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Effects and detection of Nandrosol and ractopamine administration in veal calves

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1 Effects and detection of Nandrosol and ractopamine administration in veal calves

2 Running title: Nandrosol and Ractopamine treatment of veal calves

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17

18 **Abstract**

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

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

29 In conclusion, the anatomo-histophatological observations and the MYH mRNA up-
30 regulation in *Longissimus dorsi* muscle confirm the androgenic treatment in experimental
31 animals. Unfortunately, the biosensor assay was not enough sensitive to detect residues in
32 urines and only the direct chemical analysis of urine samples confirmed both β-agonist and
33 SARM treatment.
34

35

36 **Keywords**: SARM, Nandrosol; ractopamine; veal calves; histopathology; gene expression;
37 bioassay; LC-MS/MS.

1. Introduction

The large production demands 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 rapid 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

63 (Sinha-Hikim, Roth, Lee, & Bhasin, 2003) and rodents (Joubert & Tobin, 1995). These
64 treatments are sometimes associated with severe androgenic side effects. In human
65 medicine, in order to avoid treatment induced alterations, the employ of selective androgen
66 receptor modulators (SARMs) is a promising alternative to natural or synthetic pure
67 androgen hormone administration (Yarrow, McCoy & Borst, 2010)
68 SARMs are molecules with anabolic effects similar to steroids like testosterone, but they
69 lack a lot of the negative side effects. Nandrolone, a direct metabolite of nandrolone
70 phenylpropionate, also called Nandrosol (NA), is a SARM, (Bhasin & Jasuja, 2009).
71 Also β-agonists have anabolic effects in skeletal muscle (Stoffel & Meyer, 1993). β-
72 agonists act as 're-partitioning agents' that increases muscle protein deposition by
73 inhibition of proteolysis and enhanced lipolysis. Ractopamine (RA) administration
74 increases muscle mass with minimal effects on adipose tissue (Mersmann, 1998).
75 Gonzalez, Carter, Johnson, Oullette, and Johnson, (2007) and O'Connor, Butler, Hogue, and
76 Beermann, (1991) noted that although there was an increase in skeletal muscle mass, the
77 DNA content of skeletal muscle fiber was not changed following β-adrenergic agonist
78 administration. This suggests that the increase in muscle mass that is characteristic of β-
79 adrenergic agonist administration is due to changes in protein synthesis and degradation
80 rather than to satellite cell proliferation and incorporation into existing muscle fibers.
81 Satellite cells, which are quiescent myoblasts, can be regulated by a variety of alterations
82 to the surrounding environment in the muscle, including mechanical, growth factor, and
83 hormonal signaling (Bischoff, 1990). Androgen actions in skeletal muscle are mediated by
84 the cytosolic androgen receptor (AR), which translocates to the nucleus, where it regulates
85 gene transcription. Satellite cell activation is tightly regulated by the helix-loop helix
86 myogenic regulatory factor (MRF) family of DNA binding proteins. This family includes
87 myogenic differentiation 1 (MYOD), myogenic factor 5 (MYF5), myogenin (MYOG), and
88 myogenic regulatory factor 4 (MRF4) (Zanou & Gailly, 2013).

89 MRFs control the transcription of important muscle-specific proteins, such as myosin
90 heavy chain (MYH) and muscle creatine kinase. Different growth factors, including insulin-
91 like growth factors (IGFs), are secreted during muscle regeneration and hypertrophy.
92 MRFs could be induced in response to IGF stimulation, and, inversely, IGF expression
93 may also be regulated by MRFs (Zanou & Gailly, 2013). Hence a transcriptomic analysis in
94 target tissues could help to detect an illicit administration of growth promoters.
95 Therefore, screening methods to detect these compounds are required to ensure food safety: in
96 particular it is necessary to develop indirect methods, such as histological analysis of target
97 organs and/or transcriptomic analysis in target tissues, which could help to detect an illicit
98 administration of growth promoters.
99 Another approach to detect illicit drug treatment in cattle consists of direct methods
100 of analysis: in this work, a screening test based on a biosensor was applied for the
101 assessment of androgen molecules. Biosensors represent a direct screening method and
102 have already been used by several laboratories. The biosensor used for this aim is derived
103 from genetically modified yeast (Bovee, Bor, Heskamp, Lasaroms, Sanders, & Nielen, 2009).
104 The assay consists of a recombinant yeast strain that stably expresses the human AR and
105 a yeast enhanced green fluorescent protein (yEGFP) as a reporter protein of AR
106 activation.
107 The first step in the research for new screening methods for the detection of illegally
108 treated animals should be to detect significant biological effects induced by the treatment.
109 In this context, aims of the work were the evaluation of the effects of a SARM, NA and a β-
110 agonist, RA, on morphology of target organs and the study of gene regulation of selected
111 genes in skeletal muscle. On the basis of the obtained results, a biosensor assay was then
112 compared with the official LC-MS/MS approach regarding the detection of drugs'
113 metabolites in urine during the treatment.
114

