Physiopathological changes related to the use of ractopamine in swine: clinical and pathological investigations

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Physiopathological changes related to the use of ractopamine in swine: Clinical and pathological investigations

D. Catalano [a], R. Odore [a], S. Amedeo [a], C. Bellino [a], E. Biasibetti[a], B. Miniscalco [a], G. Perona [b], P. Pollicino [a], P. Savarino [a], L Tomassone[c], R. Zanatta [a], M.T. Capucchio [a],

[a] *Department of Animal Pathology, Faculty of Veterinary Medicine, University of Turin, via Leonardo da Vinci 44, 10095 Grugliasco, Italy*
[b] *Centro Interdipartimentale Servizio Ricovero Animali (CISRA), Faculty of Veterinary Medicine, University of Turin, via Leonardo da Vinci 44, 10095 Grugliasco, Italy*
[c] *Department of Animal Production, Epidemiology and Ecology, Faculty of Veterinary Medicine, University of Turin, via Leonardo da Vinci 44, 10095 Grugliasco, Italy*

ABSTRACT

The aim of the study was to investigate clinical and pathological modifications induced by treatment with different doses of the β2-agonist ractopamine (RAC) in pigs. Thirty-two eight-month-old LandracexLarge White pigs (mean weight, 102.8 ±6.7 kg) were treated with 0, 10 (R10), 20 (R20) and 40 (R40) mg/kg/diet of RAC for the last 35 days before slaughtering. Before and after treatment body weight was recorded, blood samples were collected for complete blood count and thyroid hormone concentration analysis, and cardiac instrumental evaluation was performed. At slaughtering, tissue samples were collected for histopathological examination. None of the animals showed any signs of clinical disease during the study period. Treatment did not induce any statistically significant difference in complete blood counts (P>0.05) among groups. A significant (Pb0.05) decrease in feed consumption was observed between R10 and R40 treated animals. A significant (Pb0.01) increase in ventricular internal dimensions and left ventricular mass was observed in R20; a significant reduction in thyroid weight (Pb0.05) was observed in the animals receiving the highest-dose treatment (R40). Urethral hyperplasia and metaplasia, and prostate hyperplasia and a significant (Pb0.05) reduction in circulating triiodothyronine levels were found in overall treated animals. Taken together, these data suggest that RAC treatment in pigs induces adaptive non-neoplastic urethral and prostatic disorders of growth and a reduction in thyroid hormone concentrations. Nevertheless, additional research on a larger group of animals is needed to define the biomolecular mechanisms underlying the observed lesions, in order to improve the surveillance tools to identify illegal treatments.

1. Introduction

Ractopamine (RAC) is a ß-adrenergic agonist (ß-agonist) which at repartitioning doses has been found to increase muscle growth and decrease fat deposition in different animal species (Baker et al., 1984; Mersmann, 1998; Mills and Spurlock, 2003; Ricks et al., 1984; Sillence, 2004; Watkins et al., 1990). In pigs and cattle treated during the final period of the finishing improved feed efficiency, heavier carcass weights, and higher dressing percentages have been documented (Gu et al., 1991a,b; Laudert et al., 2005a,b; Poletto et al., 2009; See et al., 2004).

The dosage required to induce "anabolic" effects is generally 10-20 times higher than that indicated for therapeutic purposes. At elevated doses, certain β2-agonists such as clenbuterol can cause morphological, histological and/or biochemical changes in various organs of exposed animals (Biolatti et al., 1994; Sillence et al., 1993). *In vivo,* β-adrenergic agonists may induce secondary events caused by hormonal or physiological responses of several tissues, involving especially endocrine and cardio-respiratory organs.

In pigs, treatment during the finishing with repartitioning doses of β-agonists has been associated with modifications of vascular microcirculation characterized by ulcerative lesions of the distal extremities (Dacasto et al., 1994; Poletto et al., 2009).

Elevated heart rate and increased peripheral catecholamine concentrations have been reported in pigs fed RAC (Marchant-Forde et al., 2003). Additionally, hyperactivity, overreactivity to transport, and difficulty in handling have been also described (Marchant-Forde et al., 2003). Treated pigs have a greater frequency of front and rear hoof lesions. They show an increase in *Enterobacteriaceae* shedding concentration at the first week of treatment, that progressively decreases until slaughter when it is less than in control animals (Poletto et al., 2009). The same Authors reported also intensified aggression, especially in gilts (Poletto et al., 2010a,b), probably due to reduced expression of serotonergic genes detected in the brain (Poletto et al., 2011). The only macroscopic modification observed so far in RAC treated pigs is a decrease in the relative weight of thyroid glands (Ungemach, 2005). The only histopathological finding reported in the same species is increased liver glycogen in periportal hepatocytes of a few pigs at high doses (Williams, 1987).

