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Development of an enhanced histopathological approach to detect low-dose dexamethasone illicit treatment in veal calves

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ABSTRACT

Dexamethasone is one of a number of synthetic corticosteroids illegally used to promote growth in food-producing animals. Since these low-level drug cocktails evade detection by currently available chemical methods, simple biological indicators that can aid in laboratory analysis are needed. In an attempt to devise an accurate biological method that could detect illicit drug treatment in food-producing animals, we characterized microscopic morphologic alterations of the thymus in veal calves administered low-dose dexamethasone versus control animals. For this purpose, 122 male calves were farmed for 6 months in controlled condition: 81 animals were orally administered dexamethasone (0.4 mg day) for 20 days during the sixth month and the remaining 41 were kept as control. Urine samples were collected systematically during the treatment period, the suspension period and at the slaughterhouse. All animals were slaughtered 10 per day starting from 10 days after the last dexamethasone administration and the thymus was sampled for histological examination. The difference between the two animal groups was evaluated by means of a non-parametric test of hypothesis. No residues were detected in the urines collected since the third day after the last administration, whereas morphometric analysis of the thoracic thymus revealed a significant decrease in the cortex: medulla ratio in the treated animals ($p < 0.0005$). We can conclude that this histological approach offers encouraging prospects as a screening method to overcome current limitations in controlling growth promoter abuse.

KEYWORDS: screening assays, health significance, drug residues, hormones, residues, veterinary drug residues, veterinary drug residues, anabolic steroids, animal products, meat

INTRODUCTION

The illegal use of chemical growth promoters in food-producing animals constitutes a potential risk for human health. The administration of synthetic corticosteroids that can mimic, block or interfere with hormone actions is increasingly widespread (Vázquez et al. 2005). Furthermore, glucocorticoids may be responsible for Cushing's syndrome or other side-effects such as hypertension, hypokalemia, hypernatremia, metabolic alkalosis and connective tissue weakness. They may also induce ulcer formation or cause permanent eye damage by inducing central serous retinopathy. Thanks to their lipolytic effect, these cocktails result in the lean meat that consumers find more appealing (Brambilla et al. 1998). The novel synthetic compounds also exert relevant biological effects: appetite stimulants in cattle lead to increased feed intake and rapid weight gain (Vázquez et al. 2005).

To date, the administration of synthetic hormones, such as dexamethasone in livestock, is approved by the European Union (EU) only for therapeutic indications, given the proven benefits of these veterinary drugs in treating inflammatory disorders. To protect consumers against illegal drug use in

food-producing animals, the EU set appropriate maximal residue limits (MRLs) in tissues and milk intended for human consumption (Council of the European Communities 1990. Council Regulation 90/2377/EEC). Depending on the particular glucocorticoid formulation, withdrawal periods of up to several weeks to prevent the accumulation of illegal residues in animal products are recommended.
To expose illicit corticosteroids use, rapid and simple analytical techniques need to be developed that can be applied in routine analysis laboratories targeting these substances or their residues. Furthermore, to accurately detect trace residues in samples of animal origin and new compounds or low-dose cocktails, novel screening tools are needed. Innovative methods employ indirect markers that can determine receptor concentration (Odore et al. 2006). Other methods that can aid in identifying animals treated with anabolic agents include the so-called omics techniques (transcriptomic, proteomic and metabolomic), based on the simultaneous detection of biomarkers predictive of administration of specific substances (Gardini et al. 2006; Toffolatti et al. 2006; Reiter et al. 2007; Courant et al. 2009).
Several studies on young animals have found that illegally administered glucocorticoids promote a physiological reduction of the thymus identifiable as gross and microscopic lesions (Groot et al. 1998; Schilt et al. 1998; Biolatti et al. 2005; Cannizzo et al. 2008). In this context, anatomo-histopathological examination may provide a valid tool as an indirect marker of illicit drug treatment. However, the growing use of low-dosage drug cocktails that escape chemical detection and cause only minimal tissue changes makes the detection of suspected alterations a difficult task for veterinary inspectors (Courtheyn et al. 2002; Biolatti et al. 2005; Carletti et al. 2007), with the inevitable consequence of increased risk for consumer health (Groot et al. 1998).
This situation further stresses the importance of identifying chemical-induced thymic atrophy and differentiating it from physiological thymic involution. Due to the potential difficulties of differentiating those latter changes from chemical-related effects, histopathological examination should be carried out by comparing treated with control animals (Elmore 2006).
The aim of this study was to determine whether morphological changes in the thymus could constitute a reliable treatment parameter following administration of low-dose glucocorticoid for growth promotion in veal calves. To do this, we devised an accurate histopathology-based biological method that may prove useful in detecting glucocorticoid compound abuse by the meat producing industry.