115 ## 2. Materials and methods

116 *2.1. Experimental design*

117 Fifteen male six-month-old Friesian veal calves were randomly divided into the following
118 two groups: the control group (n=7), named group C, received a placebo, the treated
119 group T(n=8) received four doses of NA (150 mg/animal, *im*) every 15 days for two months
120 and RA (80 mg/day/animal, *per os*) for the last 31 days (Supplementary material 1). NA
121 and RA for calf treatment were obtained from AST Farma B.V. (Oudewater, NL) and
122 Unibrom Corp. (Weifang, China), respectively. Animals were sacrificed three days after the
123 last treatment. All groups of experimental animals were kept in separate 10 m × 10 m
124 boxes, tethered, and fed with liquid milk replacer twice a day (dry matter 95% wet weight
125 basis, crude protein 23%, ether extract 21%, ash 6%, cellulose 0.1%; vit. A 25.000 IU/kg,
126 vit. C 50 mg/kg, Cu 5 mg/kg, vit. D3 5.000 IU/kg, vit. E 80 mg/kg).
127 Target tissue samples were collected at the slaughterhouse and preserved in 10% neutral
128 buffered formalin or Bouin's fluid for subsequent histological preparations or in RNA later
129 (Sigma-Aldrich, St. Louis, MO, USA) for molecular analyses. The testes, thyroid and heart
130 of each animal were weighed and relative weight was calculated as organ (g) / total animal
131 weight (Kg).
132 Urine samples were collected before NA treatment (t0) and at eleventh day after the third
133 (t1) and fourth (t2) injections. Urine samples were also collected at slaughterhouse from
134 the bladder (t3) (Supplementary material 1). This timing was elicited to simulate the
135 condition of an illicit treatment and a random sampling conducted by the sanitary officers.
136 Samples were analyzed by Yeast Androgen Bioassay (RAA), and the results were
137 confirmed by LC-MS/MS. All samples were stored at -20°C until analysis.

138 This study was approved by the Italian Ministry of Health and the Ethics Committee of the
139 University of Turin. The carcasses of treated animals were disposed according to proper
140 protocols.
141 All experiments were carried out according to European Economic Community (EEC)
142 Council Directive 86/609 and successive modifications (Directive 2010/63/EU) as
143 recognized and adopted by the Italian Government.

144 *2.2. Tissue sampling and processing*

145 Prostate, bulbo-urethral glands and testes were collected from each animal. Sex
146 accessory glands samples were fixed in 10% neutral buffered formalin at room
147 temperature, whereas testis samples were fixed in Bouin's fluid. All samples were
148 processed and paraffin embedded according to routine histological procedures.
149 Representative sections of each sample were stained with hematoxylin–eosin (HE).
150 Samples of the *Longissimus dorsi* (LD), *Vastus lateralis* (VL), and *Biceps brachii* (BB)
151 muscles were collected from each animal after slaughter. Sections weighing 150-200 mg
152 were immediately frozen in liquid nitrogen and kept at −80ºC for molecular studies.

