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Effects and detection of Nandrosol and ractopamine administration in veal calves

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ABSTRACT

The present study describes different effects of the selective androgen receptor modulator (SARM) nandrolone phenylpropionate (Nandrosol) and the β-agonist ractopamine administration in veal calves, and it investigates different strategies applied to trace these molecules.

Morphological changes of gonads and accessory glands attributed to androgen effects, such as testicular atrophy, seminiferous tubule diameter reduction and hyperplasia of prostate epithelium, were detected, although SARMs are not described to cause these lesions. The gene expression analysis showed an anabolic activity of Nandrosol in *Longissimus dorsi* muscle, where myosin heavy chain (MYH) was significantly up-regulated. An IGF1 increase was weakly significant only in *Vastus lateralis* muscle.

In conclusion, the anatomo-histopathological observations and the MYH mRNA up-regulation in *Longissimus dorsi* muscle confirm the androgenic treatment in experimental animals. The biosensor assay was not enough sensitive to detect residues in urines and only the direct chemical analysis of urine samples confirmed both β-agonist and SARM treatment.

1. Introduction

The large production demand of the beef industry promotes, in many cases, the use of illicit pharmacologically active substances, to improve animal performance and increase profits. Natural or synthetic growth promoters are widespread in this field, particularly β-agonists, glucocorticoids, and sex hormones. These molecules are used, alone or in "smart" combinations at very low individual doses, so that they can be rapidly metabolized by animals.

The presence of growth promoters in animals earmarked for human consumption is verified by chemical analyses of animal urine and blood. These methods are referred to as official methods and are used to detect specific chemical residues in various samples of animal origin.

The scientific community promotes the use of new approaches of investigation, including direct and indirect methods of analysis (Nebbia et al., 2011). Recently, the Italian National Residues Monitoring Plan included histological test as a screening method to detect target organ alterations induced by administration of growth promoters (Biolatti et al., 2003; Imbimbo et al., 2012). Furthermore, new biological tests, based on alterations induced by growth promoters on transcriptomics or proteomics, were developed for specific target tissues or biological fluids.

Groot and Biolatti (2004) histologically investigated the prostate, bulbo-urethral gland and testes of veal calves which were found to be positive for 17β-boldenone residues in the urine. Their findings showed hypersecretion and cyst formation in the prostate and bulbo-urethral gland. In the testes, reduced development and degeneration of the germinal epithelium were also observed.

Androgens exert anabolic effects in skeletal muscle (Antonio, Wilson, & George, 1999). In particular, testosterone administration can increase satellite cell number in both humans (Sinha-Hikim, Roth, Lee, & Bhasin, 2003) and rodents (Joubert & Tobin, 1995). These treatments are sometimes associated with severe androgenic side effects. In human medicine, in order to avoid treatment induced alterations, the employ of selective androgen receptor modulators (SARMs) is a promising alternative to natural or synthetic pure androgen hormone administration (Yarrow, McCoy, & Borst, 2010).

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SARMs are molecules with anabolic effects similar to steroids like testosterone, but they lack a lot of the negative side effects. Nandrolone, a direct metabolite of nandrolone phenylpropionate, also called Nandrosol (NA), is a SARM, (Bhasin & Jasuja, 2009).

Also β-agonists have anabolic effects in skeletal muscle (Stoffel & Meyer, 1993). β-Agonists act as 're-partitioning agent' that increases muscle protein deposition by inhibition of proteolysis and enhanced lipolysis. Ractopamine (RA) administration increases muscle mass with minimal effects on adipose tissue (Mersmann, 1998). Gonzalez, Carter, Johnson, Oullette, and Johnson (2007) noted that although there was an increase in skeletal muscle mass, the DNA content of skeletal muscle fiber was not changed following β-adrenergic agonist administration. This suggests that the increase in muscle mass that is characteristic of β-adrenergic agonist administration is due to changes in protein synthesis and degradation rather than to satellite cell proliferation and incorporation into existing muscle fibers.

Satellite cells, which are quiescent myoblasts, can be regulated by a variety of alterations to the surrounding environment in the muscle, including mechanical stimulation, growth factors, and hormonal signaling (Bischoff, 1990). Androgen actions in skeletal muscle are mediated by the cytosolic androgen receptor (AR), which translocates to the nucleus, where it regulates gene transcription. Satellite cell activation is tightly regulated by the helix-loop helix myogenic regulatory factor (MRF) family of DNA binding proteins. This family includes myogenic differentiation 1 (MYOD), myogenic factor 5 (MYF5), myogenin (MYOG), and myogenic regulatory factor 4 (MRF4) (Zanou & Gailly, 2013).

MRFs control the transcription of important muscle-specific proteins, such as myosin heavy chain (MYH) and muscle creatine kinase. Different growth factors, including insulin-like growth factors (IGFs), are secreted during muscle regeneration and hypertrophy. MRFs could be induced in response to IGF stimulation, and, inversely, IGF expression may also be regulated by MRFs (Zanou & Gailly, 2013). Hence a transcriptomic analysis in target tissues could help to detect an illicit administration of growth promoters.