Ractopamine-induced changes have also been investigated in cattle. To date, no adverse effects of RAC supplementation on cattle behaviour have been reported (Baszczak et al., 2006). No changes on blood gas, blood electrolyte, or blood metabolite values have been observed (Abney, 2006). Decrease of ruminal ammonia and amino acid concentration affecting fermentation of ruminal microflora have been recently reported (Walker and Drouillard, 2010). The aim of this study was to investigate clinical, macroscopic and histopathological changes induced by RAC treatment in finishing pigs to identify possible biomarkers of illegal treatment and, therefore, to improve the surveillance methods at slaughtering.

2. Materials and methods

The study protocol adhered to national Animal Welfare Guidelines. The experiment was authorized by the Italian Ministry of Health and Welfare (D.M. 02/2007-B; 11/01/ 2007).

2.1. Animals and treatment

A total of 32 eight-month-old Landrace x Large White pigs were used for the experiment. They were randomly divided into 4 groups of 8 animals, composed of 4 neutered males and 4 females each.

The animals were acclimated for one month (pre-treatment period); at the beginning of this period a parasitosis treatment was performed, using Ivermectin at a concentration of 10 mg/ 30 kg. At the end of the pre-treatment period (T0; -1 day prior the feeding trial), each group of pigs was allocated into 1 pen composed of 8 adjacent boxes, which housed 1 pig per box. Each box measured 2.2x3.0 m. All the pens were located in the Experimental Enclosure CISRA, of the Faculty of Veterinary Medicine, University of Torino, approved by Ministry of Health (authorization DM no 86/01-A) according to DL 116/92.

The initial body weight within the groups was similar (BW: 102.8±6.7kg) (Table 1). Pigs were provided water and feed *ad libitum.* A table with detailed diet composition provided to the finishing pigs is reported (Table 2).

Table 1 Body weight (kg; means ±SEM) in pigs treated with different RAC

RAC dosages	Body weight (Kg)	
	$T0^{b}$	$T1^{c}$
R0	99.7 ± 1.5^{x}	130 ± 2.5^{y}
R10	103.5 ± 2.5^{x}	136 ± 3.6^{y}
R20	105 ± 2.1^{x}	141 ± 2.1^{y}
R40	103 ± 3.0^{x}	138.4 ± 3.7^{y}

R0: control, 0 ppm RAC.
R10: 10 ppm RAC.
R20: 20 ppm RAC.
R40: 40 ppm RAC.
x vs. y $= P<0.05$, Tukey Multiple Comparison Test.
[a] RAC: ractopamine.
[b] T0: –1 day prior the feeding trial.
[c] T1: 35th day of the feeding trial.

Table 2

Diet composition provided to the finishing pigs.

Ingredient, %	
Corn	55
Barley	20
Soybean meal, 44%	9
Bran	14
Dicalcium phosphate	0,5
Vitamins and mineral premix 1–2	1,5
Calculated analysis (dry matter)	
ME, kcal/kg	3034
CP, %	16,26
Fiber, %	5,17
Fat, %	3,56
Ca, %	3,05
Lysine, %	0,61
Methionine, %	0,31
Methionine + cysteine, %	0,55
Tryptophan, %	0,17
Ca, %	0,7
P, %	0,5

Ractopamine (Unibrom Corp., RM 906, Xinjianye Bldg, NO. 303 Dongfeng East Street, Weifang 261041, P.R. China) was assigned randomly: one untreated group served as control, whereas the other groups received different RAC doses for 5 weeks.

Dietary treatment (as-fed basis) was supplemented as follows: controls R0 (0 mg/Kg diet/die [ppm] of RAC), R10 (10mg/Kg/die [ppm] of RAC), R20 (20mg/Kg/die [ppm] of RAC) and R40 (40 mg/Kg/die [ppm] of RAC) during the last 35 days of the finishing period. Throughout the experimental period, the animals underwent daily clinical observation to evaluate their health state.

At T0 (— 1 day prior the feeding trial) and at the end of the experiment (T1; 35th day of the feeding trial) blood samples were collected and immediately after pigs were anesthetized with an intramuscular injection of tiletamine-zolazepam (Zoletil®, Virbac, Milan, Italy) at a dose of 4.0 mg•animal^{-1} and xilazine (Rompum®, Bayer Healthcare LLC, Shawnee Mission, KS, USA) at a dose of 2.0 mg•animal^{-1} to perform cardiographic evaluation.

At T1 animals were then euthanized in accordance with ethical guidelines.

2.2. Blood collection and chemistry analysis

At T0 and T1, 10 ml of blood samples were collected in test tube for serum, to evaluate pre-treatment and post-treatment complete blood count (CBCs), thyroid stimulating hormone (TSH) and triiodothyronine (T$_3$) blood levels.

Blood samples were centrifuged at 3000 *g* force for 10 minutes at room temperature, and serum aliquots were stored at — 80 °C for further analysis.

