Histological and Molecular Approaches to Detect Illegal Use of Anabolic Steroids in Lambs

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Abstract

Since 1996, the European legislation has prohibited the use of growth-promoting hormones in livestock production because of their potential risk to developing cancer in human consumers. The main goal of this study was to define and validate a screening test suitable for detection of anabolic treatments in lambs using histological, immunohistochemical and molecular approaches. The resulting information could be used to develop preventive and containment measures to avoid problems similar to those already revealed in bovine production. In particular, we have inspected the effects of estrogen treatment in sheep, a poorly studied species whose consumption has recently increased in Italy. Histological modifications induced by hormonal treatment (17β-estradiol) in target tissues (prostate, bulbourethral glands, ovary, udder, Bartolin's glands, thymus, heart, lung, liver, spleen, adrenal glands and striated muscle) were investigated. Histologic and immunohistochemical (IHC) analyses revealed a clear squamous metaplasia of the urethra, with a marked hyperplasia of both urethra and prostate gland, in treated animals. The IHC for cytokeratin 5/6 showed a higher expression than in control (untreated) animals and metaplasia of both urethra and glandular ducts in treated animals, due to the proliferation of basal cells. Furthermore, RT-PCR assay showed a significant up-regulation of four estrogen-regulated genes (IGF-I, IGF-IR, PR and TFF3) in treated animals. These results suggest that the potential of a histologic screening test could be improved by its combination with measurement of molecular biomarkers, eventually leading to detect the illegal use of hormonal treatment of animals with a higher accuracy.

Keywords: Lambs; Anabolic steroids; Histological analysis; Immunohistochemistry; RT-PCR; Estrogen-regulated genes

Introduction

Since 1996, the European legislation has prohibited the use of growth-promoting hormones in livestock production because of their potential risk to developing cancer in human consumers. The main concern about the use of anabolic products in livestock production in other non-EU countries, including Canada, USA and South Africa [1], is mainly related to cancer risk through the repeated consumption of red meat. This association has been supported, though indirectly, by epidemiological studies [2] reporting highest incidence rates of breast and prostate cancer in meat consumers in those countries where the use of growth-promoting substances is legal. In Italy the use of growth-promoting hormones or other anabolic agents is forbidden and the National Government has implemented control plans (D. Lgs. n. 158/2006) which constantly provide field surveillance and laboratory tests in order to monitor any possible food-borne health risk in our country. The main target of this national surveillance plan are beef cattle farms where illegal treatment have sometimes been discovered by the local veterinary services in charge of food control.

One limit of the Italian current national plans is represented by its limited range of investigation in the number of either products monitored or species involved (only large ruminants/sheep calves). Other domestic species (small ruminants, pigs, horses) have never been properly monitored for the presence of growth promoters, even though they represent an important source of protein for the Italian food market. Despite the ban [3], the use of anabolic steroids is still practiced and specific and sensitive diagnostic methods for detecting anabolic treatments are still needed. Surveillance programs have been developed in order to guarantee food safety and to protect public health. Analysis of residues in livestock production is mainly based upon chemical [4], immunochromical or biological [5,6] tests and an additional mass spectrometry test as a confirmatory method.

The current laboratory approach is based on looking for “well known” compounds, or those more frequently identified, whereas the real scenario may include new cocktails, natural compounds or even new molecules which often escape the official analytical techniques. These agents are commonly used in minimal concentrations and may therefore escape detection being under the lower limits established by the Veterinary Public Authority. Furthermore, new more effective synthetic steroids have been produced that are often administered as esterified molecules and illegally used as growth-promoting agents in meat production [7].

In Italy histological screening has been introduced since 2009 to detect illegal use of anabolic drugs because it has been proved that sex hormones induce specific, permanent morphological changes in steroid target organs [8], especially those of the genital tract, being, in some cases, visible by the inspector at abattoir [9–11]. In an effort to overcome the technical difficulties mentioned above, gene expression analysis could represent an attractive new method to detect the illegal use of growth-promoting substances. Several studies have demonstrated significant changes in mRNA levels of selected genes in tissues of bovine treated with growth promoters [12–14]. In the present study we have investigated histological modifications and gene expression variations induced in the genital tracts/target organs of lambs by treatment with 17β-estradiol, eventually leading to develop an accurate screening method to identify estrogen-treated animals.
Materials and Methods

Estrogen Treatment

Eighty healthy Valle del Belice x Comisana weaned lambs were used in the study. After one week of an acclimatization period, they were divided into 4 homogeneous groups according to age (55 ± 10 days), sex and body weight. All lambs were fed with vetch and oat hay offered ad libitum and 520 g/head/d of commercial concentrate; fresh water was always available.

