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The transcriptome of muscle and liver is responding differently to a combined trenbolone acetate and estradiol implant in cattle

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ABSTRACT

We investigated the transcriptomic signature of some anabolic steroids in cattle. Our main objective was to evaluate the effect of a combined trenbolone acetate (TBA, 200 mg) and estradiol-17β (E2, 40 mg) implant (Revalor-XS®, REV) on the transcriptome of muscle (target tissue for anabolic steroids) and liver (main biotransformation site). Transcriptomic profiling was performed on 60 samples (30 per tissue) representing 2 groups of animals: REV (sustained release implant for 71 days, $n = 15$), and a control group (CTR, $n = 15$). The analyses (REV vs. CTR) evidenced the differential expression of 431 (down-regulated) and 503 transcripts (268 up-regulated and 235 down-regulated) in muscle and liver tissues, respectively. Functional annotation showed the enrichment of several ion transport systems (cation, metal ion and potassium ion transport) in muscle, while revealing the enrichment of carbohydrate, protein and glycoprotein metabolism and biosynthesis mechanisms in the liver. Both tissues had 20 genes commonly expressed in-between. Seven randomly-selected genes showed positive correlation with their corresponding microarray data upon a qPCR cross-validation step. In muscle, but not the liver, Principal Component Analysis (PCA) on the microarray data resulted in the separation of treated animals from the untreated ones (first 2 components = 97.87%.). Overall, the identification of different genes, pathways and biological processes has illustrated the distinctive transcriptomic profile of muscle and liver in response to anabolic steroids. Moreover, it is becoming more clear that anabolic steroids are working through a complex interaction of numerous pathways and processes incorporating different tissues.

1. Introduction

Beef cattle production is a strong animal industry worldwide. The economic challenges and the profit-oriented strategies in this business are, logically, favoring the cost-reduction procedures. Growth promoters (GPs), such as anabolic steroids, are used to increase animal productivity and feed conversion rates; hence, reducing costs. Although they are legally and widely used in the USA, anabolic steroids are prohibited by law in the EU [1,2] because of their public health concerns. Despite this ban, GPs are still illicitly employed in bovine meat production due to their economic benefits resulting from the improved animal growth [3–6]. Subcutaneous implantation of androgenic and estrogenic compounds, such as trenbolone acetate (TBA) and β-estradiol (E2), administered alone or in combination, is one of the most common ways of anabolic steroid applications for cattle, due to its sustained release that can last up to 200 days [7].

On a monitoring-and-drug-abuse-discovery basis, some studies have been performed for the indirect detection of TBA and E2 in cattle in Europe, in which investigations on transcriptomic profiling of thymus [8], uterine endometrium [9], vaginal smears [10] and muscle [11,12] have been conducted. In the same context, other groups have investigated the use of alternative "omic" techniques, such as proteomics [13] and metabolomics [14,15].

Being the main target organ of such anabolic steroids, muscle has been given the main attention in the few "omic" studies that investigated the effect of TBA and/or E2 on muscle either in a physiological [11] or detection and monitoring context [8,9,12,13,16]. However, despite the well-documented effectiveness of anabolic steroid implants in increasing bovine muscle growth, information on the mechanism of action in nonmuscle tissues (e.g. Liver) have not been extensively studied [17]. Moreover, the liver has never been considered in a global gene expression microarray analysis following TBA or E2 administration, and was only considered either in direct residue detection studies [18–20] or mRNA expression of a set of pre-defined genes [6,21,22]. The fact that anabolic

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steroids can cause a number of serious side effects, including immunity and liver dysfunction [23] was a further reason for the inclusion of liver in our study.

In the present study, we conducted a comparative investigation on bulls treated by a combination of anabolic compounds (sexual steroids) forbidden in the European Union, and untreated bulls. We used a commercially available ear implant containing 200 mg of TBA and 40 mg of E2 (Revalor-XS®, Merck Animal Health, USA) that is approved in the United States in bovine breeding. To the best of our knowledge, this was the first comparative global gene expression profiling study to be performed on both muscle and liver of cattle implanted with Revalor-XS®. The primary aim of this study was to compare, via transcriptomic approach, the response of both muscle and liver to anabolic steroids, and to point out the main differences and similarities between both matrices. In addition, a second aim was to list out the main differentially expressed (DE) genes that can be used as a "pool" for biomarker investigations in the future.

2. Materials and methods

2.1. Animals and experimental design

Animals in this study were part of a transcriptomic–proteomics comparative trial, where 30 Charolais cattle, all males, aged 10–14 months, were randomly divided into two groups of 15 animals each. The first group received no treatment and served as a control (CTR), while the second was exposed for 71 days to the steroid hormone subcutaneous implant Revalor-XS® (group REV). The beef cattle were weighed at the beginning and the end of the treatment. The average daily gain (ADG) was calculated as the difference between two subsequent body weights. The procedure was checked and approved by the Animal Experimentation Ethics Committee of the University of Bologna on January 31, 2011 (PROT: 8134-X/10 and 4783-X/10). Health status was monitored daily by recording all individual pathological events and medical treatments. The experimental workflow is shown in Fig.1.