Therefore, screening methods to detect these compounds are required to ensure food safety: in particular it is necessary to develop indirect methods, such as histological analysis of target organs and/or transcriptomic analysis in target tissues, which could help to detect an illicit administration of growth promoters.

Another approach to detect illicit drug treatment in cattle consists of direct methods of analysis: in this work, a screening test based on a biosensor was applied for the assessment of androgen molecules. Biosensors represent a direct screening method and have already been used by several laboratories. The biosensor used for this aim is derived from genetically modified yeast (Bovee et al., 2009). The assay consists of a recombinant yeast strain that stably expresses the human AR and a yeast enhanced green fluorescent protein (yEGFP) as a reporter protein of AR activation.

The first step in the research for new screening methods for the detection of illegally treated animals should be to detect significant biological effects induced by the treatment. In this context, aims of the work were the evaluation of the effects of a SARM, NA and a β-agonist, RA, on morphology of target organs and the study of gene regulation of selected genes in skeletal muscle. On the basis of the obtained results, a biosensor assay was then compared with the official LC-MS/MS approach regarding the detection of drugs' metabolites in urine during the treatment.

2. Materials and methods

2.1. Experimental design

Fifteen male six-month-old Friesian veal calves were randomly divided into the following two groups: the control group (n = 7), named group C, received a placebo, the treated group T(n = 8) received four doses of NA (150 mg/animal, *im*) every 15 days for two months and RA (80 mg/day/animal, *per os*) for the last 31 days (Supplementary material 1). NA and RA for calf treatment were obtained from AST Farma B.V. (Oudewater, NL) and Unibrom Corp. (Weifang, China), respectively. Animals were sacrificed three days after the last treatment. All groups of experimental animals were kept in separate 10 m × 10 m boxes, tethered, and fed with liquid milk replacer twice a day (dry matter 95% wet weight basis, crude protein 23%, ether extract 21%, ash 6%, cellulose 0.1%; vit. A 25.000 IU/kg, vit. C 50 mg/kg, Cu 5 mg/kg, vit. D3 5.000 IU/kg, vit. E 80 mg/kg).

Target tissue samples were collected at the slaughterhouse and preserved in 10% neutral buffered formalin or Bouin's fluid for subsequent histological preparations or in RNA later (Sigma-Aldrich, St. Louis, MO, USA) for molecular analyses. The testes, thyroid and heart of each animal were weighed and relative weight was calculated as organ (g)/total animal weight (kg).

Urine samples were collected before NA treatment (t0) and at eleventh day after the third (t1) and fourth (t2) injections. Urine samples were also collected at slaughterhouse from the bladder (t3) (Supplementary material 1). This timing was elicited to simulate the condition of an illicit treatment and a random sampling conducted by the sanitary officers. Samples were analyzed by Yeast Androgen Bioassay (RAA), and the results were confirmed by LC-MS/MS. All samples were stored at −20 °C until analysis.

This study was approved by the Italian Ministry of Health and the Ethics Committee of the University of Turin. The carcasses of treated animals were disposed according to proper protocols.

All experiments were carried out according to European Economic Community (EEC) Council Directive 86/609 and successive modifications (Directive 2010/63/EU) as recognized and adopted by the Italian Government.

2.2. Tissue sampling and processing

Prostate, bulbo-urethral glands and testes were collected from each animal. Sex accessory glands samples were fixed in 10% neutral buffered formalin at room temperature, whereas testis samples were fixed in Bouin's fluid. All samples were processed and paraffin embedded according to routine histological procedures. Representative sections of each sample were stained with hematoxylin–eosin (HE).

Samples of the *Longissimus dorsi* (LD), *Vastus lateralis* (VL), and *Biceps brachii* (BB) muscles were collected from each animal after slaughter. Sections weighing 150–200 mg were immediately frozen in liquid nitrogen and kept at −80 °C for molecular studies.

2.3. Morphometric analysis

Morphometric analyses on testis samples were performed on HE stained sections, and digital images were obtained with a Nikon DS-Fi1 color digital camera (Nikon Instruments). The testes were imaged by light microscopy at 200× magnification, and at least 40 randomly selected complete tubules per animal were examined using Image-Pro-Plus software (Media Cybernetics). Seminiferous tubular equivalent diameters (STED) and mean tubular areas (MTA) were evaluated. The STED (μm) of each seminiferous tubule was calculated as $\frac{4\times area}{perimeter}$.

2.4. Total RNA extraction and relative quantification of MYH, MRFs, and IGF1 gene expression by qPCR

Total RNA from each muscle sample was extracted using TRI Reagent (Sigma-Aldrich,) according to the manufacturer's protocol.

RNA quantity was determined by UV–visible spectrophotometry, and the RNA integrity was verified by automated gel electrophoresis (Experion Instrument, BioRad, Hercules, CA, USA). cDNA was synthesized from 1 μg of total RNA according to the manufacturer's instructions using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), which included a DNase digestion.