2.3. Cardiac instrumental evaluation

At T0 and T1 the anesthetized pigs were submitted to electrocardiographic (ECG-P-power 40, Esaote Biomedica, Milan, Italy) and echocardiographic evaluation (My Lab 30, Esaote Biomedica). A right short axis view was used to evaluate left ventricular dimensions (interventricular septum [IVS], left ventricular internal dimensions [LVID] and left free wall dimension [LFW] in diastole [d] and systole [s] - IVSd, IVSs, LVIDd, LVIDs, LFWd and LFWs, respectively), left ventricular mass (LVM), ejection (EF) and shortening fraction (SF). Doppler and color-Doppler evaluation were performed in order to detect the presence and clinical relevance of valvular leakage and/or stenosis. Differences in heart rate (HR) were evaluated.

2.4. Pathological investigations

A complete necropsy of the pigs was performed. Skin, fat, muscle *(longissimus dorsi),* tonsils, thymus gland, mammary gland, thyroid, trachea, esophagus, heart, lung, stomach, small intestine, colon, liver, spleen, pancreas, kidney, adrenal gland, mesenteric lymph node, ovary, prostate, pituitary gland and eye were examined macroscopically and samples of each tissue were collected. Thyroids were measured by means of a calliper, and weighed. Tissue samples were stored in 10% neutral buffered formalin for further evaluation.

After fixation and removal of extracardiac structures, total heart weight was measured. The total length (from the atrioventricular sulcus to the apex) and the right and left ventricular thickness at the insertion of the papillary muscle were estimated. Cross-sections of the left and right ventricular wall and the IVS at the insertion of the papillary muscle were prepared for histological examination.

All formalin-fixed tissues were processed for histological examination. Briefly, samples were dehydrated using ethanol at increasing concentrations and xilol solutions. At this stage they were paraffin embedded and 4-5 μm sections were obtained using a microtome (Leica Microsystems, Wetzlar, Germany).

The sections were then stained with hematoxylin/eosin and examined under a light microscope by two pathologists. Observed lesions were classified using a semiquantitative scoring system.

Eosinophilic cell infiltrate severity was graded as follows: no lesions (0), low number of focal to multifocal lesions/cells (1), moderate number of lesions/disseminated cells (2) or diffuse severe lesions/cells (3). Cellular hyperplasia of the urethral and prostatic epithelium of the males (16 pigs)

was graded by counting the layers of epithelial cells in 20 fields at 400xas follows: 0 (transitional normal epithelium); 1 ($\leq$ 5 layers); 2 (5-15 layers); 3 (> 15 layers).

Urethral metaplasia was graded according to the presence and distribution of metaplastic cells.

Histochemical staining of the liver samples with the periodic acid-Schiff (PAS) method was performed to demonstrate glycogen distribution in hepatocytes. A semi-quantitative assessment of glycogenosis was estimated on the basis of a scale from 0 to 5 by counting the number of affected hepatocytes observed in20 fields at400x: 0 (none); 1 (b50 cells); 2 (50-150 cells); 3 (150-300 cells); 4 (300-400 cells); 5 (>400 cells).

2.5. Blood chemistry

CBC was performed using an ADVIA®120 Hematology System (Siemens Diagnostics, Erlangen, Germany).

Plasma TSH and T_3 concentrations were measured using commercial radioimmunoassay kits (Izotop, Budapest, Hungary). The intra- and inter-assay coefficients of variations were 4.6 ±0.7% and 7.5 ±1.2% for T_3 and 2.7 ±0.1% and 8.5 ± 1.6% for TSH, respectively.

2.6. Statistical analysis

To assess normal distribution of data, the Shapiro-Wilk Normality test was performed. Metric data are presented as means ± standard error of mean (SEM).

To investigate for possible differences in scores attributable to histopathological lesions, a non-parametric Kruskal-Wallis test was performed. Correlation between histopathological lesions and groups (RAC dosages) was evaluated using the non-parametric Spearman Rank Correlation Test (Rs).

Tukey Multiple Comparison Test was applied to test for differences among groups in the means of hematological and echocardiodiographic values, thyroid weight, animal weight, feed consumption and blood hormone levels at the different RAC treatment dosages.

Statistical analysis was performed using a freeware statistical software package (R2.10.1)[a] (R Development Core Team, 2008-http://www.R-project.org). The significance limit was set at P≤0.05.

3. Results

3.1. Clinical observations

None of the animals showed any signs of clinical disease during the study period.

No significant difference in weight increase among the treatment groups was observed (P = 0.27) (Table 1).

By contrast, a significant reduction in feed consumption between groups R10 and R40 was

observed when considering all the experimental animals (Pb0.05) (Fig. 1). No statistically significant difference in CBCs was observed among groups.