Lambs were monitored daily for clinical signs and behavioral abnormalities occurring during the period of the experimental trial. Two groups of 20 lambs of both sexes (20 male and 20 female lambs) were treated with 0.5 ml/head solution of oil Depot Estradiol® (containing 5 mg of 17β-estradiol valerate) by intramuscular injection into the thigh. The estradiol administration was repeated four times weekly. Two control group lambs of both sexes (20 male and 20 female lambs) received placebo (0.5 ml saline solution). Thirty days after the last treatment lambs were slaughtered and tissues collected for pathology, immunohistochemical examination and RT-PCR analysis.

At necropsy, target organs (prostate, bulbourethral glands, ovary, udder, Bartholin’s glands) and thymus, heart, lung, liver, spleen, adrenal glands, striated muscle were collected and examined. After pathological evaluation, samples were immediately fixed in 10% buffered formalin and processed for histology. Once tissues were paraffin embedded, they were sectioned at 5 μm, and stained with hematoxylin-eosin (HE) by standard procedures. Sections were examined by light microscopy in order to evaluate any histological changes resulting from the anabolic treatment. All animals have received human care in compliance with the “Guide for the Care and Use of Laboratory Animals” and experimental procedures were designed in order to avoid any animal distress according to the European (EC Directive 86/609/ EEC for animal experiments) and Italian animal health regulation.

Histological Examination and Immunohistochemistry

Tissues were fixed with 10% phosphate-buffered formalin and embedded in paraffin. Sections (4 μm thick) were cut and stored at room temperature until required. Routine histology (hematoxylin-eosin staining) was performed in order to evaluate basic histological features of tissues. The avidin-biotin-peroxidase complex (LSAB, Dako®) method was used for immunohistochemical study. Tissue sections were sequentially dewaxed through a series of xylene, graded alcohol and water immersion steps. Antigen was retrieved in citrate buffer (pH 6.0) microwave digestion (2 cycles of 5 min each at 750W). Endogenous peroxide activity was suppressed by 10 minutes of hydrogen peroxide incubation. Sections were rinsed in PBS, all tissue sections were incubated with 1% bovine serum albumin for 30 minutes at room temperature. After two x 5-minute rinses with PBS, all tissue sections were incubated with 1% bovine serum albumin for 30 minutes at room temperature. A monoclonal antibody was used to detect keratins with high molecular weight, expressed in the basal and intermediate cell layers of stratified epithelia (Cytokeratin 5/6, Dako®, clone D5/16 B4, monoclonal mouse, cod. M7237); Primary antibody was diluted in PBS (1:100) with 0.1% normal albumin serum and sections were further incubated overnight at 4°C. After incubation, slides were rinsed three times with PBS for 5 minutes, and a secondary biotinylated immunoglobulin (LSAB, Dako) was applied for 30 minutes at room temperature. After two x 5-minute rinses with PBS, tissue sections were incubated in PBS for 1 hour at room temperature with streptavidin, horseradish peroxidase conjugate. All tissue sections were rinsed three times with Tris-buffer saline (TBS), incubated with the chromogen 3-3′-diaminobenzidine tetrahydrochloride (DAB; Dako) diluted 0.035% in TBS for 1 minute, rinsed in tap water, and finally counterstained with Mayer’s hematoxylin. The specific primary antibody was replaced with PBS in tissue sections used as negative controls.

Tissue sections were also stained for TFF3 (trefoil factor family) using a rabbit polyclonal antibody (Santa Cruz Biotec., Dallas, TX) raised against amino acids 1-80 representing the full length TFF (Intestinal trefoil factor) of human origin. The TFF peptides are a family of small regulatory proteins consisting of three members [15]. TFF peptides are involved in the mucosal maintenance and over expressed during inflammatory processes and cancer progression [16,17]. Primary anti-TFF3 antibody was diluted in PBS (1:100) with 0.1% normal albumin serum and tissue sections incubated overnight at 4°C. After incubation, slides were rinsed three times with PBS for 5 minutes and a secondary biotinylated immunoglobulin (LSAB, Dako) was applied for 30 minutes at room temperature. After two x 5-minute rinses with PBS, tissue sections were incubated in PBS for 1 hour at room temperature with streptavidin, horseradish peroxidase conjugate. All tissue sections were rinsed three times with Tris-buffer saline (TBS), incubated with the chromogen 3-3′-diaminobenzidine tetrahydrochloride (DAB; Dako) diluted 0.035% in TBS for 1 minute, rinsed in tapwater, and counterstained with Mayer’s hematoxylin. The specific primary antibody was replaced with PBS in tissue sections used as negative controls. Images of all stained slides were captured using Leica DMR microscope equipped with a Leica DFC 320 digital camera.

