME[2)] (kcal/kg)	2,826	3,157	3,380
Lysine/ME ratio[3)]	0.37	0.38	0.37

[1)] See Hamano et al. 1995.
[2)] Calculated values.
[3)] Calculated values as g/kcal × 100

The abdominal fat breast muscle and leg muscle (*gastrocnemius* and *peroneus longus*) were removed and weighed after the 32-day-old chickens were killed. The collected muscles were stored at −85℃ until used for chemical analysis of protein, DNA and RNA concentrations. Protein content in muscle was determined by the modified Lowry method (Markwell et al., 1978). Bovine serum albumin was used as standard for the protein assay. DNA content was analyzed by fluorometric procedure (Labraca and Paigen, 1980) using Hoechst 33258 reagent (bisbenzimidazole, Sigma Chemical, MO, USA), after samples were homogenized in phosphate buffer containing 2M NaCl (pH 7.4). Muscle RNA was isolated by the method of Shibko et al. (1967), and orcinol reaction was used for a colorimetric procedure (Lin and Schjeide, 1969). Calf thymus DNA and purified yeast RNA were used as standards for DNA and RNA determinations, respectively.

All data were analyzed by a two-way analysis of variance according to a 3×2 factorial scheme. Significant differences between control and clenbuterol treatments in each dietary group were tested using Student's t-test when a significant interaction between dietary treatments was detected for a parameter.

RESULTS

The effects of clenbuterol on body weight gain, feed intake, feed efficiency, protein consumption, energy consumption and protein efficiency ratio in broiler chicks fed the diets containing different levels of protein are showin in table 2. No significant interaction between dietary condition and clenbuterol was detected in body weight gain, feed intake, energy consumption or protein efficiency ratio. As to significant effects of dietary condition on the performance, increasing the dietary protein contents influenced feed efficiency ($p < 0.01$) and protein consumption ($p < 0.05$). The highest feed efficiency and protein consumption were observed in chickens fed the HPM diet. However, the clenbuterol treatment did not affect the feed efficiency or protein consumption altered by the different levels of dietary protein.

The effects of clenbuterol on tissue weight in broilers fed the diets containing different levels of protein are shown in table 3. Neither dietary condition nor clenbuterol affect abdominal fat weight. While the clenbuterol feeding significantly increased muscle weight (breast muscle, $p < 0.01$; *gastrocnemius*, $p < 0.01$; *peroneus longus*, $p < 0.05$; total leg muscle, $p < 0.01$), no significant interaction between dietary condition and clenbuterol was observed. With regard to the breast muscle, the muscle weight was the heaviest in the chickens fed the HPM diet supplemented with clenbuterol. The magnitude of difference between control and

Table 2. Effects of clenbuterol (CL) on growth performance of broiler chickens fed diets containing different levels of protein (PM)

Diet[1] / Treatment	LPM		APM		HPM		Pooled SEM	Analysis of variance[2]		
	Control	CL	Control	CL	Control	CL		Diet	CL	Interaction
BW (g)	1,155	1,114	1,166	1,181	1,277	1,243	23	NS	NS	NS
BW gain (g/18d)	777	736	790	805	898	865	23	NS	NS	NS
Feed intake (g/18d)	1,322	1,204	1,183	1,195	1,223	1,206	28	NS	NS	NS
Feed efficiency[3]	0.59	0.59	0.66	0.67	0.73	0.71	0.01	**	NS	NS
Protein consumption (g/18d)	264	241	260	263	294	289	6.7	*	NS	NS
ME consumption (Mcal/18d)	3.70	3.37	3.67	3.70	4.03	3.98	0.09	NS	NS	NS
Protein efficiency ratio[4]	2.94	3.04	3.03	3.06	3.05	2.97	0.03	NS	NS	NS

[1] LPM, APM and HPM had different levels of dietary protein that retained an equivalent protein-to-energy ratio, and contained 20, 22 and 24% CP respectively.
[2] ** : $p < 0.01$, * : $p < 0.05$, NS: No significance.
[3] Values represent BW gain/feed intake.
[4] Values represent BW gain/protein consumption.

