Thymus atrophy is an efficient marker of illicit treatment with dexamethasone in veal calves: Results from a triennial experimental study

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Thymus and dexamethasone illicit treatments

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Abstract

Glucocorticoids, used in a wide range of pathologies thank to their therapeutical properties, are also illegally used as growth-promoters in animal breeding even if the European Union regulates their use to protect consumers' health from the adverse effects of residues in food. The first aim of the study was to establish the applicability of two histological parameters – atrophy and cortex-medulla ratio - to detect glucocorticoids misuse in calves. The second aim was to test the potentiality of both parameters to discriminate between treated and untreated animals.

One hundred and seventytwo male Friesian veal calves were raised for six months and divided into two groups: Group A (106 calves) was given dexamethasone *per os* for twenty days (0.4 mg/day), Group B (66 calves) used as control. Thymic samples were microscopically examined. Fat infiltration was evaluated and a degree of atrophy from 1 to 3 (mild, moderate, severe) was attributed; thymic cortex-medulla ratio was calculated too. Fisher's exact test and a Wilcoxon–Mann–Whitney test were performed to investigate the differences in thymic atrophy and cortex-medulla ratio between the groups.

Results demonstrate that the thymic atrophy grading was significantly increased in group A ($p= 0,006$), whereas the cortex-medulla ratio was decreased ($p<0,004$) when compared to group B; moreover, the parallel testing with fixed degree of atrophy and cortex-medulla ratio cut-off thresholds optimize the sensitivity (90%) in the detection of glucocorticoids anabolic treatments.

These data suggest that microscopic thymus analysis represent a valid tool for the screening and monitoring of glucocorticoid illicit treatments.

Keywords: glucocorticoids; illegal treatment; dexamethasone; calves; thymus; histological screening method

Introduction

Glucocorticoids (GCs) are widely used in bovine internal medicine for their anti-inflammatory properties, but are also illegally used, despite the European Union ban stated in the Council Directive 2003/74, in food-producing animals as growth-promoters constituting a health risk for the consumers (Botsoglou NA et al., 2001).

Natural corticosteroids are hormones secreted by the adrenal cortex that are involved in a wide range of physiological processes, such as stress response, inflammation, immune function, hydro electrolyte balance, reproduction, behaviour (Osamu N 2001; Schuerholz T et al., 2007). The discovery of their anti-inflammatory properties has led to the chemical synthesis of more active synthetic glucocorticoids, e.g. dexamethasone and prednisolone, that are used as therapeutic drugs. In veterinary medicine, the legal utilization of these compounds is strictly regulated, with withdrawal periods between treatment and slaughtering and maximal residue levels (MRLs) established for some compounds (Commission Regulation EU N. 37/2010).

In particular dexamethasone seems to be often involved in bovine illegal treatment protocols, as confirmed by the technical report published by the European Food Safety Authority (EFSA) on the "Results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products" that summarises the monitoring data collected in 2014 on the presence of residues of veterinary medicinal products in live animals and animal products all over the European Union (EFSA Report 2014) .

Dexamethasone, in fact, can be illegally used at very low dosage for a prolonged period of time before slaughtering to obtain an enhancement of bovine carcass yield at slaughterhouse.

It can be illegally used alone or in association with other growth-promoting substances to constitute unauthorized cocktails; the simultaneous administration of dexamethasone and oestrogens or β-agonists to synergize their growth promoting effects is widely reported (Abraham G. et al., 2004; Odore R. et al., 2007; Lopparelli RM et al., 2011).

Moreover this molecule, if fraudulently used, has the advantage of having a high and rapid rate of urine excretion that makes it not detectable by the official analytical chemical methods already a few days after the last administration (Vincenti M. et al., 2009; Ferranti C. et al., 2011).

Given the powerful pharmacological action carried out by dexamethasone, the illegal use of this molecule poses a serious issue for consumers' health: the possible accumulation of residues of dexamethasone in edible organs of cattle is, in fact, potentially dangerous. Among all the adverse effects, the most dangerous for human health include: the immunosuppressive action and the alterations of glucose metabolism, with increased glycemia and resistance to insulin (Lupu F. et al., 2001). Furthermore, the transfer of GCs and their metabolites through the placental barrier to the foetus is associated to abortions, premature births, adrenal insufficiency of the newborn and delays in skeletal growth and brain development (Allen DB 1996). Even the exposure through breast milk may hesitate in a series of negative effects, such as decreased growth, metabolic disorders and bone mineralization impairment (Yeh TF. et al., 2004).