Echocardiographic assessment at both T0 and T1 revealed tricuspid insufficiency in nine pigs and aortic insufficiency in five. None of the valvular insufficiencies was hemodynamically significant, the regurgitant jet (evaluated with color-Doppler) was very small, end peak velocity flow very low. A minimal amount of pleural effusion was detected in two pigs at the
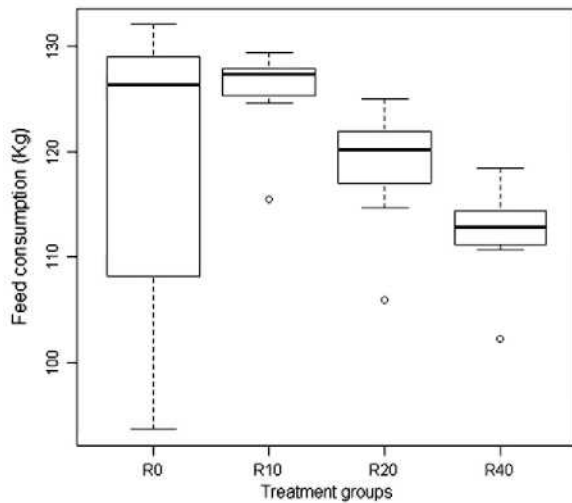

Fig. 1. Boxplotof the total feed consumption (kg) in pigs treated with different RAC dosages for 35days (R0: control, 0ppm RAC; R10: 10ppm RAC; R20: 20 ppm RAC; R40: 40 ppm RAC).

end of the study. No arrhythmias or differences in heart rate (HR) were found in any group at either assessment. There was no statistically significant difference in echocardiographic parameters among groups at T0 (P>0.05; Table 3).

The ventricular internal dimensions were significantly (P = 0.01) increased at T1 as compared with T0 values in group R20, as was LVM, with significant increases noted in both group R20 (P = 0.01) and group R10 (P = 0.05).

Inter-group evaluation showed a significant increase in left ventricular internal dimensions in group R10 with respect to group R40 (P = 0.02) and group R0 (P = 0.01) (Table 3).

Table 3

Echocardiographic parameters in pigs treated with different RAC[a] dosages.

		R0[b]	R10[b]	R20[b]	R40[b]
IVSd	T0[d]	12.8±0.9	12.7±0.7	12.3±0.4	13.2±0.6
mm[c]	T1[e]	12.2±0.6	13.2±0.4	12.8±0.5	13.2±0.4
LVIDd[f]	T0	46.8±1.7	50.0±3.2	46.7±1.4	46.8±1.4
	T1	52.1±1.2	54.2±1.7	53.4±0.7	49.0±1.5
LFWd[g]	T0	9.6±0.7	9.6±0.6	10.3±0.6	11.5±1.0
	T1	11.3±0.3	10.3±0.5	11.6±0.5	10.9±0.78
IVSs	T0	17.7±1.3	18.6±1.6	18.2±1.0	17.2±1.7
	T1	18.4±1	16.0±0.6	18.2±1.0	19.1±0.8
LVIDs	T0	26.2±1.8	28.5±2.4	26.3±1.9	28.8±1.8
	T1	28.0±0.6**	32.8±0.9**	30.8±0.5*	29.2±1.0**
LFWs	T0	17.4±0.6	17.4±1.0	18.6±0.8	17.5±0.7
	T1	20.3±0.5	17.9±1.2	19.9±0.7	17.9±0.7
EF[h]	T0	74.7±3.3	72.2±4.3	73.2±4.1	67.4±4.7
	T1	76.7±1.5	69.1±1.8	71.0±2.5	71.3±3.0
SF[i]	T0	44.4±3.1	42.9±3.9	43.7±4.0	38.5±3.9
	T1	45.9±1.4	39.1±1.5	40.9±1.9	41.3±2.5
LVM[j]	T0	228.1±25.4	246.8±20.7	228.1±10.3	262.0±25.2
	T1	292.2±23.0	313.4±28.4*	305.6±13.7*	279.1±13.5
HR[k]	T0	126±5.7	132±7.1	135±9.6	121±12.1
	T1	119±7.1	116±4.2	110±7.8	120±8.5

*Intragroup T0–T1 Gr0 (LFWs = 0,01)–G10 (MVS = 0,05)–Gr20 (LVIDs = 0,01) (LVM = 0,001), Tukey Multiple Comparison Test.
** Intergroup LVIDs Control/RAC10 (0,01), RAC10/RAC40 (0,02), Tukey Multiple Comparison Test.
s = systolic.
d = diastolic
 [a] RAC: ractopamine.
 [b] R0: control, 0 ppm RAC; R10: 10 ppm RAC; R20: 20 ppm RAC; R40: 40 ppm RAC.
 [c] IVS = interventricular septum thickness.
 [d] T0: −1 day prior the feeding trial.
 [e] T1: 35th day of the feeding trial.
 [f] LVID = left ventricular internal dimensions.
 [g] LFW = left free wall thickness.
 [h] EF = ejection fraction.
 [i] SF = shortening fraction.
 [j] LVM = left ventricular mass.
 [k] HR = heart rate.