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL DESIGN

A total of 122 male veal calves, aged between 15 and 35 days, were bought by local breeders and randomly divided in two groups, homogenous for body weight and age, and farmed in two boxes in the same condition for 6 months. Each box had its own crib, multiple drinking troughs and a dedicated automated milk feeder system. To protect the animals against infections, all were vaccinated against IBR, Para influenza (PI3), BRSV and BVDV (CATTLEMASTER® 4; Pfizer Animal Health, New York, USA). Clinical controls were carried out daily by a veterinarian and treatments for occurring infections were performed without using hormonal active substances.
All animals had free access to fresh water and were fed standard milk replacer with an automatic milk feeder until 4 months of age, then 0.5 kg of corn was added to the diet twice a day. Before administration, all feeds, milk replacer and corn were analyzed with an ELISA test to exclude the presence of hormonal active substances.
During the sixth month, 81 randomly selected animals received dexamethasone 21 disodium phosphate (0.4 mg day per os) mixed with the milk for 20 days while the remaining 41 animals were used as controls and administered milk and placebo mixed too. The study was designed to achieve a statistical power of 80% with a confidential interval 95%; an expected frequency of disease in the treated group of 27% and a frequency of 5% in the control group were considered. On this basis, a

simple random and representative sample of 40 controls and 80 treated animals was evaluated to be consistent with a detection of an odds ratio of 7.
The trial was conducted in a blinded manner.
The animals were slaughtered 10 per day in an EC-certified slaughterhouse starting from 10 days after the last drug administration. All experiments were carried out according to European Economic Community Council Directive 86/609, recognised and adopted by the Italian Government (D.L. 27/01/1992 no. 116).

CHEMICAL ANALYSIS

Urine samples of 10 treated animals and 10 controls were collected when spontaneous urination occurred, the day before the first administration, 10 days later, and the last day of treatment; urine was also collected after the last treatments, 1, 2, 5 and 10 days later, and at the slaughterhouse. Each sample, without preservative, was divided into 6-ml aliquots and stored at −80°C until use.

SAMPLE PREPARATION

Urine samples (5 ml) spiked with triamcinolone acetonide-d_6 used as the internal standard (I.S.) were added with acetate buffer solution 1 M, pH 4.8 and β-glucuronidase-arylsulphatase enzyme solution (*Helix pomatia*). The samples were then incubated for 12 h at 37°C, centrifuged and purified by solid phase extraction (SPE) using an Oasis hydrophile/lipophile balance (HLB) cartridge (60 mg, 3 ml) (Waters, Milford, MA, USA). After elution with methanol, the solvent was removed at 40°C under nitrogen stream and the residue was dissolved in 100 µl of methanol and injected into the liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) system.

LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY/MASS SPECTROMETRY ANALYSIS

Analyses were carried out using a liquid chromatography system (Perkin Elmer Series 200 Micro Pump with a PE Series 200 autosampler; Perkin Elmer, Waltham, MA, USA). The chromatographic separations were obtained under gradient conditions at room temperature using a reversed-phase HPLC column (250 × 4.60 mm I.D., 4 mm POLAR-RP 80 A) Synergi C_{18} (Phenomenex, Torrance, CA, USA) with a C_{18} guard column (4 × 2 mm I.D.) (SecurityGuard, Phenomenex). The mobile phase was composed of 1% acetic acid and acetonitrile and the flow rate was 0.8 ml min.
The API 3000 triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI)-heated nebulizer (HN) source was set in negative ionization mode with a source temperature of 400°C and a needle current of −3 µA. Ultra-pure nitrogen was used as curtain and collision gas, while ultra-pure air was used as nebulizer and auxiliary gas.
Decision limit (CCα) and detection capability (CCβ) for dexamethasone, calculated in accordance with Commission Decision 2002/657/EC [16], were 0.3 and 0.4 ng ml, respectively.

HISTOPATHOLOGY

SAMPLE PREPARATION

After slaughtering, the central area of the thoracic thymus of each animal was sampled, fixed in 4% buffered formaldehyde at room temperature for 2 days, routinely processed, embedded in paraffin wax, sectioned at 3–5 µm and stained with hematoxylin and eosin (HE). Routine histology by light microscopy was performed on HE-stained paraffin sections.

HISTOPATHOLOGICAL CHARACTERIZATION

Histopathologic examination was blindly performed. The presence of fat, as a possible indirect indicator of the degree of gland atrophy, was evaluated and a score from 1 to 3 (mild, moderate, severe) was attributed to the pattern. The patterns of the optical and dyeing parameters were evaluated based on the histopathologist's experience rather than by statistical methods.

MORPHOMETRY

The thymus sections were examined at low magnification (4×) using a digital microimaging device (Leica DMD108 Digital microimaging device for clinical diagnostics labs) to evaluate cortex and medulla thickness. For each slide, five functional lobules, composed of an outer cortex and inner medulla and surrounded by connective tissue, were randomly selected and measured against a graduated line, starting and ending at the interlobular connective tissue; a second parallel line was drawn to measure medulla thickness.
All data were recorded on a spreadsheet; the cortex thickness was obtained by subtracting the second from the first value and the cortex: medulla ratio was calculated.

STATISTICAL ANALYSIS

The results of the microscopic observations were entered into an ad hoc database. Two different tests of analysis were carried out. To demonstrate the difference between treated and control animals with regard to the cortex: medulla ratio, we performed a test of hypothesis for non-parametric data (Wilcoxon rank-sum test), as the data were not normally distributed, assuming as the null hypothesis that there would be no difference between the two groups. To verify the presence of a significant difference in the degree of thymic atrophy between the two groups, a test for linear trend was performed and the difference between three increasing degrees of atrophy was expressed as odds ratios (OR).

RESULTS AND DISCUSSION

CHEMICAL ANALYSIS

Dexamethasone residues (>CCα) were detected in all the urine samples collected from the treated bovines at the 10th and 20th day of treatment and the day after the last administration (21st day). At the 22nd and 25th day of sampling, dexamethasone residues were found in 90% and 50% of the analyzed samples, respectively (Table 1). No residues were detected in the samples collected the day before the treatment, 10 days later, or in the samples collected from the urinary bladder at the slaughterhouse. These findings showed that the drug disappeared shortly after treatment, precluding a long-term survey.

Table 1. Dexamethasone concentration (ng ml) in urine samples of treated and control calves.

Sampling day	0th	10th	20th	21st	22nd	25th	30th	30th slaughterhouse
Treated								
Average	n.d.	2.0	2.1	1.2	1.0	0.4	n.d.	n.d.
Concentration interval	–	0.5–9.1	0.3–5.9	0.4–3.1	n.d.–2.5	n.d.–2.5	–	–
>CCα (0.3 ng ml^{-1})	0/7	10/10	10/10	10/10	9/10	5/10	0/10	0/10
Control								
Average	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Concentration interval	–	–	–	–	–	–	–	–
>CCα (0.3 ng ml^{-1})	0/8	0/9	0/10	010	0/10	0/10	0/8	0/9

No dexamethasone residues were detected in the control animals; this precluded any further statistical analysis.
Data were determined by averaging the urine concentration values of dexamethasone obtained from the treated and control animals at each time interval.