For all the reasons stated above, many research groups are focusing their efforts in overcoming the limits of official control methods through shifting the target from the detection of the illegally-administrated molecules, to the study of their biological effects. New approaches have been recently investigated: tissue and serum biomarkers, biosensors, -omic techniques (Biancotto G. et al., 2013; Bovee TF. et al., 2013; Cacciatore G et al., 2009;

Courant F. et al., 2009 ; Divari S. et al., 2010; McGrath TF. et al., 2013; Nebbia C. et al., 2011; Pezzolato M. et al., 2013; Pirro V. et al. 2015)

In the attempt to develop an accurate biological method to detect illicit glucocorticoids treatments in food-producing animals, microscopic modifications of the thymus induced by the administration of low-dose dexamethasone were preliminary investigated in veal calves. The results of this previous works were obtained on a limited number of calves and data published needed to be confirmed in order to be applicable in a routine-based workflow (Biolatti B. et al., 2005; Bozzetta E. et al., 2011).

The main purpose of this experimental trial was to study thoroughly and concurrently histological alterations, i.e. fat infiltration and cortex-medulla ratio, induced by low-dose dexamethasone treatment as robust and reliable parameters to accurately evaluate the performance of a relatively simple and not time consuming analytical approach, in order to correctly identify treated calves. The objective was to implement the Italian Histological Residues Control Plan that has been successfully applied since 2008 in the context of the Italian Residues Control Plan. Therefore the final aim of this work is to protect the consumers against the possible adverse effects caused by the ingestion of glucocorticoids' residues.

Materials and Methods

Animals and experimental design

The study was set up as a randomised controlled blind clinical trial. The whole experimental trial was carried out in accordance with the European Council Directive 86/609, recognised and adopted by the Italian Government (DLgs 27/01/1992 no. 116). The experiment was authorized by the Italian Ministry of Health and the Ethics Committee of the University of Turin. At the end of the sampling procedure, the carcasses of the treated animals were destroyed according to the law in force (Directive 2003/74/EC)

Due to the wide number of animals included in the experiment, the study was developed during three years (from may to October) with three cycles of farming.

The sample size was calculated to detect a statistically significant difference in Cortical/Medulla ratio between group A (treated) and group B (control) with a power of 95% and level of significance of 5%.

Overall 180 male veal calves were recruited, randomly divided into two groups (group A and B) and raised in multiple pens for 5 months under the same conditions. Each pen had its own crib, multiple drinking troughs, and a dedicated automated milk feeder system. The calves were vaccinated against Bovine Infectious Rhinotracheitis (IBR), Parainfluenza3 (PI3), Bovine Syncytial (BRS) and Bovine Viral Diarrhoea Viruses (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 hormonally active substances.

The calves were fed through an automatic milk feeder; corn silage was increasingly added up to 1 Kg/day during the fourth month according to the indications suggested by European Commission Decision 97/182. Before administration, all feeds, milk replacer and corn were analyzed with an Enzyme-Linked Immuno Assay (ELISA) to exclude the presence of hormonally active substances.

During the sixth month, the animals without insurgence of clinical signs, hence did not require medical treatments, entered the experiment (n=172). Calves belonging to Group A (n = 106) were given a daily dose of 0,4 mg of dexamethasone-21-disodium-phosphate *per os per capita* (dexadreson) for 20 consecutive days orally, according to a presumed anabolic protocol of treatment. Animals belonging to Group B (n = 66) were used as control.

The animals were all slaughtered ten a day in an EC certified slaughterhouse about 10 days after the last drug administration, control animals were slaughtered after the treated ones.

Histopathology

Sample preparation

At the slaughthouse the central portion of the thoracic thymus of each animal was sampled, fixed in 4% buffered formaldehyde at room temperature for about three days, routinely processed, embedded in a paraffin wax, sectioned in 3-5 µm slices and stained with haematoxylin and eosin (HE).

Histopathological characterization of thymus atrophy

The morphology of the thymus parenchyma was evaluated by two expert pathologists using light microscopy in two different session works and in blind.