To determine the relative amounts of specific transcripts, the cDNA was subjected to qPCR using the IQ5 detection system (BioRad) and the IQ SYBR Green Supermix (BioRad). Primer sequences for MYH and IGF1 genes were designed using Primer3-web (vers. 4.0.0) (Koressaar & Remm, 2007; Untergrasser et al., 2012) (Table 2), and the MRF primers were designed as described by Shibata et al. (2006). In LD and VL muscle samples, the cyclophilin A (PPIA) gene was used as a housekeeping gene (De Maria et al., 2010). For the BB muscles, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene.

The levels of gene expression were calculated using a relative quantification assay based on the comparative C_q method ($\Delta\Delta C_q$ method) after verifying similar efficiencies of both the target and housekeeping gene amplifications. Then, the relative abundance of each transcript, normalized to the endogenous housekeeping gene and relative to the control sample, was recorded as $2^{-\Delta\Delta Cq}$ (fold increase).

2.5. Steroid extraction from calf urine samples

Ten-milliliter calf urine aliquots from experimental groups C and T were adjusted to pH 4.8 before addition of 20 μL of β-glucuronidase/arylsulfatase from *Helix pomatia* (3 U/mL) (Roche Diagnostics GmbH, Boehringer Manheim, Germany). Enzymatic deconjugation was carried out overnight in a water bath at 37 °C. Successive samples were subjected to solid phase extraction (SPE) on 1000 mg C18 and 500 mg NH2 columns (Supelco, Sigma-Aldrich, St. Louis, MO, USA) using methods previously described by Bovee et al. (2009).

2.6. Yeast androgen bioassay (RAA)

Saccharomyces cerevisae transformants expressing the AR were grown on selective minimal medium plates supplemented with l-leucine. Supplemented minimal medium (MM/L) consisted of yeast nitrogen base without ammonium sulphate or amino acids (1.7 g/L), dextrose (20 g/L), ammonium sulphate (5 g/L) and supplementation with L-leucine (6 mg/L) (Sigma-Aldrich, St. Louis, MO, USA).

The RAA was performed as described previously (Bovee et al., 2009). In short, 10 mL MM/L was inoculated with a single colony of the recombinant yeast and grown overnight at 30 °C in an orbital shaking incubator at 125 rpm.

For exposure of the yeast to sample extracts, 200-μL aliquots of the yeast cultures were pipetted into each well of a 96-well plate already containing the dried extracts as described by Bovee et al. (2009). For exposure to the standard compounds such as 17β-testosterone and the NA metabolites α- and β-nandrolone (α-ND and β-ND), 200-μL aliquots of the yeast cultures were pipetted into each well of a 96-well plate and 2 μL from the standard stock solutions dissolved in DMSO were added (final concentration about 4 to 1000 ppb).

The yeast and samples were incubated together for 24 h and the yeast fluorescence was measured directly in a Victor 31420 Multilabel Counter (PerkinElmer, Waltham, MA, USA) using excitation at 485 nm and emission measurement at 530 nm. Differences in the fluorescence emission at 24 and 0 h of yeast exposure to the samples were calculated and corrected according the blank control values, thus providing the final androgenic activity data for each sample. We defined the mean signal of 20 blank samples plus three times the corresponding standard deviation as the decision limit CCα (α = 1%) (EC Decision 2002/657). Urine samples that had fluorescence values greater than those of the CCα contained androgenic molecules.

Table 1
Relative weight of testes, thyroid and heart of group C and T animals. Data are represented as mean ± SEM.

	Relative weight (g/kg animal)	
	C	T
Testes	0.54 ± 0.03	0.26 ± 0.02***
Thyroid	0.10 ± 0.01	0.13 ± 0.01*
Heart	5.02 ± 0.17	4.93 ± 0.20

* $p < 0.05$;
*** $p < 0.001$.

2.7. LC-MS/MS

Analyses of α-ND, β-ND, and RA were carried out using a Thermo Finnigan HPLC system (Thermo Fisher, San Josè, CA, USA) with Surveyor pump equipped with degasser and a Surveyor AS autosampler equipped with a column oven and a Rheodine valve. The mass spectrometer used was a Thermo Finnigan TSQ Quantum triple quadrupole that utilizes an electrospray ionization source (ESI) as the interface (Thermo Fisher, San Josè, CA). Data were analyzed using Xcalibur software (Thermo Fischer). All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid 98–100% was obtained from Riedel-de Haën (Sigma-Aldrich, St. Louis, MO, USA). α-ND and β-ND and their internal standard 17β-nandrolone-d3 were purchased from LGC Standards (Teddington, UK), and RA and its corresponding internal standard isoxsuprine were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.7.1. α-ND and β-ND determination by LC-MS/MS