HISTOPATHOLOGICAL CHARACTERIZATION

On blinded analysis, the score of 1 and 2 (mild and moderate), indicating the presence of infiltrating fat, were present in both groups, whereas the score of 3 was attributed only to the thymus of the treated animals. The amount of fat infiltrating the thymic tissue was not found to be significantly associated with the steroidal treatment. Table 2 shows the trend and the chi-square value for trend.

Table 2. Risk of gland atrophy is expressed as odds ratio (OR) and relative 95% confidential interval. There was no increase in infiltrating fat in treated versus control animals.

Fat score	Odds ratio	95% CI	
1	1	–	–
2	1.2	0.1	20.4
3	1.8	0.1	30.3
Chi-square for trend = 0.16, $p = 0.7$			

COMPARATIVE MORPHOMETRY

Microscopic examination at low magnification of the thymus glands showed a striking tinctorial change: the thymus of the controls was darker than that of the treated animals. At a higher magnification, a decrease in the cortical area in the treated animals was visible.
Qualitative overall assessment indicated that the cortical:medullary ratio was reversed in the treated animals (Figure 1) in comparison with the normal 2:1 ratio (Figure 2).

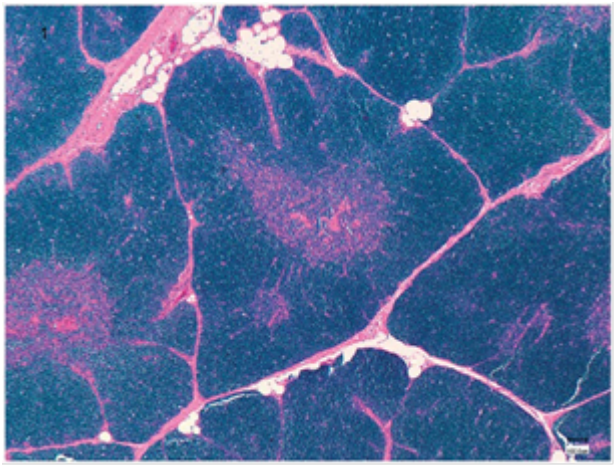

Figure 1. Photomicrograph of treated animal thymus; HE stain, 4 × magnification.

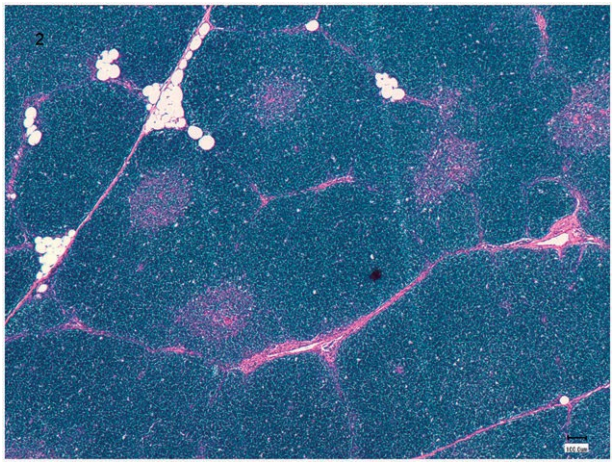

Figure 2. Photomicrograph of control animal thymus; HE stain, 4× magnification.

The two-sample Wilcoxon rank-sum (Mann–Whitney) test highlighted a significant decrease in the cortex:medulla ratio between the treated animals and the controls ($p = 0.0005$) (Figure 3).