The presence of adipose tissue, as indirect marker of thymus atrophy, was evaluated by light microscopy at low magnification (1x and 4x) and a grading was attributed to the amount of fat infiltration: grade 1 was attributed to minimal or mild invasion of adipose tissue localized within the thymus septa; grade 2 was attributed to moderate invasion of adipose tissue in septa with minimal invasion of cortex part of the thymus; grade 3 was attributed to severe invasion of adipose tissue in the cortex of the thymus with invasion of the medullar part (Fig. 1).

Morphometry

The thymus sections were also examined at low magnification (4x) using a digital microimaging device (Leica DMD108 Digital micro imaging device for clinical diagnostics labs) to evaluate cortex and medulla thickness. Assuming that the lobule is the morpho-functional unit of the thymus and that every lobule is composed of an outer cortex and inner medulla and surrounded by connective tissue, for each slide, five lobules were randomly selected and measured against a graduated line, starting and ending at level of the interlobular connective tissue; a second line was drawn just in correspondence of the first to measure

medullar diameter (Fig 2). Cortex thickness was obtained by subtracting the medullar diameter from the corresponding diameter of the entire lobule, then the cortex-medulla ratio was calculated.

All measures were recorded on a spreadsheet.

Statistical analysis

A descriptive analysis on the outcome variables was carried out.

The association between thymus fat score and group (Group A vs Group B) was assessed by Fisher's exact test.

In order to fit the Gaussian distribution of the data, a logarithmic transformation was applied. Then, the distribution of cortex-medulla ratio (C/M ratio), of the cortex and of the medulla thickness among groups was evaluated using a linear mixed model. A mixed-effects model consists of two parts, fixed effects and random effects. Fixed-effects terms are usually the conventional linear regression part and are not modelled, and the random effects represent the varying coefficients and they are associated with individual experimental units drawn at random from a population. It is a term that refers to the randomness in the probability model for the group-level coefficients. The fixed effect was the group of animals while the random effect was the animal nested within group; lobules, collected for each animal, represented the residual. The analysis of residuals was adopted for checking the models.

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).

Fixed cutoff thresholds optimizing sensitivity and specificity values for thymic degree of atrophy and cortex-medulla ratio were calculated using the Receiver Operating Characteristic (ROC) curves which plot sensitivity against (1 − specificity) across all possible cut-off thresholds. The combination of the two histological tests was evaluated using parallel test

interpretation and assuming conditionally dependence of the two binary tests (Gardner IA. Et al., 2000). The extent of the dependence has been calculated estimating the covariance between the two test results. The covariance in the two groups has been calculated as suggested by Dohoo et al. (2009).

All data analyses (thymus atrophy, cortex thickness, medulla thickness and cortex-medulla ratio) were performed using Stata 11.2 (Copyright 1985-2013 StataCorp LP Statistics/Data Analysis StataCorp). The P value of 0.05 was considered as the level of statistical significance.

Results

One hundred and seventy-two animals concluded the trial without insurgence of clinical signs or requiring medical treatment and entered the data analysis process.

Histopathological characterization of thymus atrophy

The results obtained by the microscopic observation showed that the scores of 1, mild, and 2, moderate infiltration of fat, were detected in both treated and control animals, whereas the score of 3, severe infiltration of fat, even if detected in both group was mainly attributed to the group of the treated animals and recorded in only four controls.

The amount of fat infiltrating the thymic tissue resulted significantly associated with the treated group by Fisher's exact test (p=0.001), even if the trend was not linear as shown in Table 1 in terms of Odds Ratio.

The Cohen's Kappa of 0.78 (95% CI = 0.69 - 0.88) established a good repeatability among pathologists .

Comparative morphometry

Microscopic examination at a high magnification showed a decrease in the cortical area of the treated animals verified by measured recorded results.

Descriptive statistics of cortex thickness, medulla thickness and cortex-medulla ratio (C/M ratio) stratified by treatment group are provided in Table 2.

The distribution of the C/M ratio between the two groups is provided in Graph 1: a significantly different distribution between the two groups ($p<0.0001$) was evident.

Table 3 shows the results of the comparison performed by the linear regression model. The three parameters taken into account (Cortex thickness, medulla thickness and C/M ratio) resulted significantly different between the two groups; In particular the model results put in evidence that the C/M ratio is one-third lower in the treated animals compared to the control ones.