3.2. Pathological lesions

Table 4 reports total heart weight and macroscopic heart measurements. A significant (Pb0.05) decrease in heart weight was observed in group R40 with respect to groups R0 and R10. Heart length was significantly increased in group R10 (Pb0.05).

A statistically significant (Pb0.05) difference in thyroid weight between groups R10 and R40 was observed when considering all the experimental animals. The mean values ± SEM for the different groups were: R0: 10.48±0.57; R10 11.09 ± 0.69; R20:9.19± 0.53 and R40:8.69 ± 0.31 g, respectively. Fig. 2 shows the gross appearance of thyroid gland of two pigs treated with R10 and R40 doses.

Histologically, no lesions potentially associated with treatment were observed in the skin, fat, muscle *(longissimus dorsi),* mammary gland, thyroid, trachea, oesophagus, heart, lung, stomach, intestine, pancreas, kidney, adrenal gland, pituitary gland, or eye.

Hepatic glycogenosis was observed in all animals, but no significant differences among groups

were seen (P>0.05) (data not shown).

Eosinophilic cell infiltrates were found in the mesenteric lymph nodes, tonsils and in the substantia medullaris of the thymus of all RAC-treated animals. Eosinophils were sometimes degranulated with focal or multifocal distribution or organized in small clusters in some cases. In some instances, eosinophilic cells were detected in the spleen and the interfollicular spaces, where rarefaction of red pulp was present. Eosinophils were also present in the ovary, particularly around the follicles at different levels of development. There was a significant linear correlation between eosinophil infiltration in the thymus and RAC treatment ($P = 0.014$; $Rs = 0.49$) (Fig. 3).

Table 4

Heart dimensions and weight in pigs treated with different RAC[a] dosages.

RAC dosages	Heart weight, g	Length, cm	Right ventricle thickness, cm	Left ventricle thickness, cm
R0[b]	485 ± 29^a	8.56 ± 0.12^A	1.4 ± 0.10	2.1 ± 0.25
R10[b]	534 ± 15^a	9.3 ± 0.20^B	1.3 ± 0.04	2.5 ± 0.20
R20[b]	480 ± 18^{ab}	9.0 ± 0.16^{AB}	1.2 ± 0.10	2.2 ± 0.05
R40[b]	405 ± 8^b	8.7 ± 0.12^{AB}	1.1 ± 0.06	2.4 ± 0.10

R10: 10 ppm RAC.
R20: 20 ppm RAC.
R40: 40 ppm RAC.
a vs. b, A vs. B = P <0.05, Tukey Kramer Multiple Comparison Test.
[a] RAC: ractopamine.
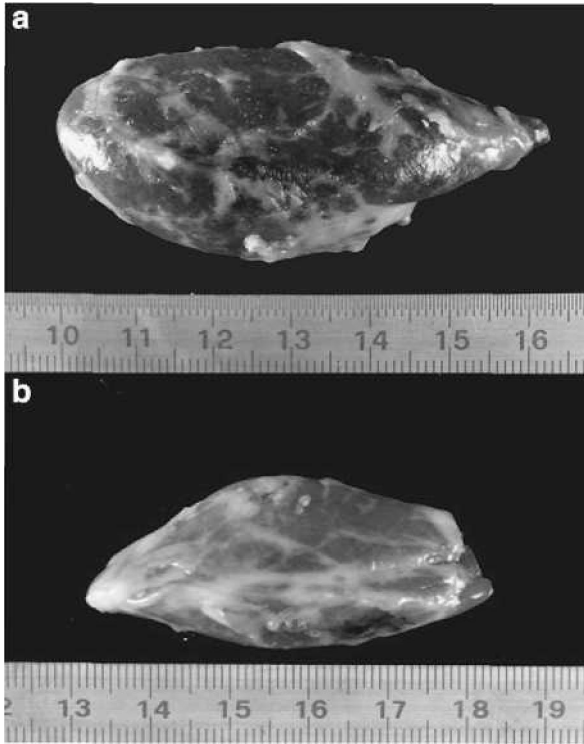[b] R0: control, 0 ppm RAC.