153 *2.3. Morphometric Analysis*

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

161 *2.4. Total RNA extraction and relative quantification of MYH, MRFs, and IGF1 gene*
162 *expression by qPCR*

163 Total RNA from each muscle sample was extracted using TRI Reagent (Sigma-Aldrich,)
164 according to the manufacturer's protocol. RNA quantity was determined by UV-visible
165 spectrophotometry, and the RNA integrity was verified by automated gel electrophoresis
166 (Experion Instrument, BioRad, Hercules, CA, USA). cDNA was synthesized from 1 μg of
167 total RNA according to the manufacturer's instructions using the QuantiTect Reverse
168 Transcription Kit (Qiagen, Hilden, Germany), which included a DNase digestion.
169 To determine the relative amounts of specific transcripts, the cDNA was subjected to
170 qPCR using the IQ5 detection system (BioRad) and the IQ SYBR Green Supermix
171 (BioRad). Primer sequences for MYH and IGF1 genes were designed using Primer3web
172 (vers. 4.0.0) (Koressaar & Remm, 2007, Untergrasser et al., 2012) (Table 2), and the MRF
173 primers were designed as described by Shibata, Matsumoto, Aikawa, Muramoto, Fujimura
174 & Kadowaki, (2006). In LD and VL muscle samples, the cyclophilin A (PPIA) gene was
175 used as a housekeeping gene (De Maria et al., 2010). For the BB muscles, glyceraldehyde
176 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene.
177 The levels of gene expression were calculated using a relative quantification assay based
178 on the comparative C_q method ($\Delta\Delta C_q$ method) after verifying similar efficiencies of both the
179 target and housekeeping gene amplifications. Then, the relative abundance of each
180 transcript, normalized to the endogenous housekeeping gene and relative to the control
181 sample, was recorded as $2^{-\Delta\Delta Cq}$ (fold increase).

182 *2.5. Steroid extraction from calf urine samples*

183 Ten-milliliter calf urine aliquots from experimental groups C and T were adjusted to pH 4.8
184 before addition of 20 μL of β-glucuronidase/arylsulfatase from *Helix pomatia* (3 U/mL)
185 (Roche Diagnostics GmbH, Boehringer Manheim, Germany). Enzymatic deconjugation

186 was carried out overnight in a water bath at 37°C. Successive samples were subjected to
187 solid phase extraction (SPE) on 1000 mg C18 and 500 mg NH2 columns (Supelco, Sigma-
188 Aldrich, St. Louis, MO, USA) using methods previously described by Bovee et al., (2009).

189 *2.6. Yeast Androgen Bioassay (RAA)*

190 *Saccharomyces cerevisae* transformants expressing the AR were grown on selective
191 minimal medium plates supplemented with l-leucine. Supplemented minimal medium
192 (MM/L) consisted of yeast nitrogen base without ammonium sulphate or amino acids (1.7
193 g/L), dextrose (20 g/L), ammonium sulphate (5 g/L) and supplementation with l-leucine (6
194 mg/L) (Sigma-Aldrich, St. Louis, MO, USA).
195 The RAA was performed as described previously (Bovee et al., 2009). In short, 10 mL
196 MM/L was inoculated with a single colony of the recombinant yeast and grown overnight at
197 30°C in an orbital shaking incubator at 125 rpm.
198 For exposure of the yeast to sample extracts, 200-μL aliquots of the yeast cultures were
199 pipetted into each well of a 96-well plate already containing the dried extracts as described
200 by Bovee et al. (2009). For exposure to the standard compounds such as 17β-testosterone
201 and the NA metabolites α- and β-nandrolone (α-ND and β-ND), 200-μL aliquots of the
202 yeast cultures were pipetted into each well of a 96-well plate and 2 μL from the standard
203 stock solutions dissolved in DMSO were added (final concentration about 4 to 1000 ppb).
204 The yeast and samples were incubated together for 24 h and the yeast fluorescence was
205 measured directly in a Victor 31420 Multilabel Counter (PerkinElmer, Waltham, MA, USA)
206 using excitation at 485 nm and emission measurement at 530 nm. Differences in the
207 fluorescence emission at 24 and 0 h of yeast exposure to the samples were calculated and
208 corrected according the blank control values, thus providing the final androgenic activity
209 data for each sample. We defined the mean signal of 20 blank samples plus three times
210 the corresponding standard deviation as the decision limit CCα (α= 1%) (EC Decision

211 2002/657). Urine samples that had fluorescence values greater than those of the CCα
212 contained androgenic molecules.