Table 3. Effects of clenbuterol (CL) on tissue weight in broiler chickens fed diets containing different levels of protein (PM)

Diet[1] / Treatment	LPM		APM		HPM		Pooled SEM	Analysis of variance[2]		
	Control	CL	Control	CL	Control	CL		Diet	CL	Interaction
Tissue weight (g/kg BW)										
Abdominal fat	13.2	9.9	12.2	12.6	13.5	10.1	0.50	NS	NS	NS
Breast muscle	53.6	59.8	55.9	58.7	55.5	62.2	0.90	NS	**	NS
Gastrocnemius	8.07	8.75	8.54	9.05	8.75	9.47	0.14	NS	**	NS
Peroneus longus	6.61	8.04	7.22	8.20	7.24	7.58	0.16	NS	*	NS
Total leg muscle[3]	14.7	16.8	15.7	17.3	16.0	17.1	0.20	NS	**	NS

[1] LPM, APM and HPM had different levels of dietary protein that retained an equivalent protein-to-energy ratio, and contained 20, 22 and 24% CP respectively.
[2] ** : $p < 0.01$, * : $p < 0.05$, NS: No significance.
[3] Muscle weights consisted of the gastrocnemius and peroneus longus.

clenbuterol treatment for the increased muscle weight was also the highest in the HPM group (+ 12%) as compared with other dietary groups.

Table 4 shows the effects of dietary protein levels and clenbuterol on muscle composition in chickens. Significant interactions between dietary condition and clenbuterol treatment were detected in protein content of the leg muscles (*gastrocnemius*, $p < 0.05$; *peroneus longus*, $p < 0.01$), but not in that of breast muscle. When chickens were fed APM or HPM diet, clenbuterol did not affect the protein content of the *gastrocnemius*. However, a decrease in the protein content of this muscle was caused by feeding the LPM diet supplemented with clenbuterol ($p < 0.05$). In the *peroneus longus* muscle, an 8% increase in protein content due to clenbuterol treatment was detected ($p < 0.05$) only in chickens fed the HPM diet. In contrast, the clenbuterol treatment induced a decrease in protein content of the *peroneus longus* muscle equivalent to the decrease in that of *gastrocnemius* muscle when chickens were fed LPM diet ($p < 0.05$). Clenbuterol had no effect on the protein content of this muscle in chickens fed the APM diet.

These results indicate that the response of muscle to clenbuterol, leading to protein accretion, is dependent on dietary levels of protein with metabolizable energy.

In breast muscle, clenbuterol reduced ($p < 0.01$) the DNA content without an interaction with the dietary condition. Significant interactions between clenbuterol and dietary condition were detected in DNA content of *gastrocnemius* ($p < 0.05$) and *peroneus longus* ($p < 0.01$) muscles. The clenbuterol treatment decreased ($p < 0.05$) the DNA content only when chickens were given the APM diet. In the *peroneus longus* muscle, a reduction in DNA content, induced by clenbuterol, was observed in the APM group ($p < 0.05$). In contrast, the treatment with clenbuterol resulted in an increase in DNA content of the *peroneus longus* only when chickens were fed the HPM diet ($p < 0.05$). No influence of clenbuterol on DNA content of this muscle was confirmed in the chickens fed the LPM diet. Neither clenbuterol nor dietary condition had any effect on RNA content in breast muscle. Although increased contents of protein and energy in the diet elevated the RNA content in *gastrocnemius* ($p < 0.05$) and *peroneus longus* ($p < 0.01$) muscles, as an interaction, clenbuterol treatment did not affect the RNA contents induced by dietary protein levels.

Table 4. Effects of clenbuterol (CL) on muscle composition of broiler chickens fed diets containing different levels of protein (PM)

Diet[1)]	LPM		APM		HPM		Pooled	Analysis of variance[2)]		
Treatment	Control	CL	Control	CL	Control	CL	SEM	Diet	CL	Interaction
Protein (mg/g wet weight)										
Breast	226.4	213.3	221.1	216.1	216.2	222.4	2.77	NS	NS	NS
Gastrocnemius	213.6[a]	176.5[b]	209.8	205.3	231.0	230.5	3.77	**	*	*
Peroneus longus	208.9[a]	184.6[b]	210.8	210.6	224.4[a]	243.2[b]	3.85	**	NS	**
DNA (mg/g wet weight)										
Breast	0.628	0.567	0.782	0.672	0.686	0.634	0.015	**	**	NS
Gastrocnemius	0.728	0.598	1.038[a]	0.771[b]	0.573	0.656	0.035	**	*	*
Peroneus longus	0.738	0.707	0.994[a]	0.629[b]	0.647[a]	1.070[b]	0.038	NS	NS	**
RNA (mg/g wet weight)										
Breast	1.12	1.14	1.38	1.29	1.19	1.21	0.03	NS	NS	NS
Gastrocnemius	1.09	0.96	1.16	1.22	1.29	1.35	0.04	*	NS	NS
Peroneus longus	1.01	0.97	1.28	1.19	1.38	1.39	0.05	**	NS	NS

[1)] LPM, APM and HPM had different levels of dietary protein that retained an equivalent protein-to-energy ratio, and contained 20, 22 and 24% CP respectively.

[2)] ** : $p < 0.01$, * : $p < 0.05$, NS: No significance.