Fig. 2. Gross appearance of a thyroid gland from a pig treated with R10 dose (10 ppm RAC) (a) (11.7 g) and a pig treated with R40 dose (40 ppm RAC) (b) (7.12 g), respectively.
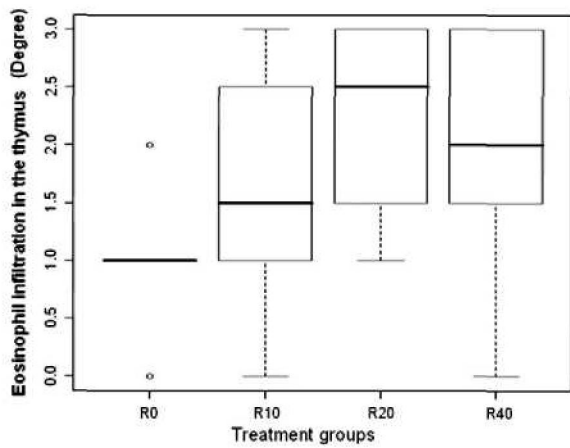

Fig. 3. Boxplot of the eosinophil infiltration in the thymus in pigs treated with different RAC dosages. (R0: control, 0ppm RAC; R10: 10ppm RAC; R20: 20 ppm RAC; R40: 40 ppm RAC).
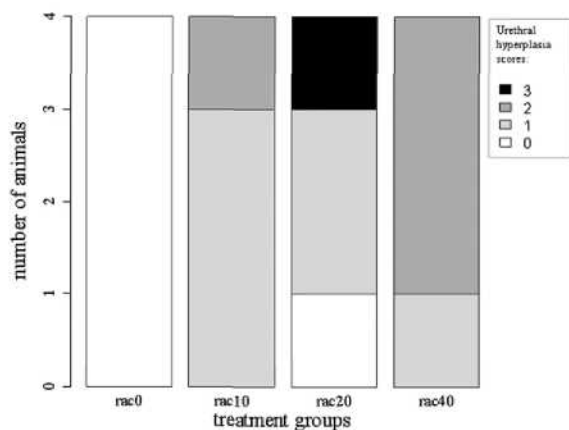
Fig. 4. Barplot of the urethral hyperplasia scores for different groups of treatment (R0: control, 0ppm RAC; R10: 10ppm RAC; R20: 20ppm RAC; R40: 40ppm RAC). 0 = no hyperplasia; 1 = (≤5 layers); 2 = (5-15 layers); 3 = (>15 layers).

scores attributed to urethral hyperplasia (P = 0.029). The median score (and 1st-3rd quartiles) was 0.0 (0.0,0.0) for R0 group, 1.0 (1.0, 1.25) for R10, 1.0 (0.75, 1.50) for R20 and 2.0 (1.75, 2.0) for R40 group.

The prostate epithelium showed grade 1 hyperplastic lesions in only six RAC-treated animals, without differences among RAC treatment dosage groups.

No other significant associations between groups and pathological features were detected (P>0.05).

No other significant correlations between groups and pathological features were detected (P>0.05).

In the RAC-treated animals, the urethral epithelium showed different grades of hyperplasia (Figs. 4 and 5). Scattered metaplastic cells associated with moderate/severe hyperplasia were also present in three RAC-treated pigs.

The Kruskal-Wallis test revealed a statistically significant difference among the RAC-treated groups for the median

3.3. Blood hormone concentrations

At T0 blood hormone concentrations were evaluated on 8 animals in order to obtain pre-treatment basal values. The mean values ± SEM for T_3 and TSH blood levels were 6.95 ± 0.4 pmol/L and 0.22 ± 0.04 µIU/ml, respectively.

AtT1, blood T_3 concentrations were significantly reduced in the RAC-treated animals; no statistically significant differences in TSH levels were observed among the RAC-treated animals (Table 5).

4. Discussion

The present study investigated the clinical, macroscopical and histopathological changes induced by RAC treatment in the finishing pigs.

The use of RAC as a feed additive for growth-promoting purposes in fattening pigs and cattle is licensed in several countries, including the United States, Canada, Japan and Mexico.

In the USA the legal label for RAC feeding ranges between 5 and 10 mg/kg (ppm) fed, for the last 18 to 40 kg before slaughter at a constant concentration or as a "step-up" feeding program.

In the European Community, the Council Directive 96/22/EC banned the use of ß-agonists in livestock as growth promoting. Nevertleless these drugs are still used illegally, particularly by livestock producers. As a consequence, the identification of markers of the use of these drugs is important to identify at the slaughter an illegal treatment.

