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Effect of dietary clenbuterol and cimaterol on muscle composition, β-adrenergic and androgen receptor concentrations in broiler chickens

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Summary

Illegal dietary supplementation with β_2-agonists has been shown to increase protein deposition and decrease fat accretion in domestic animals. In poultry the metabolic and endocrine responses to β_2-agonists are not fully elucidated. In this trial the effects of dietary clenbuterol (1 p.p.m.) and cimaterol (1 p.p.m.) on muscle composition and endocrine response of male broiler chickens were studied. Dietary clenbuterol induced a slight, but in general not significant, improvement of zootechnical performances and carcass yields. Chemical composition of muscle was not influenced by dietary treatments, even if a slight improvement of protein content was observed in treated groups. No effects on fatty acid composition of meat were detected. Both clenbuterol and cimaterol treatments caused a downregulation in testicular androgen receptors and in pulmonary, cardiac and central nervous system β-adrenergic receptors.

Introduction

In veterinary therapy β_2-adrenergic agonists have been used as bronchodilators in the treatment of pulmonary diseases and as tocolytic agents. However, due to their metabolic effects, β_2-adrenergic compounds are illegally used as repartitioning agents to improve the performance of meat-producing animals. In fact, the long-term administration of high dosages (20–30 times the therapeutic dose) induces an increase in protein content and a decrease in fat deposition (Fiems, 1987; Yang and McElligott, 1989). Nevertheless, illicit treatments cause the presence of residues in edible tissues with possible risks for consumers and can induce changes in target organs of treated animals (Re et al., 1997).

Although β_2-agonists stimulate lipolysis and inhibit lipogenesis in adipose tissue or liver (Yang and McElligott, 1989), chicken adipocytes have been shown to be quite insensitive *in vitro* to β-adrenergic stimulation (Re et al., 2000). In general, the effects induced in birds are less pronounced than in mammals and seem to be more evident in animals either very young or very old (Buttery and Dawson, 1987). The response of birds to administration of partitioning agents widely vary according to the compound, the way of administration, the dose, the duration of treatment, the sex and the age of animals (Dalrymple et al., 1984; Lamming, 1987).

The aim of this research was to investigate the influence of dietary clenbuterol and cimaterol on carcass composition and on β-adrenergic and androgen receptor concentrations in target organs of broiler chicken.

Materials and methods

Fifty-four male broiler chickens (Ross 508), 36 days old, were divided into three homogeneous groups, randomly assigned to three experimental groups and fed isoenergetic and isonitrogenous diets. Experimental animals were fed for 21 days with a standard diet containing placebo (group 1, control), 1 p.p.m. of clenbuterol (group 2) and 1 p.p.m. of cimaterol (group 3), respectively. The basal diet was a pelletted finisher diet containing 705 g of maize, 245 g of soya bean meal, 20 g of calcium biphosphate, 10 g of soya bean oil, 8 g of mineral and vitamin premix and 5 g of calcium carbonate per kg of dry matter (DM; chemical composition per kg DM: metabolizable energy 12.9 MJ, crude protein 190 g, ether extract 43 g, ash 44 g, water 120 g). The composition of mineral and vitamin premix per kg was: retinol 30 000 U.I., cholecalciferol 7500 U.I., alphatocopheril-acetate 100 mg, Cu 40 mg, Na 250 mg. Animals were housed in light–temperature-controlled room sited in the authorized Experimental Farm (C.I.S.R.A.) of the Faculty of Veterinary Medicine of the University of Torino, Italy. Feed and water were provided *ad libitum*. Individual body weight and feed intake were weekly recorded to calculate the feed conversion ratio (FCR).

At the age of 57 days birds were fasted overnight and, after anaesthesia, were killed by cervical dislocation. Eviscerated and plucked carcasses were weighed after removal of the head, neck, feet and abdominal fat to obtain ready-to-cook carcasses.

Carcasses ($n = 18$ per treatment) were stored in a cool chamber at 0–4 °C until the next day when carcass yield and pH of breast and thigh were evaluated. The m. pectoralis major of right side ($n = 10$ per treatment) was vacuum-packed and immediately frozen (−20 °C) and then analysed to determine the chemical composition as follows: moisture and ash by oven method, protein by Kjeldhal selenium catalyst method (AOAC, 1990) and total lipids (Folch et al., 1957); values are expressed as percentage of fresh matter basis.

The m. pectoralis major of left side ($n = 5$ per treatment) was stored as the right one until fatty acid analysis was performed. The fatty acid profile of diets and breast samples were determined by capillary gas chromatography. Total lipids were extracted according to Folch et al. (1957) and methyl esters of fatty acids were prepared by the methylation procedure of AOAC (1990) and analysed by means of a gas chromatograph (Perkin-Elmer 8700; Perkin-Elmer, Norwalk, CT, USA) fitted with a flame ionization detector and equipped with a 60-m fused silica capillary column. The operating conditions of the gas chromatograph were as follows: injector temperature 250 °C; initial oven temperature 180 °C for 1 min, rate of 4 °C/min up to 225 °C, final oven temperature 225 °C for 30 min; detector temperature of 270 °C.