A 1-mL aliquot of filtered urine was subjected to enzymatic hydrolysis by β-glucuronidase from *E.coli* K12 (EC 3.2.1.31) (Roche Diagnostics GmbH, BoehringerManheim, Germany). The internal standard was β-nandrolone-d3 at a concentration of 2 ppb. After purification on an Oasis HLB cartridge (3 mL, 60 mg, Waters) the methanol elute was evaporated, and the dry residue was dissolved in a mobile phase mixture prior to LC-MS/MS analysis. The mobile phase consisted of water with 0.1% formic acid and methanol at a flow rate of 250 μL/min. The appropriate gradient allowed separation of α-ND and β-ND on a reverse-phase HPLC column (Synergi Hydro RP 150 × 2.0 mm, i.d. 4μ 80 A). The mass spectrometer was operated in the positive ESI mode with the following acquisition parameters: capillary voltage set at 3000 V, ion transfer capillary temperature set a 340 °C and sheath and auxiliary (nitrogen) gases were fixed at 30 and 10 arbitrary units, respectively. The collision gas was argon at 1.5 mTorr, and the peak resolution of 0.70 Da FWHM (full width at half maximum) was used on Q1 and Q3. The signal acquisition was performed by multiple reaction-monitoring mode (MRM) with corresponding transitions for which the optimum tube lens and collision energies were established. α-ND and β-ND had the same fragmentation patterns. The precursor ion had $m/z = 275$ as $[M+H]^{+\cdot}$, which gave ion $m/z = 109$ as quantifier, whereas ions $m/z = 145$, 199 and 239 were used for conformation purposes. The deuterated β-ND gave the analog product ion arrangement with the quantification transition from parent pseudo-molecular ion ($m/z = 278$) to most abundant product ion ($m/z = 109$).

Table 2
Primer sequences for qPCR.

Gene (NCBI's RefSeq)	Forward primer (5′−3′)	Reverse primer (5′-3′)	Amplicon size (bp)
MYH (NM_174117)	ATCTGGTGAAGCAGAGGGCG	GGTTGGTCATCAGCTTATTCAGG	110
IGF1 (NM_001077828)	TGCGGGGCTGAGTTGGT	CCGTGGGCTTGTTGAAATAAA	73
MYOD (NM_001040478)	CGACTCGGACGCTTCCAGT	GATGCTGGACAGGCAGTCGA	180
MYF5 (NM_174116)	ACCAGCCCCACCTCAAGTTG	GCAATCCAAGCTGGATAAGGAG	150
MYOG (NM_001111325)	GTGCCCAGTGAATGCAGCTC	GTCTGTAGGGTCCGCTGGGA	110
MRF4 (NM_181811)	GGTGGACCCCTTCAGCTACAG	TGCTTGTCCCTCCTTCCTTGG	140
PPIA (NM_178320)	GCCCCAACACAAATGGTT	CCCTCTTTCACCTTGCCAAAG	95
GAPDH (NM_001034034)	ACACCCTCAAGATTGTCAGCAA	TCATAAGTCCCTCCACGATGC	102

2.7.2. RA determination by LC-MS/MS

Deconjugation of RA II phase metabolites was performed by adding β-glucuronidase/sulfatase from *Helix Pomatia* to 2 mL of urine. The internal standard was an RA structural isomer isoxsuprine at a final concentration of 2 ppb. After pH adjustment (8.5–9.5), the sample was treated with 5 mL of the tert-butyl methyl ether and ethyl acetate mixture (4:1 v/ v). Once shaking and centrifugation were completed, the organic phase was evaporated with subsequent resuspension of dry sample in the initial mobile phase (acetonitrile and 0.1% formic acid in aqueous solution, 10:90 v/v). The mass spectrometer was operated in the positive ESI mode. Reversed-phase LC was performed using the Synergi Hydro RP (150 × 2.0 mm, i.d. 4μ 80 A) with adequate mobile phase gradient. Acquisition parameters such as capillary potential and temperature were set at 4200 V and 360 °C, respectively. Nitrogen as sheath and auxiliary gas was set at 40 and 6 arbitrary units, respectively, while the pressure of argon as collision gas was 1.5 mTorr. Peak resolution of 0.70 Da FWHM was used on Q1 and Q3. RA and isoxsuprine identification and quantification were achieved using MRM for most specific transitions. The pseudo-molecular ion (m/z = 302) was the common parent species for both compounds, which were chromatographically separated. The RA parent ion produced m/z = 164 as a quantifier and m/z = 107, 121 and 284 as diagnostic ions, whereas isoxsuprine produced the following characteristic ions: m/z = 150 (quantifier), 105, 107 and 284.

2.8. Statistical analyses

All statistical analyses were performed using GraphPad Prism 4 (vers. 4.03) software (GraphPad Inc., San Diego, CA, USA). The STED and gene expression of target genes (ΔCq) were analyzed by unpaired *t*-test, comparing treatment group (T) against the control group (C). Normal distribution was tested by Kolmorov-Smirnov test. Grubbs' test was used to determine and exclude potential outliers.

Data are presented as the average ± SEM. A $p < 0.05$ was considered significant.

3. Results

3.1. Histopathology and morphological analysis

Macroscopic examination of the carcass did not reveal any lesions potentially associated with treatment; the accessory sex glands showed no differences between the two groups. Administration of NA and RA to veal calves induced a significant ($p < 0.001$) reduction in testis weight (Table 1). A statistically significant increase in thyroid weight of group T was observed ($p < 0.05$) whereas the heart did not present any significant relative weight change.