213 *2.7. LC-MS/MS*

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

225 *2.7.1. α-ND and β-ND determination by LC-MS/MS*

226 A 1-mL aliquot of filtered urine was subjected to enzymatic hydrolysis by β-glucuronidase
227 from *E.coli* K12 (EC 3.2.1.31) (Roche Diagnostics GmbH, BoehringerManheim, Germany).
228 The internal standard was β-nandrolone-d3 at a concentration of 2 ppb. After purification
229 on an Oasis HLB cartridge (3 mL, 60 mg, Waters) the methanol elute was evaporated, and
230 the dry residue was dissolved in a mobile phase mixture prior to LC-MS/MS analysis. The
231 mobile phase consisted of water with 0.1% formic acid and methanol at a flow rate of 250
232 μL/min. The appropriate gradient allowed separation of α-ND and β-ND on a reverse-
233 phase HPLC column (Synergi Hydro RP 150 x 2.0 mm, i.d. 4μ 80 A). The mass
234 spectrometer was operated in the positive ESI mode with the following acquisition

235 parameters: capillary voltage set at 3000 V, ion transfer capillary temperature set a 340°C
236 and sheath and auxiliary (nitrogen) gases were fixed at 30 and 10 arbitrary units,
237 respectively. The collision gas was argon at 1.5 mTorr, and the peak resolution of 0.70 Da
238 FWHM (full width at half maximum) was used on Q1 and Q3. The signal acquisition was
239 performed by multiple reaction-monitoring mode (MRM) with corresponding transitions for
240 which the optimum tube lens and collision energies were established. α-ND and β-ND had
241 the same fragmentation patterns. The precursor ion had m/z=275 as $[M+H]^{+\cdot}$, which gave
242 ion m/z=109 as quantifier, whereas ions m/z=145, 199 and 239 were used for
243 conformation purposes. The deuterated β-ND gave the analog product ion arrangement
244 with the quantification transition from parent pseudo-molecular ion (m/z=278) to most
245 abundant product ion (m/z=109).

246 *2.7.2. RA determination by LC-MS/MS*

247 Deconjugation of RA II phase metabolites was performed by adding β-
248 glucuronidase/sulfatase from *Helix Pomatia* to 2 mL of urine. The internal standard was an
249 RA structural isomer isoxsuprine at a final concentration of 2 ppb. After pH adjustment
250 (8.5-9.5), the sample was treated with 5 mL of the tert-butyl methyl ether and ethyl acetate
251 mixture (4:1 v/ v). Once shaking and centrifugation were completed, the organic phase
252 was evaporated with subsequent resuspension of dry sample in the initial mobile phase
253 (acetonitrile and 0.1% formic acid in aqueous solution, 10:90 v/v). The mass spectrometer
254 was operated in the positive ESI mode. Reversed-phase LC was performed using the
255 Synergi Hydro RP (150 x 2.0 mm, i.d. 4µ 80 A) with adequate mobile phase gradient.
256 Acquisition parameters such as capillary potential and temperature were set at 4200V and
257 360°C, respectively. Nitrogen as sheath and auxiliary gas was set at 40 and 6 arbitrary
258 units, respectively, while the pressure of argon as collision gas was 1.5 mTorr. Peak
259 resolution of 0.70 Da FWHM was used on Q1 and Q3. RA and isoxsuprine identification

260 and quantification were achieved using MRM for most specific transitions. The pseudo-
261 molecular ion (m/z=302) was the common parent species for both compounds, which were
262 chromatographically separated. The RA parent ion produced m/z=164 as a quantifier and
263 m/z= 107, 121 and 284 as diagnostic ions, whereas isoxsuprine produced the following
264 characteristic ions: m/z=150 (quantifier), 105, 107 and 284.