Immediately after slaughtering, tissue samples ($n = 18$ per treatment) of heart, lung, brain and testicles were collected from each animal and frozen (−80 °C) until they were homogenized to obtain cytosol and plasma membranes by fractioned ultracentrifugation. Briefly, frozen tissue samples were homogenized in ice-cold buffer (50 mM Tris–HCl, 1 mM EDTA, 12 mM thioglycerol, 10 mM sodium molybdate, 10% glycerol; pH 7.4). The homogenates were then filtered through a double layer of cheese-cloth and centrifuged at 3000 *g* for 20 min at 4 °C. The supernatants were centrifuged at 105 000 *g* for 45 min at 4 °C and the resulting supernatants (cytosolic fractions) were used for the measurement of androgen receptors (An-R) (Re et al., 2001). The resulting pellets (membrane fractions) were suspended using a sonicator in the incubation buffer (75 mM Tris–HCl, 25 mM $MgCl_2$; pH 7.4) at 4 °C, diluted to a final concentration of 2 mg protein per millilitre and used for the determination of β-adrenergic receptors (β-AR) (Re et al., 1995). Protein concentrations were measured according to the method described by Lowry et al. (1951).

Androgen receptors and β-AR concentrations were measured using binding assays as described previously by Re et al. (1993) and by Re et al. (1999). For the measurement of β-AR concentrations, aliquots of heart, lung and brain membrane (200 μg) were incubated in triplicated for 1 h at 37 °C with increasing concentrations (0.06–4.00 nM) of high-affinity, non-selective β-AR antagonist (−) [^{3}H]CGP 12177 in a total volume of 200 μl of

incubation buffer. Non-specific binding was measured in the presence of 100 μM isoprenaline. Incubations were stopped by the addition of 2 ml of ice cold-buffered saline (154 mM NaCl, 50 mM Tris–HCl; pH 7.4), and the resulting mixtures were immediately filtered under vacuum over pre-soaked Whatman GF/C filters. The filters were then washed with 3 × 4 ml of ice cold-buffered saline to remove free radioactivity, solubilized in 4 ml of scintillation fluid (Filter-Count; Canberra Packard, Groningen, the Netherlands) and the radioactivity was measured for 2 min using a β-counter (Tri-Carb 1600 TR, Canberra Packard) at an efficiency of 60% (Re et al., 1999). The equilibrium dissociation constants (K_d) and the maximum number of binding sites (B_{max}), expressed as nanomolar values and femtomoles of specifically bound (−) [^{3}H]CGP 12177 per milligram of membrane protein, respectively, were calculated by Scatchard analysis (Scatchard, 1949) using a computer program (GraphPad, Prism; Institute for Scientific Informatics, San Diego, CA, USA).

For the measurement of An-R concentrations, aliquots of cytosol (200 μl) were incubated overnight in triplicated at 4 °C with 50 μl of increasing concentrations of [^{3}H]mibolerone (0.13–2.6 nM). In order to avoid possible binding of mibolerone to glucocorticoid receptors, 100 nM triamcinolone acetonide was added to each incubation tube. After absorption of free hormones on dextran-coated charcoal and precipitation, 200 μl of the supernatant were added to 4 ml of scintillation liquid (Pico Fluor, Canberra Packard) and counted by scintillation spectrometry as described above for β-AR assay. The K_d (nM) and B_{max} (fmoles of specifically bound hormone per milligram of cytosol protein) were calculated as described above.

Data were analysed by one-way analysis of variance (ANOVA), the Tukey test was performed to split differences between groups (SPSS, 1997).

Results

Growth performance and carcass yields were slightly influenced by dietary administration of β_2-agonists (Table 1). Mean values were in general higher for the group treated with clenbuterol. Data of feed intake and FCR were not submitted to statistical analysis because they represent a global data for each group.

Chemical composition of breast muscle (Table 2) was affected by dietary treatment with regard to moisture only, showing the highest value of moisture in both treated groups ($p < 0.05$). A moderate, but not significant, increase of protein content in treated groups was observed. Fatty acid composition of breast muscle was not affected by treatments (Table 3).