In testis, histological evaluation showed an apparent reduction in the epithelial germ line thickness, along with reduced nuclei volume (Fig. 1b) in the treated animals. Moreover, the reductions in STED and MTA were significant ($p < 0.001$) (Supplementary material 2a and 2b) in group T. At the histological level, the prostate and bulbo-urethral glands of the treated animals showed mild epithelial hyperplasia associated with moderate hypersecretion and cystic dilatation of the ducts (Fig. 1d, f). A gland maturation delay was evident in particular in the bulbo-urethral of the treated group. The prostate urothelium presented a moderate hyperplasia in group T.

3.2. Effects of NA and RA administration on the relative expression of MRFs, MYH, and IGF1 in skeletal muscle tissue

NA and RA administration induced distinct MRF gene regulation in different skeletal muscle types. In particular, VL showed an up-regulation of MRFs involved in skeletal muscle differentiation; in fact MRF4, MYOD and MYOG gene expression levels were increased by about 2–2.5-fold ($p < 0.01$) in group T. In BB muscle, androgen treatment induced the over expression of MYOD by 1.7-fold but did not elicit any MRF expression changes in LD. On the contrary, MYH expression in LD was significantly up-regulated by 6-fold (Table 3).

In VL, IGF1 gene expression was increased approximately 2-fold compared with that of control group C.

3.3. RAA

Fig. 2 shows the dose–response curve obtained by RAA after 24-h exposure to testosterone and α-ND and β-ND. All the results are reported as the means of triplicate measurements. After 24 h of yeast exposure to different concentrations of the standard molecules, no differences in yeast growth could be detected indicating that no toxic effects on the yeast were observed. The data shows that testosterone and β-ND demonstrate full dose-response curves with similar sensitivities to those reported in the literature (Bovee et al., 2009). α-ND was less active as expected to since changing the OH-group at position 17 from 17β to 17α strongly decreases the potency of the androgen (Bovee et al., 2009).

A decision limit, CCα, was calculated from the corrected fluorescence signals of the 20 blank urine samples. The mean value of these blank urine samples was 1908, and the standard deviation was 2058; therefore, the CCα was 8083. Samples giving a signal lower than CCα were classified as compliant or negative. Samples giving a signal higher than the decision limit CCα were classified as suspected of containing androgen molecules.

Supplementary material 3 represents the group T urine samples that gave signals below the CCα and were thus classified as negative.

3.4. LC-MS/MS

Urine samples from groups C and T were screened in LC-MS/MS for α-ND, β-ND and RA. Group C samples were negative for α-ND