[a,b]: Significant difference between control and clenbuterol treatment in each dietary protein group ($p < 0.05$)

The ratios of protien/DNA and RNA/DNA in muscles are shown in table 5. The protein/DNA ratio of breast and *gastrocnemius* muscles had no interaction between dietary condition and clenbuterol treatment, although clenbuterol increased ($p < 0.05$) this ratio of breast muscle regardless of dietary levels of protein. As a significant interaction ($p < 0.01$), the protein/DNA ratio of the *peroneus longus* was increased ($p < 0.05$) by clenbuterol when the chickens were given the APM diet. Conversely, clenbuterol reduced ($p < 0.05$) this ratio in the *peroneus longus* of chickens fed the HPM diet. With regard to the RNA/DNA ratio of breast and *gastrocnemius* muscles, the clenbuterol treatment had no interrelationship with dietary protein level. The response to clenbuterol in the RNA/DNA ratio of the *peroneus longus* muscle was altered by the different levels of dietary protein. Although in LPM and APM diet groups, the RNA/DNA ratio of the clenbuterol-treated chickens was unchanged as compared with controls, the clenbuterol treatment markedly reduced ($p < 0.05$) this ratio only when chickens were fed the HPM diet.

Table 5. Effects of clenbuterol (CL) on protein/DNA and RNA/DNA in muscles of broiler chickens fed diets containing different levels of protein (PM)

Diet[1]	LPM		APM		HPM		Pooled SEM	Analysis of variance[2]		
Treatment	Control	CL	Control	CL	Control	CL		Diet	CL	Interaction
Protein/DNA (mg/mg)										
Breast	361	372	284	321	318	352	8	**	*	NS
Gastrocnemius	306	305	205	277	204	176	12	**	NS	NS
Peroneus longus	284	282	212[a]	349[b]	349[a]	231[b]	13	NS	NS	**
RNA/DNA (mg/g)										
Breast	1.78	2.06	1.80	1.90	1.76	1.96	0.06	NS	NS	NS
Gastrocnemius	1.58	1.61	1.22	1.62	1.17	1.03	0.07	*	NS	NS
Peroneus longus	1.38	1.45	1.30	1.94	2.15[a]	1.32[b]	0.09	NS	NS	**

[1] LPM, APM and HPM had different levels of dietary protein that retained an equivalent protein-to-energy ratio, and contained 20, 22 and 24% CP respectively.
[2] ** : $p < 0.01$, * : $p < 0.05$, NS: No significance.
[a,b] : Significant difference between control and clenbuterol treatment in each dietary protein group ($p < 0.05$).

DISCUSSION

A previous study indicated that the clenbuterol-fed chickens gain more body weight and this is dependent on a certain level of protein intake, in accordance with increased protein consumption (Hamano et al., 1998). In contrast, a remarkably high protein-to-energy ratio, in protein levels in excess of 30% CP, was responsible for a lack of the growth-promotion in broilers (Hamano, 1996). Kim et al. (1991) confirmed that the greatest body weight gain and feed efficiency of cimaterol-fed broilers occurred at level of 19% CP with 3,200 kcal ME/kg, when broilers were provided diets containing 19 or 17% CP with various energy levels (3,200, 2,900, 2,600 kcal ME/kg) during the period of 4 to 7 weeks of age. The effect of dietary protein state on the performance of β-agonist-fed animals appears to be also associated with the protein-to-energy ratio in the diet (Reeds and Mersmann, 1991). In the present study, the results of body weight gain, feed intake, feed efficiency and protein efficiency ratio observed no influence of various dietary protein levels. This would be attributable in part to the treatment being applied for too short a period. With regard to body weight gain, more distinct increase in consumption of protein and energy than normal may be necessary to draw the maximal performance of broilers received β-agonist (Hamano et al., 1998).

Dietary administration of β-agonists had been shown to increase skeletal muscle mass in several species (Reeds and Mersmann, 1991; Yang and McElligott, 1989), but the responsiveness of the muscles to the agents is different between muscle types. Namely, the concentration of β-adrenergic receptors on the muscle would involve the responsiveness leading to muscle hypertrophy. Thus, the β-adrenergic response of leg muscles that possess high proportion of red fiber appears to be greater than that of breast muscle (Gwartney et al., 1992; Morgan et al., 1989), because the red fiber would possess high β-adrenergic receptor density (Watson-Write and Wilkinson, 1986). In this study, the clenbuterol supplementation increased both breast and leg muscle mass. The increased breast muscle weight in clenbuterol-fed chickens was the highest in the HPM diet group (a significant interaction between dietary condition and clenbuterol was not observed statistically). Thus, the hypertrophic effects of β-agonist on muscle would vary not only with alteration of the β-receptor function, but also with the metabolic response to the nutritional states.