To assess the combined performance of thymic degree of atrophy and cortex-medulla ratio to detect glucocorticoids illicit treatments the ROC curves were examined to select optimal fixed cutoff thresholds for each histological test. In table 4 test sensitivity, specificity and the cutoff values are reported for the two histological methods. Parallel testing, with fixed cutoff thresholds of ≥ 2 thymic degree of atrophy and ≤ 1.24 cortex-medulla ratio, increases sensitivity to 88% (95% CI 80%-93%) at the expense of specificity (63% - 95%CI:54% - 78%).

Considering the conditionally dependence of the two tests, the sensitivity and specificity are, respectively, 90% and 61%.

Discussion

Our findings confirm that the administration of low-dose dexamethasone as a growth promoter in veal calves, according to a protocol often illegally adopted in farm practice (Biolatti B. et al., 2005), can induce morphologic changes in the thymus, resulting in a significant reduction in the cortex-medulla ratio (Bozzetta E. et al., 2011). In 1998 Schilt (Schilt R . et al., 1998) first reported this finding as a valuable feature to distinguish between beclomethasone treated and

untreated veal calves. Later Elmore in 2006 included this parameter among criteria to distinguish between chemical-induced and age or stress-related thymic atrophy. Here we show the performance of this parameter calculated on a large number of animals raised under controlled conditions. Cortical atrophy and fat infiltration has been previously reported by several research groups (Biolatti B, et al., 2005; Cannizzo FT. et al., 2008; Groot MJ. Et al., 1998) as co-existing finding in dexamethasone-fed veal calves; our experiment confirms that fat infiltration and cortex-medulla ratio could be associated with low-dose dexamethasone treatment, with high statistical support.

Prolonged treatment with glucocorticoids at low doses causes perceivable changes in thymus architecture.

In a previous work, two histological parameters i.e. fat infiltration and cortex-medulla ratio, were investigated and results showed that the cortex-medulla ratio performed well in discriminating treated versus untreated calves (Bozzetta E, et al., 2011). Nevertheless, in contrast with data reported in literature, no statistically significant differences were found regarding fat infiltration between the two groups (Biolatti B. et al., 2005; Cannizzo FT. et al., 2010; Vascellari M. et al., 2012; Groot MJ, et al., 1998)

This work was set up in order to investigate the performance of the histological method in order to evaluate its efficacy as screening method in the field of the histological part of the Italian Residues Control Plan. Final results from the analyses of all animals' thymus strengthened the preliminary results obtained on the reliability of cortex-medulla ratio, that was decreased in treated animals according to the results obtained by Bozzetta et al., 2011 even if in the latter thymus atrophy of treated calves was not supported by the statistical analysis. At the end of our triennial experimental study results are different and the statistical analysis confirms that treated calves can be successfully separated by untreated animals considering the atrophy parameter.

The parallel testing analysis allowed to evaluate the detection performance for the combined evaluation of the thymus degree of atrophy and cortex-medulla ratio.

Parallel testing with fixed cutoff thresholds of ≥2 for thymus degree of atrophy and ≤1.24 for cortex-medulla ratio maximizes the ability to detect anabolic treatments increasing the sensitivity of the method. The results of the combined employment of the two parameters shows the possibility to use the parallel analysis in the screening phase of the glucocorticoids illicit treatments monitoring.

Conclusion

This scientific and statistically robust finding can finally confirm that this simple parameter is able to distinguish the presence of illicit treatment achieved with synthetic glucocorticoids and can be successfully applied in the Italian Residue Control Plan. The European Food Safety Agency recommends that all the efforts to improve the control of illegal use of growth promoters in cattle should include novel screening methods, able to highlight the biological effects of growth promoters in livestock rather than measuring their residues concentration in food or feed (European Food Safety Authority 2013).

For that reason and based on these results we can conclude that histological investigations i.e. specifically, the assessment of thymus morphometry represent a standardized and powerful method for screening purposes.

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388

Table 1. The linear trend in the degree of thymic atrophy. The trend is not statistically significant. Score test for trend of Odds *p=0.26*

FAT SCORE	OR	CI95%	
1	-	-	1
2	1.43	0.09	2
3	5.63	0.32	3

Table 2. Descriptive statistics for the parameters of thymus samples

PARAMETER	GROUP	MEAN	MEDIAN	SD	Min	Max
Cortex thickness	Treated	666.9	634.8	261.1	153.8	1795
	Control	909	858.4	356.1	169.6	2175.4
Medulla thickness	Treated	685	610.3	297.8	81.4	2100
	Control	581.1	550	208.6	117.5	1300
Cortex-Medulla ratio	Treated	1.1	1	0.6	0.2	5.3
	Control	1.7	1.5	0.9	0.3	7.5

Table 3. Results of the linear mixed models. The dependent variables are the features of the thymus and are expressed as log. All the variables resulted significantly different between the two groups.