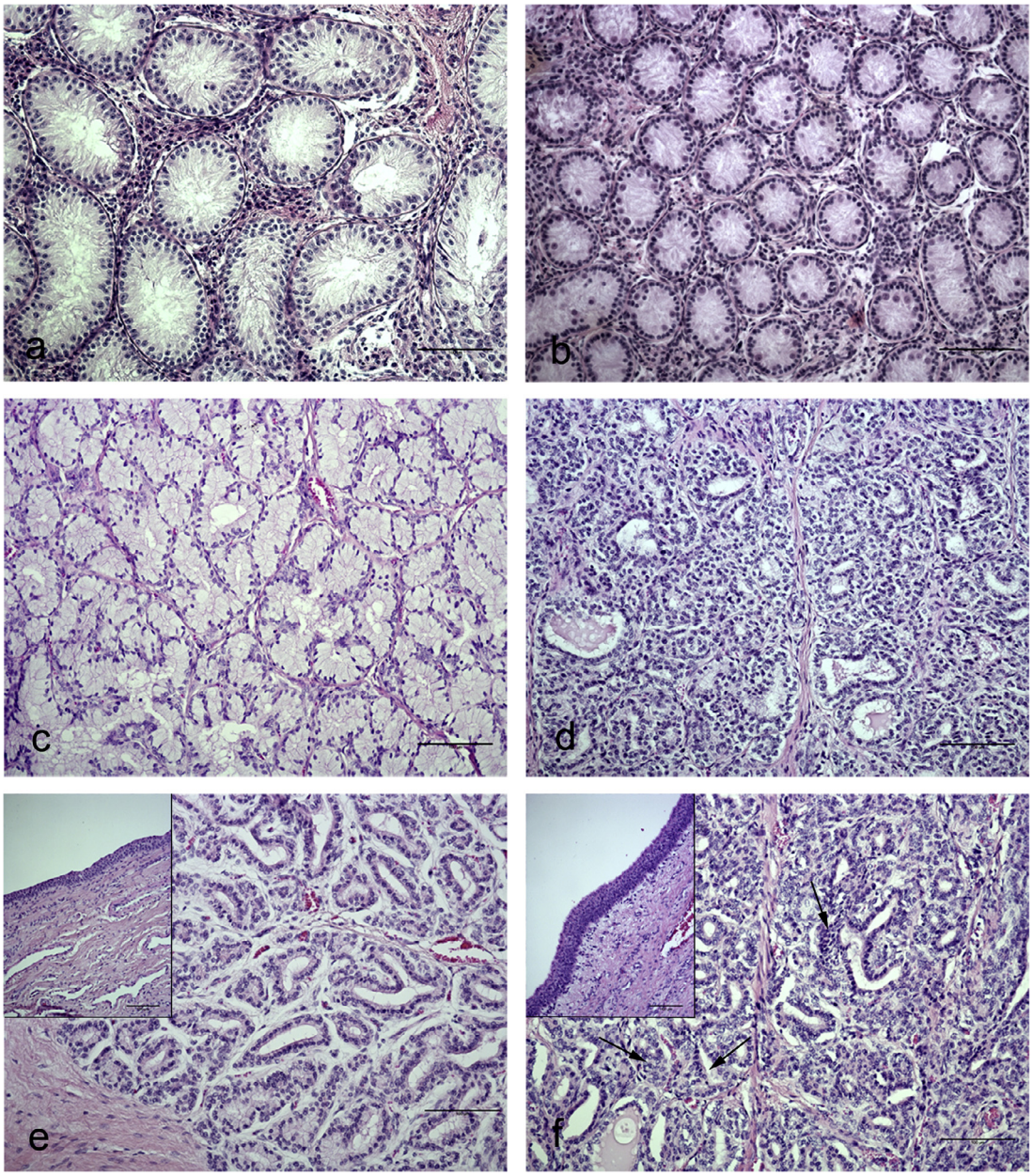

Fig. 1. Testis of a) control animal and b) treated animal. Note the significant reductions in seminiferous tubular diameters and areas. Histological evaluation showed an apparent reduction in the epithelial germ line thickness, along with reduced nuclei volume (b) in the treated animals. c and e represent the bulbo-urethral gland and prostate of control animals, respectively. The bulbo-urethral gland and prostate of treated animals (d and f respectively), showed mild epithelial hyperplasia (arrows) associated with moderate hypersecretion and cystic dilatation of ducts. A gland maturation delay was evident in particular in the bulbo-urethral glands of the treated group (d). The prostate urothelium (inserts) presented a moderate hyperplasia in group T (insert in f). (HE, × 200 magnification; bars: 100 μm).

Table 3
MRFs, MYH, and IGF1 gene expression in different skeletal muscle types of group T animals.

Gene	Normalized fold increase ($2^{-\Delta\Delta Cq}$)[†]		
	BB	VL	LD
MYF5	1.21 ± 0.36	1.48 ± 0.26	0.79 ± 0.08
MYOD	1.77 ± 0.29*	1.91 ± 0.22**	1.31 ± 0.22
MYOG	1.99 ± 0.61	2.29 ± 0.25**	1.44 ± 0.17
MRF4	1.19 ± 0.22	2.01 ± 0.30**	1.27 ± 0.12
MYH	0.98 ± 0.07	1.43 ± 0.39	6.02 ± 0.46**
IGF1	0.75 ± 0.14	2.42 ± 0.10**	1.09 ± 0.08

[†] The results are presented as the means ± SEM of fold gene expression changes ($2^{-\Delta\Delta Cq}$) versus control group C. A value of 1 was assigned to mean of samples from control group C. (*$p < 0.05$; **$p < 0.01$).

and β-ND as well as for RA (data not shown). Table 4 shows the results for group T urine samples. The samples were analyzed to identify free nandrolone molecules and after hydrolysis to identify total nandrolone molecules (free and conjugate). Throughout the NA treatment, the presence of α-ND, a principal metabolite of NA, was observed. In the same way, RA was detected in group T urine samples during the treatment (Table 4).

4. Discussion

The rapid kinetics of the hormones used in animal doping and the practice of applying treatments at low doses result in an underestimation of the problem of illicit growth promoter administration in husbandry, both in Italy and in the rest of Europe.

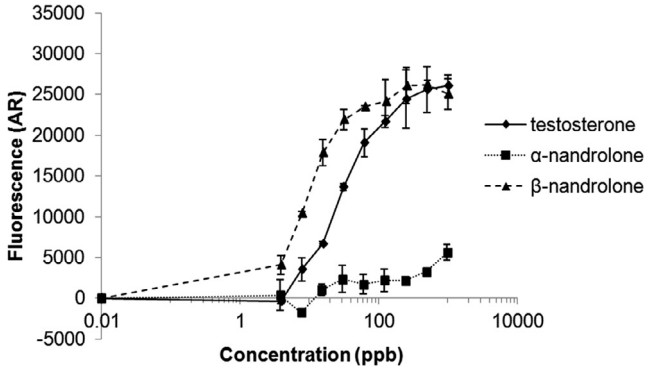

Fig. 2. RAA results after 24-h exposure to testosterone, α-ND and β-ND. Fluorescence signals are the means ± SEM of a triplicate experiment and are corrected for the signal of reagent blank.

Table 4
Mean ± SEM of total (free and conjugated) α-ND and RA levels measured in urine samples from group T during the treatment (t1, t2, t3).