265 *2.8. Statistical analyses*

266 All statistical analyses were performed using GraphPad Prism 4 (vers. 4.03) software
267 (GraphPad Inc., San Diego, CA, USA). The STED and gene expression of target genes
268 (ΔCq) were analyzed by unpaired t-test, comparing treatment group (T) against the control
269 group (C). Normal distribution was tested by Kolmorov-Smirnov test. Grubbs' test was
270 used to determine and exclude potential outliers.
271 Data are presented as the average ± SEM. A $p<0.05$ was considered significant.

272

273

274 **3. Results**

275 *3.1. Histopathology and morphological analysis*

276 Macroscopic examination of the carcass did not reveal any lesions potentially associated
277 with treatment; the accessory sex glands showed no differences between the two groups.
278 Administration of NA and RA to veal calves induced a significant ($p<0.001$) reduction in
279 testis weight (table 1). A statistically significant increasing in thyroid weight of group T was
280 observed ($p<0.05$) whereas the heart did not present any significant relative weight
281 change.
282 In testis, histological evaluation showed an apparent reduction in the epithelial germ line
283 thickness, along with reduced nuclei volume (figure 1b) in the treated animals. Moreover,
284 the reductions in STED and MTA were significant ($p <0.001$) (Supplementary material 2a
285 and 2b) in group T. At the histological level, the prostate and bulbo-urethral glands of the
286 treated animals showed mild epithelial hyperplasia associated with moderate
287 hypersecretion and cystic dilatation of the ducts (figure 1d, f). A gland maturation delay
288 was evident in particular in the bulbo-urethral of the treated group. The prostate urothelium
289 presented a moderate hyperplasia in group T.

290 *3.2. Effects of NA and RA administration on the relative expression of MRFs, MYH, and*
291 *IGF1 in skeletal muscle tissue*

292 NA and RA administration induced distinct MRF gene regulation in different skeletal
293 muscle types. In particular, VL showed an up-regulation of MRFs involved in skeletal
294 muscle differentiation; in fact MRF4, MYOD and MYOG gene expression levels were
295 increased by about 2-2.5-fold ($p<0.01$) in group T. In BB muscle, androgen treatment
296 induced the over expression of MYOD by 1.7-fold but did not elicit any MRF expression

297 changes in LD. On the contrary, MYH expression in LD was significantly up-regulated by
298 6-fold (Table 3).
299 In VL, IGF1 gene expression was increased approximately 2-fold compared with that of
300 control group C

301 *3.4. RAA*

302 Figure 2 shows the dose–response curve obtained by RAA after 24-h exposure to
303 testosterone and α-ND and β-ND. All the results are reported as the means of triplicate
304 measurements. After 24 h of yeast exposure to different concentrations of the standard
305 molecules, no differences in yeast growth could be detected indicating that no toxic effects
306 on the yeast were observed. The data shows that testosterone and β-ND demonstrate full
307 dose-response curves with similar sensitivities to those reported in the literature (Bovee et
308 al., 2009). α-ND was less active as expected to since changing the OH-group at position
309 17 from 17β to 17α strongly decreases the potency of the androgen (Bovee et al., 2009).
310 A decision limit, CCα, was calculated from the corrected fluorescence signals of the 20
311 blank urine samples. The mean value of these blank urine samples was 1908, and the
312 standard deviation was 2058; therefore, the CCα was 8083. Samples giving a signal lower
313 than CCα were classified as compliant or negative. Samples giving a signal higher than
314 the decision limit CCα were classified as suspected of containing androgen molecules.
315 Supplementary material 3 represents the group T urine samples that gave signals below
316 the CCα and were thus classified as negative.

317 *3.5. LC-MS/MS*

318 Urine samples from groups C and T were screened in LC-MS/MS for α-ND, β-ND and RA.
319 Group C samples were negative for α-ND and β-ND as well as for RA (data not shown).
320 Table 4 shows the results for group T urine samples. The samples were analyzed to

321 identify free nandrolone molecules and after hydrolysis to identify total nandrolone
322 molecules (free and conjugate). Throughout the NA treatment, the presence of α-ND, a
323 principal metabolite of NA, was observed. In the same way, RA was detected in group T
324 urine samples during the treatment (Table 4).