In muscle composition, the number of birds being used too small would be unable to define the distinct interrelationships between dietary condition and clenbuterol. Clenbuterol administration did not increase protein content of breast muscle, in spite of the increased muscle weight. However, the present results revealed manifest differences of protein in leg muscle, as the response to clenbuterol, among the three levels of dietary protein. Feeding the LPM diet negated the ability of clenbuterol to stimulate protein accretion in leg muscles. An insufficient supply of protein resulted in failure of the growth-promoting impact of β-agonist in pigs (Bracher-Jakob and Blum, 1990) and rats (Perez-Llamas et al., 1991). A similar trend has been exhibited in broilers, previously (Hamano et al., 1994). Thus, because the LPM diet contained less protein and energy than normal (APM diet),

this diet was interpreted as a poor nutritional state for muscle protein accretion of the treated chickens. This indicates that feeding the diet containing lower levels of protein and energy than normal throws the repartitioning action of clenbuterol, leading to protein accretion, into disorder.

In pigs, muscle protein accretion induced by a β-agonist, salbutamol, was intensified by feeding a high protein diet (Oksbjerg et al., 1994). Moreover, the impact of ractopamine on protein and lipid metabolism in pigs is associated with energy availability (Mitchell et al., 1991). In the *peroneus longus*, a pronounced increase in protein content was observed in chickens fed the HPM diet with clenbuterol supplementation. These findings, including results of the LPM condition, further supported the argument that a greater supply of energy than normal is simultaneously necessary for the protein-sparing impact of β-agonist. Likewise, energy status with consideration of the protein-to-energy ratio in the diet would affect the interrelation between protein requirement and β-agonist effect on protein accretion.

Supplementation with β-agonists has been shown to decrease muscle DNA content per wet weight with an increase in the protein/DNA ratio, resulting in muscle hypertrophy (Hamano et al., 1995; Morgan et al., 1989; Yang and McEligott 1989; Gwartney et al., 1992). With regard to leg muscle, the significantly enhanced protein/DNA ratio was confirmed only in the APM diet group. Although DNA content of breast muscle was reduced with clenbuterol administration, in leg muscle a similar trend was observed only in the APM feeding group. The depressed protein/DNA ratio with increased DNA content in the *peroneus longus* occurred in chickens given the HPM diet. The muscle DNA isolated not only had the expected number of cells, but made up the total exactly from non-muscle cells as adipocytes, satellite cells and fibroblast. Grant et al. (1990) indicated that two types of β-agonist, ractopamine and isoproterenol, stimulated proliferative activity of satellite cells isolated from embryonic chick breast muscle. Furthermore, satellite cells in clenbuterol-treated skeletal muscle of mice began proliferating earlier than in controls (Roberts and McGeachie, 1992). Although in the present study, cell type, led to increased DNA content, and the reason for the different response to clenbuterol between muscle types were obscure, the clenbuterol treatment coupled with feeding HPM might stimulate satellite cell proliferation in muscle.

Muscle RNA and RNA/DNA were unchanged by the clenbuterol administration. Muramatsu et al. (1990) reported that in White Leghorn chickens, a protein accretion of breast muscle, induced by clenbuterol, was not attributable to the increased fractional synthesis rate of muscle protein. The β-agonist supplementation to chickens, resulting in the muscle hypertrophy, reduced fractional degradation rate (Hamano et al., 1994; Morgan et al., 1989; Wellenreiter, 1991). Dietary levels of protein or energy influence the protein turnover rate (Funabiki et al., 1987; Simon, 1989). Although the effect of clenbuterol administration on the fractional rates of muscle protein was unclear in this study, the clenbuterol-induced protein accretion of *peroneus longus* probably resulted from a reduced rate of protein degradation. Moreover, the increased and reduced protein accretion of leg muscles due to the clenbuterol treatment might be caused by an interaction with metabolic response of muscle to the protein levels that possibly resulted in an increased or lowered fractional synthesis rate.

Overall, a possible response of broilers to clenbuterol was affected by dietary concentration of protein and energy rather than protein-to-energy ratio. Namely, the growth-promoting impacts of β-agonist involved in dietary protein levels, even though protein (lysine)-to-energy ratio in the diet was equivalent to normal ratio. The repartitioning action of clenbuterol is responsible for stimulation of protein accretion with sparing protein. This elevated protein utilization is capable of improving meat production and its quality, but the β-agonist-fed broilers would increase dietary requirement of protein or energy. Therefore, the suitable nutritional states in the chickens received β-agonists should be considered to elicit the maximal ability of the repartitioning agents.

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