RNA Extraction from Prostate Tissues and Reverse Transcription

Prostate tissues were obtained from 5 month-old lambs from both treated and control groups. All tissue samples were collected immediately after slaughter, snap refrigerated in RNA-later solution at 4°C and stored at 80°C until analysis.

Total cellular RNA was isolated using TRI Reagent (Sigma Chemical; St. Louis, MO). Briefly, 100 mg of prostate tissues were homogenized in 1 ml of TRI Reagent and incubated at room temperature for 5 min. After addition of 0.2 ml chloroform, tissue homogenates were incubated at room temperature for 10 min. The homogenates were then centrifuged for 15 min at 4°C at 12,000 g. The aqueous layer containing the RNA was precipitated using isopropanol and brief centrifugation. The RNA pellet was washed with 70% ethanol and suspended in RNase-free water. The extracted amounts of RNA were determined by measuring the absorbance at 260 nm and the RNA integrity was assessed by non-denaturing agarose gel electrophoresis. All the RNAs were treated with RNase-free DNase I to remove potential contamination of genomic DNA. The cDNAs were synthesized in the presence of random hexamer primer, using Super Script II reverse transcriptase (Invitrogen) and stored at -20°C until semi-quantitative real-time PCR was performed.

Conventional and Semi-Quantitative “Multiplex” (SM) Polymerase Chain Reaction (PCR)

The PCR was conducted with a GeneAmp® PCR System 9700 (Applied Biosystems). β-actin was used as internal standard for PCR analysis of insulin-like growth factor 1 (IGF-I) [18], insulin-like growth factor receptor 1 (IGF-IR) [18], progesterone receptor (PR) [19], trefoil factor 3 (TFF3) [20], estrogen receptor alpha (ERα) [21], aromatase (Aro) [22], androgen receptor (AR) [23], 3α-hydroxysteroid dehydrogenase (3α-HSD) [24]. Conventional amplification was performed using 35 cycles at 95°C for 30s, specific temperature of annealing (52-59°C) for 45s, 72°C for 45s, followed by 72°C for 5 min respectively. The SM-PCR analysis was performed using conditions whereby PCR products accumulate exponentially and their quantity increases in an
mRNA-dependent manner, as previously reported [25]. All PCR products were analyzed by gel electrophoresis on 2% agarose gel with ethidium bromide staining, followed by fluorescence digitization using the software "ImageJ 1.38X" (National Institutes of Health, USA).

Expression levels of each transcript were quantified relative to β-actin expression level and expressed as arbitrary units. Discrete cDNA bands were semi-quantitated by digitized evaluation of their optical density, after subtraction of background. The results were expressed as ratios of the intensity of the band of the investigated transcript(s) to the intensity of the band used as internal standard. For semi-quantitative analysis of the amplified products, a suitable number of PCR cycles for each gene and β-actin were determined so that it was within the exponential phase. Exponential regression equations fitted to the curves were used to calculate the number of cycles necessary to reach a normalized intensity threshold value = 1 for each sample. The relative difference in abundance between two samples was taken as 2n where n is the difference between the numbers of cycles required by the samples to reach the threshold. Three different RNA preparations from each experimental condition were pooled to make more significant the differences between the expression levels, if any.

In order to identify a set of potentially estrogen-regulated genes in the prostate of lambs, expression data were collected from published studies that were performed on human prostate carcinoma cell lines or other human endocrine-related tumors. A set of 8 estrogen-regulated genes belonging to different functional categories were selected. Oligonucleotide primer pairs were designed for Ovisaries IGF-I, IGF-1R, PR, TFF3, ERα, Aro, AR, 3β-HSD, using published literature or sequence information contained in the National Center for Biotechnology Information GenBank database. Oligonucleotide primers were tested using BLAST software to confirm gene specificity and to determine exon locations.

Statistics

The data were expressed as mean ± SD. Analyses were performed using computerized statistical software with the ANOVA test. When ANOVA revealed p < 0.05 the data were further analyzed by Dunnet’s t-tests. Differences were considered statistically significant at p < 0.05. The primers used for RT-PCR are reported in table 1.

Results

Pathological Examination and Histology

Gross examination showed a general enlargement of target glands in all treated lambs: bulbourethral glands and prostate for males, ovaries and genital tract for females. Identification and inspection of Bartolin’s glands was difficult or inconclusive because these glands in sheep are rarely present and identifiable only in a small number of animals.