325

326 ## 4. Discussion

327 The rapid kinetics of the hormones used in animal doping and the practice of applying
328 treatments at low doses result in an underestimation of the problem of illicit growth
329 promoter administration in husbandry, both in Italy and in the rest of Europe.
330 Therefore, to support official methods of investigation, the scientific community promotes
331 the use of new approaches, including direct and indirect methods of analysis (Nebbia et
332 al., 2011). Among these, screening tests are targeted to detect direct or indirect
333 parameters linked to growth promoter abuse. Laboratories use these methods as a
334 screening test during sample analysis. These methods may allow the resolution of the
335 investigation if the results are negative, but they cannot be exhaustive for positive or
336 uncertain outcomes. In these cases, further investigations (confirmation methods) are
337 required. Therefore, screening methods cannot replace official analysis methods (Nebbia
338 et al., 2011), but they allow a preliminary screening of thousands of samples, thus
339 increasing the effectiveness of the official controls. Screening methods are characterized
340 by high productivity and low cost per analyzed unit.
341 In this study, a combination of a SARM (NA) with a β-agonist (RA) was investigated with
342 respect to their detection with screening and confirmation methods in veal calves.
343 An important step is to define the biological processes governed by these hormones and
344 the cell type(s) in which they exert their anabolic effects.
345 The dosages and combinations used in this experiment were based on the partial
346 knowledge of the application in the field and the results of previous studies (Groot, Schilt,
347 Ossenkoppele, Berende, & Haasnoot, 1998).
348 The testes showed reduced development according to what could be expected for the age
349 of the animals, as suggested by reductions in testis weight and in the tubular seminiferous
350 dimensions respect to those of the control group.

351 Thyroid and heart weight reduction was detected in swine following RA administration
352 (Catalano et al., 2012). On the contrary, in the present experiment, the heart relative
353 weight of veal calves did not undergo any change whereas the thyroid weight increased.
354 This finding could be attributable to the NA and RA combination and to the species
355 difference. The prostate and bulbo-urethral gland showed mild hyperplasia, fibrosis and
356 hypersecretion, along with a delay in gland maturation most likely due to NA
357 administration. These androgenic effects were smaller than those observed following
358 androgen hormones administration, confirming the SARM action of NA. In fact, Cannizzo,
359 Zancanaro, Spada, Mulasso, & Biolatti, (2007) reported a significant hypersecretion and
360 reduced testicular development as consequences of androgen administration.
361 In previous experiments with anabolic steroids like testosterone and estrogen, reduced
362 testicular development and increased stromal proliferation were observed (Groot & Arts,
363 1991). Similar findings were reported in lamb and calf testes from animals implanted with
364 estradiol and trenbolone (Rodriguez Barbudo, Mendez Sanchez, & Blanco, 1991).
365 In addition, the prostates of treated veal calves showed vacuolar degeneration that could
366 be ascribed to β-agonist RA; in the same way RA could be responsible of urethral
367 epithelium hyperplasia (Groot et al., 1998, Catalano et al., 2012). However, these effects
368 are not specific of β-agonist treatment, since also an estrogen administration may induce
369 similar lesions (Biolatti et al., 2003, Imbimbo et al., 2012).
370 RA is a β-adrenergic agonist that can be defined as a repartitioning agent that redirects
371 and increases nutrient flow from fat deposition towards muscle deposition (Ricks,
372 Dalrymple, Baker & Ingle, 1984). β-agonists, in both male and female cattle, influence
373 functional and morphological aspects of the gonads and genital tract, directly or via the
374 hypothalamo-pituitary axis. In vivo, β-adrenergic agonists may induce secondary events
375 caused by the hormonal or physiological responses of several tissues, especially involving
376 the endocrine and cardio-respiratory systems (Groot et al., 1998). Skeletal muscle mass

377 increases during postnatal development through a process of hypertrophy, i.e.,
378 enlargement of individual muscle fibers, and a similar process may be induced in adult
379 skeletal muscle in response to contractile activity, such as strength exercise, and by
380 specific hormones, such as androgens and β-adrenergic agonists.

