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

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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 concurrently test the potentiality of both parameters to discriminate between treated and untreated animals.

One hundred and seventy-two 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, ranging 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.

1. Introduction

Glucocorticoids (GCs) are widely used in bovine internal medicine for their anti-inflammatory properties, but are also illegally used, despite the Directive 2003/74/EC of the European Parliament and of the Council, 2003, in food-producing animals as growth-promoters constituting a health risk for the consumers (Botsoglou and Fletouris, 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, 2001; Schuerholz 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 of 22, 2009).

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

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over the European Union (European Food Safety Authority (EFSA), 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 et al., 2004; Odore et al., 2007; Lopparelli 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 et al., 2009; Ferranti 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 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, 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 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 et al., 2013; Bovee et al., 2013; Cacciatore et al., 2009; Courant et al., 2009; Divari et al., 2010; McGrath et al., 2013; Nebbia et al., 2011; Pezzolato et al., 2013; Pirro 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 et al., 2005; Bozzetta 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.

2. Materials and methods

2.1. 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.

3. Histopathology

3.1. 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).

3.2. 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 (1 × and 4 ×) 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).

3.3. Morphometry

The thymus sections were also examined at low magnification (4 ×) using a digital microimaging device (Leica DMD108 Digital micro imaging device for clinical diagnostics labs) to evaluate cortex and