Table 1. **Growth performances, carcass yields and pH (n = 10) (mean ± SEM)**

	Control	Clenbuterol	Cimaterol	p
Final body weight (g)	2755.94 ± 92.29	2827.83 ± 81.76	2739.65 ± 84.82	0.27
Daily feed intake (g)[1]	173.36	167.04	164.70	–
FCR (35–57 days)[1]	2.5	2.3	2.4	–
Carcass weight (g)	1979.43 ± 58.41	2064.42 ± 62.11	1939.04 ± 80.49	0.41
Carcass yield (%)	70.95 ± 1.10	72.11 ± 0.33	72.43 ± 0.75	0.37
Breast weight (g)	434.13 ± 19.27	481.53 ± 19.58	435.14 ± 22.56	0.18
Breast yield (%)	21.83 ± 0.56	23.24 ± 0.36	22.45 ± 0.53	0.14
Thigh weight (g)	290.94 ± 8.85	309.43 ± 9.84	281.74 ± 52.89	0.05
Thigh yield (%)	14.69 ± 0.21	14.98 ± 0.18	14.53 ± 0.13	0.02
Breast pH_{24h}	5.86 ± 0.11	5.82 ± 0.04	5.80 ± 0.10	0.75
Thigh pH_{24h}	6.16 ± 0.0.08	6.17 ± 0.09	6.16 ± 0.09	0.77

[1] For each group

Dietary administration of clenbuterol induced significant decreases in β-AR concentrations in heart ($p < 0.001$), lung ($p < 0.05$), brain ($p < 0.001$) and of An-R levels in testicles ($p < 0.001$) (Table 4).

In the same way, cimaterol induced significant downregulation of β-AR concentrations in heart ($p < 0.01$), lung ($p < 0.05$), brain ($p < 0.01$) and of AnR in testicles ($p < 0.001$) (Table 4).

Table 2. **Chemical composition of breast meat (% on fresh matter basis) ($n = 10$) (mean ± SEM)**

	Control	Clenbuterol	Cimaterol	p
Moisture	71.71 ± 2.91	73.14 ± 1.87*	73.04 ± 1.90*	0.02
Crude protein	22.93 ± 0.66	23.66 ± 0.53	23.60 ± 0.85	0.15
Lipid	1.38 ± 0.27	1.29 ± 0.12	1.39 ± 0.21	0.14
Ash	1.18 ± 0.10	1.20 ± 0.05	1.22 ± 0.11	0.72

* $p < 0.05$

Table 3. **Fatty acid composition of diets and breast meat (% of fatty acid acyl esters) ($n = 5$) (mean ± SEM)**

	Diets			Breast meat			
	Control	Clenbuterol	Cimaterol	Control	Clenbuterol	Cimaterol	p
14:0	0.69	1.00	0.89	0.64 ± 0.00	0.71 ± 0.12	0.63 ± 0.01	0.73
14:1	0.20	0.26	0.20	ND	ND	ND	–
16:0	15.50	15.72	15.89	25.59 ± 0.12	25.50 ± 0.49	24.05 ± 0.52	0.04
16:1	2.07	1.90	1.99	6.48 ± 0.49	6.24 ± 0.22	6.22 ± 0.26	0.85
18:0	4.87	4.85	4.30	8.63 ± 0.42	9.24 ± 0.26	9.82 ± 0.55	0.18
18:1	28.37	28.68	28.87	35.04 ± 0.50	33.73 ± 0.73	33.65 ± 0.90	0.35
18:2 n6	45.83	44.96	45.01	15.90 ± 0.74	16.36 ± 0.24	16.15 ± 0.52	0.84
18:3 n3	2.45	2.62	2.73	0.67 ± 0.14	0.63 ± 0.01	0.60 ± 0.01	0.85
20:1	ND	ND	ND	0.53 ± 0.01	0.49 ± 0.01	0.63 ± 0.14	0.59
20:4 n6	ND	ND	ND	3.03 ± 0.49	3.38 ± 0.13	3.89 ± 0.52	0.38
SFA	21.07	21.57	21.17	34.86 ± 0.41	35.45 ± 0.52	34.50 ± 0.18	0.26
MUFA	30.64	30.84	31.07	45.53 ± 0.63	44.17 ± 0.61	44.87 ± 0.88	0.43
PUFA	48.28	47.58	47.75	19.61 ± 0.74	20.37 ± 0.24	20.63 ± 0.76	0.52
n3	2.45	2.62	2.73	0.67 ± 0.14	0.63 ± 0.01	0.60 ± 0.01	0.85
n6	45.83	44.96	45.01	18.93 ± 0.66	19.74 ± 0.25	20.04 ± 0.01	0.43
n3/n6	0.05	0.06	0.06	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.74

ND, not detected; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

Table 4. **β-AR (fmol/mg of protein) and An-R (fmol/mg of protein) concentrations ($n = 18$) (mean ± SEM)**

	Control	Clenbuterol	Cimaterol
β-AR			
Heart	244 ± 10	137 ± 11***	177 ± 18**
Lung	906 ± 16	728 ± 28*	721 ± 75*
Brain	393 ± 28	253 ± 21***	259 ± 23**
An-R			
Testicles	33 ± 2	8 ± 0.4***	8 ± 0.3***