381 To confirm the anabolic effects of NA and RA at the molecular level, mRNA expression in
382 three different types of skeletal muscle was analyzed. Androgen action in skeletal muscle
383 is mediated through binding to cytosolic AR and translocation to the nucleus, where this
384 complex can regulate gene transcription. Androgens can regulate MRFs and other
385 regulatory factor genes are transcriptional target genes of androgens (Lu et al., 1999,
386 2000).

387 Recently, Diel et al. (2008) described the up-regulation of myostatin and AR genes in the
388 *Gastrocnemius* muscle of rats treated with 19-norandrostenedione and testosterone
389 propionate. They also demonstrated local modulation of the mRNA expression of distinct
390 growth factors like IGF-1(Matsakas, Nikolaidis, Kokalas, Mougios, & Diel, 2004).

391 Satellite cell activity is also regulated by testosterone and its synthetic derivatives (Ishido,
392 Kami & Masuhara, 2004).

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

402 LD muscle reacted differently to NA and RA; no MRF gene regulation was observed but an
403 important up-regulation of the MYH gene was evident in the present experiment.
404 Some studies report changes in the local expression of IGF1 in human muscle samples
405 from patients receiving anabolic androgens (Sheffield-Moore, 2000). IGF1 stimulates
406 satellite cell proliferation and promotes muscle hypertrophy (Musarò & Rosenthal, 1999);
407 therefore, it is possible that androgens regulate muscle mass through these mechanisms.
408 Pampusch et al. (2003, 2008) studied trenbolone acetate and estradiol effect,
409 administered together or alone by implant, in LD of steers. IGF1 mRNA was up-regulated
410 in particular by estradiol during the treatment. In the same way, Walker et al. (2007)
411 observed a IGF1 mRNA increase in LD after trenbolone acetate/estradiol implant of steers.
412 However IGF1 mRNA decreased after addiction of RA.
413 RA and androgens had opposite effect on serum concentrations of IGF-I and
414 mRNA expression of IGF-I in LD. In the present study this effect was confirmed in LD and
415 BB; differently, IGF1 mRNA was up-regulated by 2.42 fold in VL.
416 This finding is probably due to different types of metabolism among the collected muscles.
417 In fact, VL in cattle have an intermediate fiber composition, also called fast oxidative fibers,
418 and therefore, the main pathway for ATP production is oxidative phosphorylation in which
419 IGF1 is involved.
420 Androgens induce increase in muscle mass, and this increment is partly due to muscle
421 fiber hypertrophy, reflected by an increase in myonuclear numbers and cross-sectional
422 areas of both slow and fast type muscle fibers (Dubois, Laurent, Boonen, Vanderschueren &
423 Claessens, 2012).
424 In the present experiment, due to the combination of androgen and β-agonists
425 administered, it was possible to observe alterations in muscle gene expression levels
426 different from what has been reported in the literature.

427 In fact, clenbuterol in cattle induced a slow to fast transition of MYH isoforms (Polla et al.,
428 2001); in swine, MYH genes are differentially regulated by RA, and the βadrenergic
429 agonist-induced repartitioning effect is, in part, mediated by changes in muscle fiber type-
430 specific gene expression (Gunawan, Richert, Schinckel, Grant & Gerrard, 2007).
431 On the contrary, Chung, Baxa, Parr, Luqué, and Johnson (2012) specifically described
432 MYHs mRNA abundance in LD of steers steroidal implanted. They conclude that the treatment
433 had no effect on proportion of the three MYH isoforms mRNA but it only decreased the adipogenic
434 genes expression.
435 To confirm the presence of NA residues and RA in urine samples from the animals during
436 the experiment, a direct screening test such as RAA and an official analytical method (LC-
437 MS/MS) were applied.
438 RAA is a yeast androgen bioassay (Bovee et al., 2009) that expresses yEGFP as
439 measurable reporter protein in response to androgens.
440 NA is a synthetic ester of 19-nortestosterone that was developed to minimize undesirable
441 androgenic side effects. NA is quickly metabolized by the liver and its direct metabolites
442 (α- and β-ND) can be detected in urine.
443 In recent years, it was possible to directly identify esters of anabolic steroids only in hair
444 samples (Groot et al., 2012). Because physiological steroids do not occur naturally in an
445 ester form, detection of intact steroid esters in hair was considered as a prove of illegal
446 administration of exogen steroids but actually, β-ND, which was considered exogenic, was
447 discovered to occur naturally in some species (Scarth et al., 2009). Furthermore, α-and β-
448 ND can occur naturally in the urine of injured male cattle (Glenn Kennedy et al., 2009).
449 All urine samples were analyzed for the presence of α- and β-ND.
450 RAA did not detect androgens in any urine samples; in particular, RAA was employed to
451 test α- and β-ND presence, but it resulted not specific for α-ND. This characteristic did not