Histological changes in treated animals showed marked metaplasia of epithelium while histology of prostate and bulbourethral glands in untreated group confirmed what has previously been described [9]. Immunohistochemical analysis of cytokeratin 5/6 (specific for the basal layer of epithelium) displayed metaplasia of epithelium in urethra and glandular ducts (both in prostate and bulbourethral glands) in treated animals, due to the proliferation of basal cells (Figure 3). Conversely, positivity for cytokeratins 5-6 was localized only in the basal layer of glandular epithelia in control animals (Figure 3). Interestingly enough, the expression of TFF3 (TIF) was significantly greater in both urethral epithelium and bulbourethral glands of treated animals with respect to control, untreated lambs (Figure 4).

Expression of Selected Estrogen-Regulated Genes

To determine the potential impact of E2 treatment on gene expression, we analyzed the expression of 8 selected estrogen-regulated genes in 16 lamb prostate samples (8 untreated animals and 8 treated animals). Estradiol was administered four times weekly and animals were slaughtered thirty days after the last injection. The prostate tissues of treated animals showed a significant up-regulation of the IGF-I gene (2.8 fold), IGF-1R gene (4.2 fold) and PR gene (2.1 fold). In addition, TFF3 gene was expressed in all treated prostate samples, but was not detected in the untreated prostate tissues (see Figure 5).

The mRNA encoding for ERα and AR was weakly expressed in all samples and no significant variation was observed between treated and untreated animals. Furthermore, Aro and 3βHSD genes were undetectable in all samples tested (data not shown). It is noteworthy that over-expression of selected genes was observed in the treated animals for thirty days after treatment with E2 had been suspended, suggesting that the biological effects of E2 administration are persistent.

Discussion and Conclusions

Estrogens are the most frequently used illegal growth-promoting agents in animal production [26]. Continuous improvement of detection methods is necessary to follow the rapid and constant development of new unknown anabolic substances. The purpose of this study was to detect histological modifications and variations in gene expression induced by estrogen treatment in lambs, eventually leading to develop a screening approach for the identification of animals illegally treated with growth-promoting hormones, notably estrogens.

The prostate and urethra of lambs treated with estrogens showed typical histological changes of epithelial metaplasia and hyperplasia. Moreover, the proliferating cells of both prostate and urethra epithelium of treated animals were strongly positive for cytokeratin 5/6, indicating a significant proliferation of cell basal layer. Histological screening methods based on the histopathological effects of anabolic treatment on target tissues have been developed and successfully implemented [8,10]. The use of indirect biomarkers in target tissues for the detection of illegal administration of anabolic steroids in livestock animals should be considered a complementary, rather than alternative, method to histological analysis, since their combination might allow identifying not only individual anabolic agents, but also the administration of anabolic cocktails. In this framework, it is of utmost importance to implement a validated combination of screening and confirmatory methods to deal with a relatively large number of samples and to secure a high reproducibility of results.

Recently, it has been shown that specific genes are differentially responsive to various combinations of growth promoters (boldenoneundecylenate and estradiol benzoate, testosterone

Table 1: List of primers used for RT-PCR of estrogen-regulated genes. The sequences of forward (for) and reverse (rev) primers, the annealing temperature and the accession number for each gene are reported. [Abbreviations: IGF-I: Insulin-Like Growth Factor I; IGF-IR: Insulin-Like Growth Factor Receptor I; PR: Progesterone Receptor; TFF3: Trefoil Factor 3; ERα: Estrogen Receptor Alpha; ARO: Aromatase; AR: Androgen Receptor; 3β-HSD: 3β-Hydroxysteroid Dehydrogenase].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'-3')</th>
<th>Annealing</th>
<th>Accession No</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>for - TTGGTGGATGCTCTCCAGTTC&lt;br&gt;rev - AGCAGCACTCATCACAGATTTC</td>
<td>57°C</td>
<td>NM_001009774</td>
</tr>
<tr>
<td></td>
<td>IGF-IR</td>
<td>57°C</td>
<td>AJ162434</td>
</tr>
<tr>
<td></td>
<td>for - AAGAACCATGCTGCAAGAGG&lt;br&gt;rev - GGATCTCGATGCTGCGGATCATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>for - ACTGCCCAGCATGCTGACTTT&lt;br&gt;rev - CCACTGGCTGTTGGAGGCA</td>
<td>59°C</td>
<td>Z66555</td>
</tr>
<tr>
<td>TFF3</td>
<td>for - GTGGGCTTGGCAGGAAACCA&lt;br&gt;rev - AAGCAGCAGCGCGGCTTGTT</td>
<td>59°C</td>
<td>DQ152995</td>
</tr>
<tr>
<td>ERα</td>
<td>for - CTGCCGCGCCGCTTAAAGG&lt;br&gt;rev - CTGCTGGCTGCTTGCCTC</td>
<td>59°C</td>
<td>Z49257</td>
</tr>
<tr>
<td>ARO</td>
<td>for - CTTCCCCTTCTCTGGGAATT&lt;br&gt;rev - TCGGATATGTAAGCATGCTGCT</td>
<td>55°C</td>
<td>AJ012153</td>
</tr>
<tr>
<td>AR</td>
<td>for - GCCCGCTGCTGCTTTCATCA&lt;br&gt;rev - TCCGCGACAGCGCTGTACCA</td>
<td>59°C</td>
<td>AF105713</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>for - AACAACGGCATCTCTGAC&lt;br&gt;rev - AAGCCCGGCTTCTTGCTC</td>
<td>52°C</td>
<td>NM_001135932</td>
</tr>
<tr>
<td>β-Actin</td>
<td>for - CCAGCAGTGAAGATCAAG&lt;br&gt;rev - ATCTGCTGGAGGCTGAC</td>
<td>56°C</td>
<td>HM067830</td>
</tr>
</tbody>
</table>