	α-ND (ppb)	RA (ppb)
t1	9.23 ± 5.40	34.38 ± 9.89
t2	8.81 ± 3.93	819.57 ± 71.07
t3	8.98 ± 3.77	212.13 ± 51.77

Free α-ND and total and free β-ND were not present during the treatment. Before treatment (t0) all animals resulted negative (<0.5 ppb).

Therefore, to support official methods of investigation, the scientific community promotes the use of new approaches, including direct and indirect methods of analysis (Nebbia et al., 2011). Among these, screening tests are targeted to detect direct or indirect parameters linked to growth promoter abuse. Laboratories use these methods as a screening test during sample analysis. These methods may allow the resolution of the investigation if the results are negative, but they cannot be exhaustive for positive or uncertain outcomes. In these cases, further investigations (confirmation methods) are required. Therefore, screening methods cannot replace official analysis methods (Nebbia et al., 2011), but they allow a preliminary screening of thousands of samples, thus increasing the effectiveness of the official controls. Screening methods are characterized by high productivity and low cost per analyzed unit.

In this study, a combination of a SARM (NA) with a β-agonist (RA) was investigated with respect to their detection with screening and confirmation methods in veal calves.

An important step is to define the biological processes governed by these hormones and the cell type(s) in which they exert their anabolic effects.

The dosages and combinations used in this experiment were based on the partial knowledge of the application in the field and the results of previous studies (Groot, Schilt, Ossenkoppele, Berende, & Haasnoot, 1998).

The testes showed reduced development according to what could be expected for the age of the animals, as suggested by reductions in testis weight and in the tubular seminiferous dimensions respect to those of the control group.

Thyroid and heart weight reduction was detected in swine following RA administration (Catalano et al., 2012). On the contrary, in the present experiment, the heart relative weight of veal calves did not undergo any change whereas the thyroid weight increased. This finding could be attributable to the NA and RA combination and to the species difference. The prostate and bulbo-urethral gland showed mild hyperplasia, fibrosis and hypersecretion, along with a delay in gland maturation most likely due to NA administration. These androgenic effects were smaller than those observed following androgen hormones administration, confirming the SARM action of NA. In fact, Cannizzo, Zancanaro, Spada, Mulasso, and Biolatti (2007) reported a significant hypersecretion and reduced testicular development as consequences of androgen administration.

In previous experiments with anabolic steroids like testosterone and estrogen, reduced testicular development and increased stromal proliferation were observed (Groot & Arts, 1991). Similar findings were reported in lamb and calf testes from animals implanted with estradiol and trenbolone (Rodriguez Barbudo, Mendez Sanchez, & Blanco, 1991).

In addition, the prostates of treated veal calves showed vacuolar degeneration that could be ascribed to β-agonist RA; in the same way RA could be responsible of urethral epithelium hyperplasia (Catalano et al., 2012; Groot et al., 1998). However, these effects are not specific of β-agonist treatment, since also an estrogen administration may induce similar lesions (Biolatti et al., 2003; Imbimbo et al., 2012).

RA is a β-adrenergic agonist that can be defined as a repartitioning agent that redirects and increases nutrient flow from fat deposition towards muscle deposition (Ricks, Dalrymple, Baker, & Ingle, 1984). β-Agonists, in both male and female cattle, influence functional and morphological aspects of the gonads and genital tract, directly or via the hypothalamo-pituitary axis. In vivo, β-adrenergic agonists may induce secondary events caused by the hormonal or physiological responses of several tissues, especially involving the endocrine and cardio-respiratory systems (Groot et al., 1998). Skeletal muscle mass increases during postnatal development through a process of hypertrophy, i.e., enlargement of individual muscle fibers, and a similar process may be induced in adult skeletal muscle in response to contractile activity, such as strength exercise, and by specific hormones, such as androgens and β-adrenergic agonists.

To confirm the anabolic effects of NA and RA at the molecular level, mRNA expression in three different types of skeletal muscle was analyzed. Androgen action in skeletal muscle is mediated through binding to cytosolic AR and translocation to the nucleus, where this complex can regulate gene transcription. Androgens can regulate MRFs and other regulatory factor genes are transcriptional target genes of androgens (Lu, Jenster, & Epner, 2000).

Recently, Diel et al. (2008) described the up-regulation of myostatin and AR genes in the *Gastrocnemius* muscle of rats treated with 19-norandrostenedione and testosterone propionate. They also demonstrated local modulation of the mRNA expression of distinct growth factors like IGF-1.

In the present study, to determine the myogenic effects of NA and RA, the gene expression of MRFs and MYH was analyzed. MRF-like MYOD is a key mediator of initial myogenesis, whereas MYH is a marker of mature fibers (Sellers, 2000; Tapscott & Weintraub, 1991). Up-regulation of MRFs involved in myogenic differentiation was detected in VL and, in part, in BB muscles; this response could be important for the increase in muscle mass and protein content typical of androgen effects on cattle skeletal muscle. Zhao, Hu, Zhu, and Du (2011), described an *in vitro* experiment wherein trenbolone promoted myogenesis in cultured bovine cells. This effect was at least partially mediated by the AR and trenbolone increased the AR expression at both the protein and mRNA levels.