Fig. 5. Urethra. Control pig showing a normal histological pattern (a). Different grades of hyperplasia in the RAC treated pigs (b,c,d). (b) grade 1 (≤5 layers); (c) grade 2 (5-15 layers); (d) grade 3 (>15 layers). Hematoxylin and eosin stain, 20x.
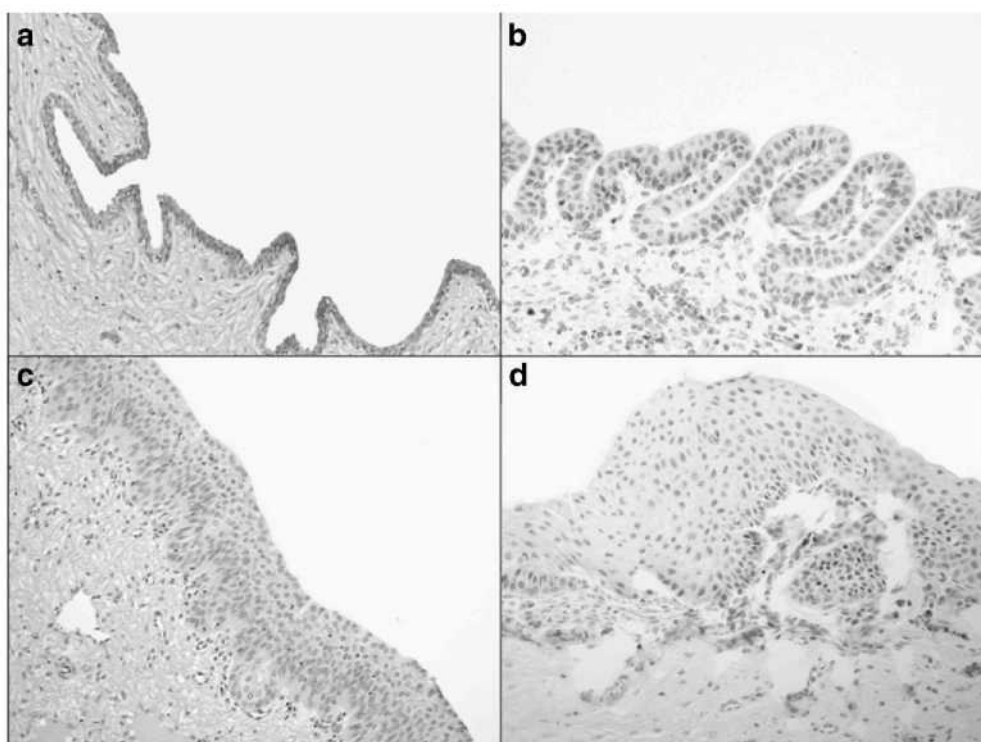
The results of the present study suggest that high doses of RAC in pigs do not induce a significant increase in body weight, by contrast a statistically significant decrease in feed consumption was observed. This finding is in line with previously published data and confirms, in part, the growth-promoting effects of RAC in this species (Apple, 2007; Fernandez-Duenas et al., 2008). The limited improvements in live weight could depend to the high dose of the β-agonist for a long period as it was reported by Abney (2006) in cattle. In fact, in these species short-term treatment causes improvements in weight gain more positive than studies of longer duration. These findings might indicate that longer exposure to β-agonists may induce desensitization and thereby minimize any apparent advantages in average daily gain (ADG) (Abney, 2006).

Table 5

Blood T3[1] and TSH[2] concentrations (means ±SEM) in pigs treated with different RAC[3] dosages

RAC[3] dosages	Blood T3[1] concentrations pmol/L	Blood TSH[2] concentrations μU/ml
	$T0 = 6.95 \pm 0.4^x$	$T0 = 0.22 \pm 0.04$
	T1[4]	T1[4]
R0[5]	7.1 ± 0.6^{ax}	0.24 ± 0.02
R10[5]	4.6 ± 0.5^{by}	0.33 ± 0.09
R20[5]	4.7 ± 0.5^{by}	0.28 ± 0.07
R40[5]	4.6 ± 0.2^{by}	0.15 ± 0.02

R10: 10 ppm RAC.
R20: 20 ppm RAC.
R40: 40 ppm RAC.
a vs. b, x vs. y = P<0.05, Tukey Kramer Multiple Comparison Test.
[1] T_3: triiodothyronine;
[2] TSH: thyroid stimulating hormone;
[3] RAC: ractopamine.
[4] T1: 35th day of the feeding trial.
[5] R0: control, 0 ppm RAC.

Short-term treatment causes improvements in weight gain more positive than studies of longer duration. These findings might indicate that longer exposure to β-agonists may induce desensitization and thereby minimize any apparent advantages in average daily gain (ADG) (Abney, 2006).

The modifications in heart weight and length observed in RAC-treated animals may be attributed to long-term stimulation of the adrenergic system. In rodents and other mammals, sustained activation of the β-adrenergic system by chronic administration of β-adrenergic agonists is a typical and well-characterised model of left ventricular hypertrophy (Mikusova et al., 2009; Osadchii et al., 2007). In our study, however, the cardiac morphological changes were dose-independent. Echocardiographic evaluation showed an increase in left ventricular mass (LVM) in groups R10 and R20 at T1 but not in group R40. Although our data suggest that low-dose RAC treatment can induce eccentric hypertrophy, variations in heart weight and dimension cannot be specifically ascribed to RAC treatment or considered as a marker of illegal treatment with the drug. The dose-independent effect on heart weight and length could be partially due to a phenomenon of desensitization or down-regulation of cardiac beta-adrenoceptors. The down regulation of cardiac beta-adrenoceptors has in fact already been observed in other species such as cattle (Badino et al., 2008; Odore et al., 2007).