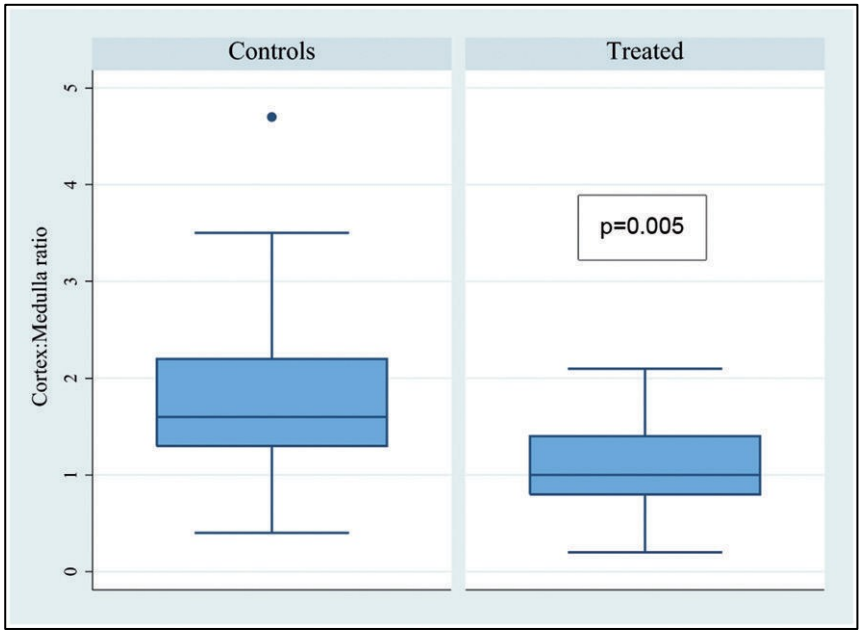

Figure 3. Box plots representing the significant difference in the cortex: medulla ratio between treated and control animals.

Our findings demonstrate that low dosages of dexamethasone administered as a growth promoter in veal calves, according to a protocol often illegally adopted in farm practice (Biolatti et al. 2005), can induce morphologic changes in the thymus, resulting in a significant reduction in the cortex: medulla ratio. In 1998, Schilt et al. (1998) first reported this finding as a valuable feature to distinguish between beclomethasone treated and untreated veal calves. Later Elmore (2006) included this parameter among criteria to distinguish between chemical-induced and age or stress-related thymic atrophy. Other possible causes of thymus atrophy, as autoimmune phenomenon and immunodeficiency syndromes, have been shown to affect cellular density with symmetrical reduction of thymic cortical and medullary areas as well (Maxie 2007). Here, we show for the first time significant values for this parameter. Cortical atrophy and fat infiltration has been variously reported (Groot et al. 1998; Biolatti et al. 2005; Cannizzo et al. 2008) as co-existing findings in dexamethasone-fed veal calves. In our experiment, fat infiltration could not be associated with low-dose dexamethasone treatment, as it was detected in both animal groups.

CONCLUSIONS

Examination of illegal preparations and results of specific investigations have exposed the depth and breadth of the continuing illegal use of growth promoters in livestock production (Courtheyn et al. 2002). Dexamethasone and other corticosteroids are frequently used for this purpose, although it has long been recognised that large doses of synthetic glucocorticoids reduce growth rates and lead to muscle atrophy. Once often combined with beta-agonists and/or anabolic steroids, more recently corticosteroids are administered alone, because even low doses of glucocorticoids have been found to improve feed intake, increase live weight gain, reduce feed conversion ratio, reduce nitrogen retention and increase water retention and fat content (Istasse et al. 1989). Yet, precisely owing to their use at low dosage, their detection in matrices of biological origin is far from evident.
One way to improve the efficiency of residue testing programmes might be to apply screening methods that measure indirect parameters. Our findings show that histopathological assessment of the cortex: medulla ratio is a simple and objective parameter for the detection of corticosteroid abuse and suggest that it may be a useful tool in monitoring illicit drug treatment. No false

negatives occurred with our method, even in thymus samples collected after the 10-day withdrawal period, which is known to limit the capability of detection by classical chemical analysis.
We can conclude that efforts to improve the control of illegal use of growth promoters in cattle should include screening methods based on histological investigations; specifically, assessment of the thymus parameter (thymus morphometry) could represent a standardizable and reproducible method for screening purposes and following the validation it could be considered as a new promising approach in term of screening to evidence the diffusion of corticosteroids illicit treatment in farm animals to orientate controls by confirmatory methods.

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