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 5. **K_d (nM) values (n = 18) (mean ± SEM)**

	Control	Clenbuterol	Cimaterol
β-AR			
K_d (heart)	0.30 ± 0.05	0.28 ± 0.02	0.22 ± 0.04
K_d (lung)	0.70 ± 0.04	0.64 ± 0.03	0.60 ± 0.06
K_d (brain)	0.24 ± 0.04	0.24 ± 0.04	0.20 ± 0.02
An-R			
K_d (testicles)	0.45 ± 0.09	0.30 ± 0.05	0.30 ± 0.03

The values of K_d for (−) [^{3}H]CGP 12177 and [^{3}H]mibolerone were not significantly affected by treatments in any of the examined tissue (Table 5).

The linearity of the Scatchard plots was demonstrated by the values of the correlation coefficient ($r > 0.9$).

Discussion

Rehfeldt et al. (1997) showed that the enhancement of muscle growth in birds receiving clenbuterol is realized through the hypertrophic response of muscle fibres, but not through the formation of new fibres or the additional incorporation of myonuclei by satellite cell proliferation. This mechanism may explain the moderate improvement of breast and thigh muscle weight, especially observed in animals treated with clenbuterol. Dietary administration of β_2-agonists induced a non-significant reduction in fat composition of breast muscle, thus confirming that the lipolytic effects in poultry may be regulated by different metabolic pathways (e.g. glucagon release enhancement) (Re et al., 2000). Moreover, β_2-agonists treatment did not influence the mechanism of captation of fatty acid by breast muscle, as revealed by the fatty acid composition of meat, that did not change among the three experimental groups.

Data obtained in the present study demonstrated that the dietary administration of clenbuterol and cimaterol as partitioning agents induced a significant downregulation of β-AR in the heart, lung and brain of treated animals. Similar results have been observed in other species such as veal calves, which have been chronically treated with dietary clenbuterol (Re et al., 1995, 1997). Long-term administration of β_2-agonist in poultry induced a significant downregulation of An-R, as previously observed in another study regarding the effects produced by clenbuterol treatment on oestrogen and progesterone receptor expression in the genital tract of female broiler chickens (Cerruti Sola et al., 1996). Strikingly, this effect is not in line with those observed in the reproductive system of mammals, such as veal calves (Re et al., 1995), rats (Re et al., 1993) and pigs (Dacasto et al., 1994).

After long-term exposure to β-adrenergic compounds, a decrease of sensitivity has been observed in several target tissues and it has been demonstrated that desensitization processes are associated with a failure of β-AR structures that act prior to cAMP production (Housdorff et al., 1989; Pitcher et al., 1992). *beta*-Adrenergic receptor downregulation could be attributed to different mechanisms. Initially, long-term exposure to an agonist enhances the sequestration of the receptor to a location that may be represented by a vescicular pool. In this state, the receptor is accessible only to ligands in a highly hydrophobic form. Subsequently, downregulation is induced by a variety of cellular processes including enhancement of protein degradation and a destabilization of mRNA encoding β-AR (Collins et al., 1992; Milligan et al., 1994). Moreover, as a binding assay was used, in the present study, a possible occupation of (−) [^{3}H]CGP 12177 binding sites by clenbuterol should be considered as partially responsible for the reduction (Kenakin, 1993).

As far as the effects induced by dietary clenbuterol on the central nervous system (CNS) β-AR are concerned, they could be related to some behavioural modifications (Gorman and Dunn, 1993). For example, reduced food consumption (which is associated with partitioning effects on animal carcass composition responsible for a significant improvement of feed efficiency) could be ascribed to excess β_2-adrenergic stimulation at the CNS (Borsini et al., 1982; Ordway et al., 1987).

At present, the mechanism of An-R downregulation observed in the testicles of broilers treated with β_2-agonist still remains quite unclear. As clenbuterol is not able to bind significantly to steroid hormones receptors (Re et al., 1995), probably, the regulation of An-R may occur in an indirect way, possibly mediated by β-AR. This theory is supported by the observation that the concomitant exposure to propranolol inhibits the regulation of ER and PgR expression exerted by clenbuterol in the female reproductive system (Re et al., 1993). This result suggests the hypothesis that adrenergic and steroid receptorial system could share some cellular mechanism (e.g. cellular messengers, protein phosphorylation or genome activation), which interfere mutually on their control processes as already demonstrated in laboratory animals (Vivat et al., 1992).

Finally, repeated β_2-adrenergic agonist exposure did not induce any significant modification in K_d values for both β-AR and An-R in any tested tissues, thus confirming that the treatment did not alter the affinity of the receptors for specific ligands, as previously observed in poultry (Cerruti Sola et al., 1996), veal calves (Re et al., 1995, 1997), pigs (Dacasto et al., 1994) and rats (Re et al., 1993).

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