Figure 1: Histological examination of lambs untreated and treated with 17β-estradiol. Metaplasia of prostatic tissue and bulbo-urethral glands surrounding urethra lumen is here illustrated in treated animals as compared to control, untreated animals. HE, 5X; for methodological details see text.
Figure 2: Histological changes in the bulbo-urethral glands of treated lambs as compared to untreated animals. HE, 5X; for methodological details see text.

Figure 3: Immunohistochemistry of cytokeratin 5/6 in epithelium in prostate and bulbo-urethral glands of lambs untreated and treated with 17β-estradiol. For methodological details see text.
**Figure 4:** Immunohistochemical assay of TFF3 (ITF) in urethral epithelium and bulbo-urethral glands of control animals and lambs treated with 17β-estradiol. Scale bar 50 µm. For methodological details see text.

**Figure 5:** RT-PCR of estrogen-regulated genes in lambs untreated and treated with 17β-estradiol. For RT-PCR analysis of estrogen-regulated genes (IGF-I, IGF-IR, PR, TFF3, ERα, ARO, AR, 3β-HSD) RNA was extracted from prostate tissues of both untreated (control) animals and lambs treated with 17β-estradiol, as described in Materials and Methods. For amplification, β-actin was used as a control for cDNA synthesis.
estrogens show typical histological changes of the epithelium, including both metaplasia and hyperplasia [27]. It has been reported that treatment of the bovine prostate stromal cell cultures (BPSCs) with E2 induces over-expression of FGFR1, FGFR2 and FGFR3 genes, along with a 30-fold increase in the expression of PR [28]. Similar results have been obtained in studies on the mouse prostate, in which E2 stimulated a significant over-expression of PR mRNA and protein [29].

In the present study, the gene expression profile observed in the prostate glands of lambs treated with E2 showed a significant increase in the expression of genes encoding for IGF-I, IGF-IR and PR (respectively of 2.8- 4.2- and 2.1-fold), while levels of ERα and AR mRNA remained unchanged. This evidence is in line with the implication of estrogen and estrogen-sensitive genes, including IGF growth factors and their receptors, as a pivotal axis in the regulation of proliferation, apoptosis, adhesion, migration and differentiation of epithelial cells from male and female reproductive tissues (mammary and/or prostate gland) [30,31]. Interestingly, the TFF3 gene was expressed only in prostate tissues of animals treated with E2, while very low or undetectable levels were detected in prostates of control untreated animals. This evidence was corroborated by immunohistochemistry, showing that TFF3 staining was significantly stronger in both bulbourethral glands and urethral epithelium of treated animals with respect to controls. In recent years, various studies have indicated that TFF3 is an estrogen-regulated, estrogen receptor-associated gene that may have a role in malignant transformation and tumor progression of estrogen target tissues, including endometrium, breast and prostate gland [19,32,33]. Furthermore, TFF3 gene over-expression can be detected for up to 30 days after the treatment is discontinued, while chemical methods can detect residues of growth-promoting agents for only a few hours. This combined evidence suggests that TFF3 might be used as a specific estrogen-related tissue biomarker and that its measurement, combined with standard histological and immunohistochemical methods, may be of value to detect the illegal use of estrogen as a growth-promoting agent in livestock production.

Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

References