DEPENDENT VARIABLE	ESTIMATE	STANDARD ERROR	ADJUSTED *p*
Cortex thickness	-0.31	0.34	0.000
Medulla thickness	0.15	0.32	0.000
Cortex-Medulla ratio	-0.47	0.44	0.000

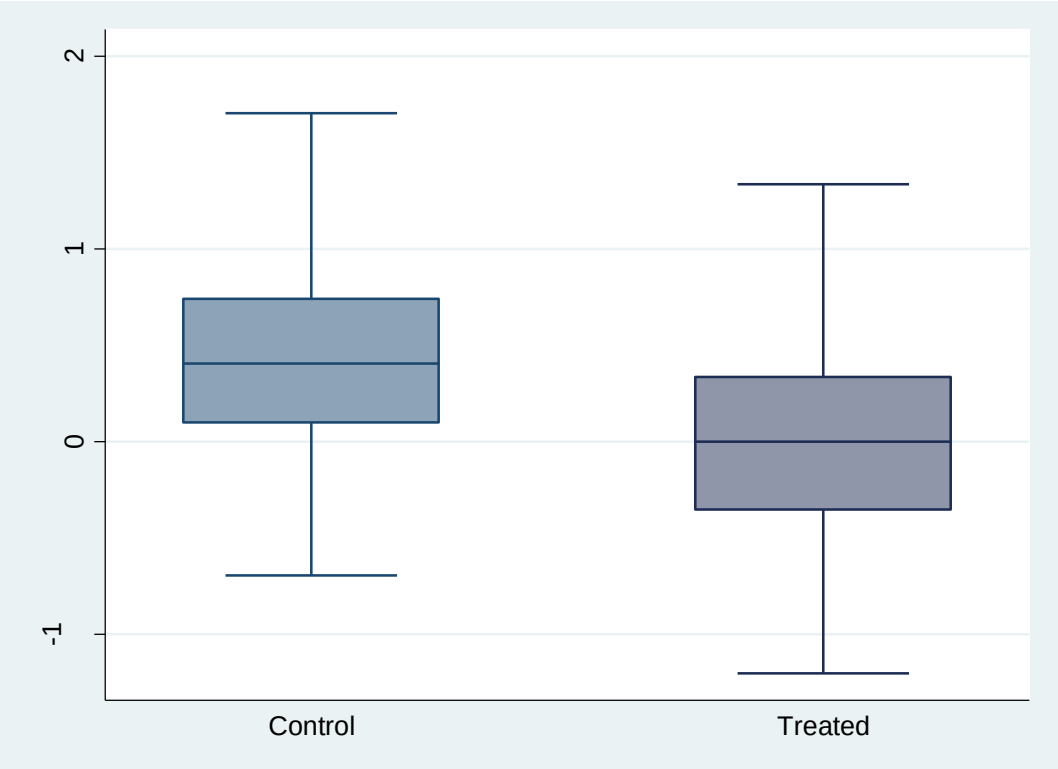
403 **Table 4. Sensitivity and specificity of the thymic degree of atrophy test and cortex-**

404 **medulla ratio test, including 95% CIs and cutoff thresholds values**

405

DIAGNOSTIC METHOD	Sensitivity	Sensitivity CI 95%	Specificity	Specificity CI 95%	CUTOFF
Thymic degree of atrophy	76%	66% - 83%	82%	70% - 90%	≥2
Cortex-medulla ratio	52%	42% - 62%	77%	65%-87%	≤1,.24

Graph 1. distribution of the C/M ratio values between the two groups. Outliers have been excluded. The difference is resulted statistically significant (*p<0.0001*).



Figures captures

Fig. 1. Thymus atrophy grading; mild (A), moderate (B), severe (C).

Fig. 2. Thymic morphometry: Extension of the cortex was measured against a graduated line (red) starting and ending at the interlobular connective or adipose tissue; a second parallel line was drawn to measure medulla thickness (green) (HE 4X).