2.2. Sample collection and RNA extraction

At the slaughterhouse, small *biceps brachii* muscle and liver specimens were sampled from all the animals. Both muscle and liver samples were immediately stored in RNAlater solution (Life Technologies, USA), then stored at −80 °C until analyses. Total RNA was isolated by TRIzol® reagent (Life Technologies, USA) and subsequently purified using the RNeasy Mini kit (Qiagen, Italy), according to the manufacturer's instructions. To avoid genomic DNA contaminations, on-column DNase digestion with the RNase-free DNase set (Qiagen, Italy) was performed. Total RNA concentration was determined using the NanoDrop ND-1000 UV–Vis spectrophotometer (NanoDropTechnologies, USA), and its quality was measured by using the 2100 Bioanalyzer and RNA 6000 Nano kit (Agilent Technologies, USA). The isolated RNAs were tested for proper concentration and integrity. All the 60 samples (30 from muscle and 30 from liver) passed the RNA quality criteria (i.e., RNA concentration $\geqslant$ 40 ng/μl and RNA integrity number (RIN) $\geqslant$ 7). Hence, they were all considered for the subsequent microarray analyses.

2.3. RNA amplification, labeling and hybridization

Sample amplification, labeling and hybridization were performed following the Agilent One-Color Microarray-Based Gene Expression Analysis protocol. Briefly, each of the 60 RNA samples was labeled with Cy3 (green) fluorescent dye label using Agilent Low Input Quick Amp Labeling kit (Agilent Technologies, USA). Ten different viral polyadenylated RNAs were used as reference "spikes" (Spike-In Mix, Agilent Technologies, USA). A purification step was applied to the labeled cRNA using the RNeasy Mini kit (Qiagen, Italy), and sample concentration and specific activity (pmol Cy3/μg cRNA) were measured. A total of 1.65 μg of labeled cRNA was fragmented using the Gene Expression Hybridization kit (Agilent, USA) according to the manufacturer's instructions, and finally diluted by the addition of 55 μL of 2× GE hybridization buffer. A final volume of 100 μL of the hybridization sample mixture was dispensed on the gasket slide and then the active side of the microarray slide was placed on the top of it to form a "sandwich slide pair". Bovine-specific oligo-arrays (Bovine V1, 4x44k G2519F, Design ID 015354, Agilent Technologies, USA) were used. The slides were firstly incubated for 17 h at 65 °C in a hybridization oven (Agilent Technologies, USA), then washed using wash buffer 1 and 2 according to the manufacturer's instructions. The hybridized slides were scanned at 5 μm resolution using a G2565BA DNA microarray scanner (Agilent Technologies, USA). The default settings were modified in order to scan the same slide twice at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). The total

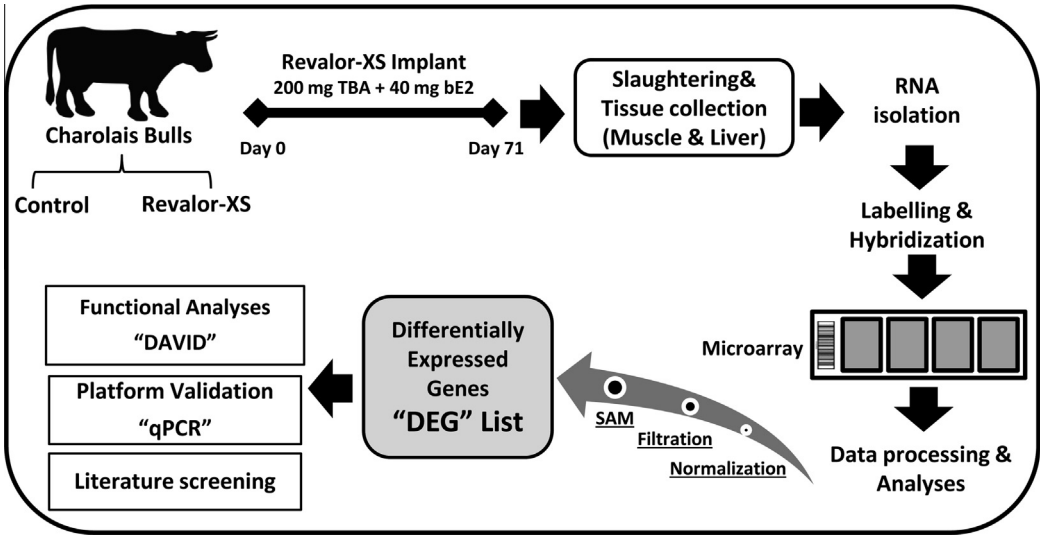

Fig. 1. General workflow of the experiment.