LD muscle reacted differently to NA and RA; no MRF gene regulation was observed but an important up-regulation of the MYH gene was evident in the present experiment.

Some studies report changes in the local expression of IGF1 in human muscle samples from patients receiving anabolic androgens (Sheffield-Moore, 2000). IGF1 stimulates satellite cell proliferation and promotes muscle hypertrophy (Musarò & Rosenthal,

1999); therefore, it is possible that androgens regulate muscle mass through these mechanisms.

Pampusch et al. (2008) studied trenbolone acetate and estradiol effect, administered together or alone by implant, in LD of steers. IGF1 mRNA was up-regulated in particular by estradiol during the treatment. In the same way, Walker et al. (2007) observed a IGF1 mRNA increase in LD after trenbolone acetate/estradiol implant of steers. However IGF1 mRNA decreased after addiction of RA.

RA and androgens had opposite effect on serum concentrations of IGF-I and mRNA expression of IGF-I in LD. In the present study this effect was confirmed in LD and BB; differently, IGF1 mRNA was up-regulated by 2.42-fold in VL.

This finding is probably due to different types of metabolism among the collected muscles. In fact, VL in cattle have an intermediate fiber composition, also called fast oxidative fibers, and therefore, the main pathway for ATP production is oxidative phosphorylation in which IGF1 is involved.

Androgens induce increase in muscle mass, and this increment is partly due to muscle fiber hypertrophy, reflected by an increase in myonuclear numbers and cross-sectional areas of both slow and fast type muscle fibers (Dubois, Laurent, Boonen, Vanderschueren, & Claessens, 2012).

In the present experiment, due to the combination of androgen and β-agonists administered, it was possible to observe alterations in muscle gene expression levels different from what has been reported in the literature.

In fact, clenbuterol in cattle induced a slow to fast transition of MYH isoforms (Polla et al., 2001); in swine, MYH genes are differentially regulated by RA, and the β-adrenergic agonist-induced repartitioning effect is, in part, mediated by changes in muscle fiber type-specific gene expression (Gunawan, Richert, Schinckel, Grant, & Gerrard, 2007).

To confirm the presence of NA residues and RA in urine samples from the animals during the experiment, a direct screening test such as RAA and an official analytical method (LC-MS/MS) were applied.

RAA is a yeast androgen bioassay (Bovee et al., 2009) that expresses yEGFP as measurable reporter protein in response to androgens.

NA is a synthetic ester of 19-nortestosterone that was developed to minimize undesirable androgenic side effects. NA is quickly metabolized by the liver and its direct metabolites (α- and β-ND) can be detected in urine.

In recent years, it was possible to directly identify esters of anabolic steroids only in hair samples (Groot et al., 2012). Because physiological steroids do not occur naturally in an ester form, detection of intact steroid esters in hair was considered as a prove of illegal administration of exogen steroids but actually, β-ND, which was considered exogenic, was discovered to occur naturally in some species (Scarth et al., 2009). Furthermore, α-and β-ND can occur naturally in the urine of injured male cattle (Glenn Kennedy et al., 2009).

All urine samples were analyzed for the presence of α- and β-ND.

RAA did not detect androgens in any urine samples; in particular, RAA was employed to test α- and β-ND presence, but it resulted not specific for α-ND. This characteristic did not allow the detection of α-ND metabolites in the urine of treated animals, testing positive in LC-MS/MS.

No NA metabolites were observed in the urine of control animals (data not shown); this allows us to conclude that veal calves from this experiment did not synthesize endogenous α- and β-ND androgens, and these metabolites were derived from NA administration. In particular, only α-ND was detected by chemical analysis and the previous metabolite, β-NA, was not observed in the urine samples from treated or control veal calves. This may be due to the sampling schedule, which was approximately 2 weeks after the day of treatment.

All urine samples were analyzed for the presence of RA by LC-MS/MS, and it was present in all the urine samples of group T during treatment. The RA values were lower at t1 and increased until slaughter.

5. Conclusions

In the present study several effects were observed in veal calves following SARM and β-agonist treatment.

In literature no specific histopathological lesions were described relative to sexual accessory glands caused by SARM molecules administration. Therefore, the results obtained from this study are totally new in particular in farm animals as calves. It was verified that NA has a mild androgen effect on veal calves testes and that treatment resulted in decreased size and weight of the gonads. The bulbo-urethral gland and the prostate showed weak histological alterations. Nevertheless, the treatment of veal calves with NA could be specifically detected by macroscopic and microscopic analysis. In contrast, RA administration in veal calves is very difficult to identify by histology or macroscopic alteration.

In parallel to anatomo-histopathological observations, it could be interesting to apply an indirect method of analysis based on different gene expression regulation.

The results showed that NA and RA influence skeletal muscle gene expression but the combination of these two molecules probably reduce the detectable effects, since there could be interplay among these molecules.

Additional *in vivo* and *in vitro* studies are necessary to better understand the mechanisms related to this complex process. At the moment, in particular for RA detection, it is necessary a direct chemical analysis to confirm its administration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.11.116.

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