452 allow the detection of α-ND metabolites in the urine of treated animals, testing positive in
453 LC-MS/MS.
454 No NA metabolites were observed in the urine of control animals (data not shown); this
455 allows us to conclude that veal calves from this experiment did not synthesize endogenous
456 α- and β-ND androgens, and these metabolites were derived from NA administration. In
457 particular, only α-ND was detected by chemical analysis and the previous metabolite, β-
458 NA, was not observed in the urine samples from treated or control veal calves. This may
459 be due to the sampling schedule, which was approximately 2 weeks after the day of
460 treatment.
461 All urine samples were analyzed for the presence of RA by LC-MS/MS, and it was present
462 in all the urine samples of group T during treatment. The RA values were lower at t1 and
463 increased until slaughter.
464

465 **5. Conclusions**

466 In the present study several effects were observed in veal calves following SARM and β-
467 agonist treatment.
468 In literature no specific histophatological lesions were described relative to sexual
469 accessory glands caused by SARM molecules administration. Therefore, the results
470 obtained from this study are totally new in particular in farm animals as calves. It was
471 verified that NA has a mild androgen effect on veal calves testes and that treatment
472 resulted in decreased size and weight of the gonads. The bulbo-urethral gland and the
473 prostate showed weak histological alterations. Nevertheless, the treatment of veal calves
474 with NA could be specifically detected by macroscopic and microscopic analysis. In
475 contrast, RA administration in veal calves is very difficult to identify by histology or
476 macroscopic alteration.
477 In parallel to anatomo-histophatological observations, it could be interesting to apply an
478 indirect method of analysis based on different gene expression regulation.
479 The results showed that NA and RA influence skeletal muscle gene expression but the
480 combination of these two molecules probably reduce the detectable effects, since there
481 could be interplay among these molecules.
482 Additional *in vivo* and *in vitro* studies are necessary to better understand the mechanisms
483 related to this complex process. At the moment, in particular for RA detection, it is
484 necessary a direct chemical analysis to confirm its administration.
485

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493

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639

Figure captions

Figure 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. Figures 1c and 1e represent the bulbo-urethral gland and prostate of control animals, respectively. The bulbo-urethral glands and prostates 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, $\times$ 200 magnification; bars: 100 μm).

Figure 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.

Supplementary material 1. Schematic of experimental procedure. Fifteen male veal calves were divided into two groups: group C (n=7) was the control group that received a placebo, and 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. Animals were sacrificed three days after the last treatment. Urine samples from the experimental groups were collected before NA treatment (t0) and at eleventh day after the third (t1) and the fourth (t2) injections. Urine samples were also collected at a slaughterhouse from bladders (t3).

Supplementary material 2. Significant reduction of STED (a) and MTA (b) was detected in veal calves treated with NA and RA. ($^{***}p<0.001$).

664 **Supplementary material 3**. RAA results after 24-h exposure to urine of group T animals
665 collected at different time points of protocol treatment (t0, t1, t2, and t3). The dotted line
666 indicates the CCα value calculated from analyses of control urine samples (without
667 exogenous androgens).

668

669 Table 1.

670 Relative weight of testes, thyroid and heart of group C and T animals. Data are

671 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

672 *p<0.05; ***p<0.001.

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

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. (*$p<0.05$; **$p<0.01$)

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).