Despite the fact that no statistically significant differences were observed in blood chemistry values among the four groups, all treated animals showed a certain degree of eosinophilic cell infiltration in the lymphoid organs. The increase in eosinophil circulating number and/or in tissue eosinophils, is associated with a wide variety of parasitic infestation, allergic responses, neoplasms, connective tissue disorders, medications and endocrinopathies (Bochsler and Slauson, 2002; Gotlib, 2005). In our study, the animals showed no clinical signs of hypersensitivity reaction and all had received antiparasitic drugs before the beginning of RAC administration. It is known that, *in vitro,* treatment with β2-agonists causes a dose-related delay in eosinophil apoptosis, whereas β-blockers reduce their number (Kankaanranta et al., 2000). On the basis of our results, the presence of eosinophil infiltrates in lymphoid organs should be carefully evaluated in cases of suspected illegal treatment with RAC, even if the role of RAC in the pathogenesis of eosinophilia should be further investigated. Macroscopically, the thyroid lesions partially confirm those described in literature (Ungemach, 2005). The thyroid glands weight of the animals treated with 20 and 40 ppm of RAC were lower as compared with the controls and 10 ppm dose, suggesting that high RAC doses decrease thyroid weight. Histologically, no lesions were observed. It has been noted that sympathetic activity in thyroids contributes to gland enlargement and may modulate tissue responsiveness to thyroid hormones (Young et al., 2005). However, whether the sympathetic effects involve changes in blood flow or in thyroid cell number or function is presently unknown.

The effects of RAC treatment on blood thyroid hormone levels have never been investigated in pigs so far. Clenbuterol administration reduces thyroid hormones in sheep and rats (Cardoso and Stock, 1998; Cardoso and Taveira, 2002) but not in broilers (Buyse et al., 1991). Our data suggest that RAC treatment at 20 and 40 ppm induces a decrease in circulating T_3 levels. It has been postulated that specific interactions between the adrenergic system and thyroid function take place (Silva and Bianco, 2008). The two systems act synergistically to allow the body to adapt to different environmental stimuli. Specifically, thyroid hormones increase the cell's capacity to respond to most catecholamine actions. On the other hand, catecholamines released by the sympathetic nerves can stimulate in a tissue-specific manner the conversion of T_4 into the more active T_3. Recently, it has been shown that thyroid hormone homeostasis regulation is mediated by the activation of both α- and β-adrenoceptors and involves the modulation of deiodinase type II activity, the enzyme responsible for the conversion of T_4 into T_3 (Kundu et al., 2009). These observations seem to disagree with the reduced T_3 concentrations found in the RAC-treated animals in our study. However, because α-adrenergic agonists inhibit the TSH-stimulated release of thyroid hormones and because of a predominance of $α_2$-adrenoceptors in porcine thyroid, the reduction in circulating T_3 levels could be due to stimulation of α-adrenoceptors (Murakietal., 1982,1984). At repartitioning dosages, β-agonist affinity for β-AR subtypes could be partially reduced. Nevertheless, due to the individual variability in hormone concentrations, further studies are needed to confirm this hypothesis.

The urethral epithelium of the RAC-treated animals showed hyperplasia, sometimes associated with metaplasia. Cytoplas-matic vacuolization of urethral cells in some tissue samples was also detected. Hyperplasia of the prostatic epithelium was less frequent and mild.

To our knowledge, there are no published data on the effects of RAC treatment on the reproductive system in swine. In cattle, reproductive system modifications have been described following treatment with steroid hormones (Biolatti et al., 2003). Moreover, prolonged clenbuterol exposure has been observed to induce pathological modifications of the female reproductive system, increased oestrogen and progesterone receptor concentrations, and a marked decrease in β-adrenoceptors in the heart, bronchial tubes and brain of veal calves (Odore et al., 2007; Re et al., 1995, 1997). By contrast, Schiavone et al. (2004) found a down-regulation of androgen receptors in the male reproductive system of broilers treated with repartitioning doses of clenbuterol. The hypothesis that the modifications induced by steroid hormone receptor concentrations could be ascribed to cross-talk between the steroid and the cate-cholaminergic systems is supported by the observation that the administration of propranolol, a β-adrenergic blocker, inhibits the up-regulation of estrogen and progesterone receptors induced by clenbuterol (Re et al., 1993).

5. Conclusion

The results of this study suggest that the most significant changes associated with the use of repartitioning doses of RAC in pigs involve the endocrine glands and the male lower urinary tract. The lesions observed in prostate and urethral epithelium can be considered as adaptative reversible lesions induced by RAC treatment. No pathological features are reported in the thyroid despite the decrease in thyroid hormone concentrations. The role of RAC in the determinism of eosinophilic infiltration in lymphoid organs and in the ovary of gilts remains to be clarified. Additional research on a larger group of pigs is needed to define the biomolecular mechanisms underlying the observed lesions.

Conflict of interest statement

None of the authors has any personal or financial relationship that could inappropriately influence or bias the content of the paper.

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