Testicular development, ultrasonographic and histological appearance of the testis in ram lambs immunized against recombinant LHRH fusion proteins

Hasan Ülker, Mehmet Kanter, Özdal Gökdal, Turgut Aygün, Ferda Karakuş, Mehmet E. Sakarya, David M. deAvila, Jerry J. Reeves

Abstract

Sixteen native ram lambs weaned at 10 wk of age were divided into two groups. Eight animals were immunized against LHRH with a mixture of two fusion proteins: ovalbumin–LHRH-7 and thioredoxin–LHRH-7. The immunized lambs received a primary immunization plus two booster immunizations at 4 and 12 wks. Animals in the control group (n = 8) were not treated. Scrotal measurements and blood samples were taken at 2-week intervals. Beginning at 25 wk of age, semen was collected and sexual behaviour was evaluated on a weekly basis. At 35 and 37 wk of age testes and accessory glands of all animals were subjected to ultrasound scanning. At 37 wk of age animals were slaughtered and testes were evaluated histologically. Serum LHRH antibodies (P < 0.01) were detected in animals of the immunized group which had reduced serum testosterone concentrations (P < 0.01). Testicular development was suppressed in the immunized animals (P < 0.01). Immunized animals exhibited mounting activity 5 wks later than control animals. No mature spermatozoa containing...
ejaculates were collected from immunized animals. Control animals had moderately echogenic ultrasonographic appearance at 37 wk age, whereas immunized animals had hypoechoic images. Mean seminiferous tubule diameter in immunized lambs was significantly smaller than that in control lambs. Basal membrane was thickened and hyalinized; there was an increase in peritubular connective tissue. No proliferating spermatogonia or mature spermatozoa were present in the tubules in these animals. There were no differences in the ultrasonographic appearance of prostate and vesicular gland between control and immunized animals. The LHRH recombinant fusion proteins were effective in immunological castration in ram lambs when started at 10 wk of age as noted by differences in serum testosterone, testicular histology and ultrasonographic appearance of testis and weight of accessory sex glands. Determining the effects of immunization on ultrasonographic appearance of the testis related to time after immunization requires further investigations.

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1. Introduction

Immunization against luteinizing hormone releasing hormone (LHRH) has been described as one of the effective means to reduce reproductive functions in farm animals and as a possible alternative to surgical castration. (Reeves et al., 1989; Bonneau and Enright, 1995; Thompson, 2000). Gonadotropin hormone concentrations, testicular development and sexual activities are suppressed in LHRH immunized animals (Robertson et al., 1982; Hoskinson et al., 1990; Adams and Adams, 1992). For the last two decades LHRH sterilization vaccines have been produced by coupling LHRH to an antigenic carrier protein using chemical conjugation techniques, however, such methods generally create a number of reaction products that require extensive purification to provide a homogenous protein. In order to overcome this problem two recombinant fusion proteins, ovalbumin–LHRH-7 and thioredoxin–LHRH-7, were developed to be used as a sterilization vaccine (Zhang et al., 1999; Quesnell et al., 2000). Genetic engineering techniques were utilized to create these recombinant protein molecules in which hormone epitopes are incorporated directly into the primary sequence of the carrier protein, by passing the need for chemical conjugation of hormone to carrier. The effectiveness of these recombinant proteins in suppressing reproductive functions was demonstrated in cattle (Sosa et al., 2000; Aissat et al., 2002) and sheep (Ulker et al., 2001). Immunization of ram lambs at 18 wk of age with these fusion proteins suppressed testicular development significantly during weeks 32–36 of the study. Additionally, severe atrophy of the seminiferous tubules and loss of spermatogenesis were observed in a majority of immunized animals (Ulker et al., 2001). However, since some tubules appeared to have matured spermatozoa, 18 wk of age for immunization might be too late to obtain complete suppression.

Ultrasonography is a non-invasive technique allowing to study changes in reproductive organs. Ultrasonographic imaging has been used in measuring size and structure of the testis in bulls (Chandolia et al., 1997b; Bailey et al., 1998; Gabor et al., 1998; Aravindakshshan et al., 2000a) and rams (Cartee et al., 1990; Ahmad et al., 1991; Chandolia et al., 1997a). Several studies to determine the changes in structure of the testis using ultrasound imaging in
pathological conditions (Ahmad and Noakes, 1995; Karaca et al., 1999), clinically healthy animals (Ahmad et al., 1991; Evans et al., 1996; Aravindakshan et al., 2000a, 2000b; Gouletsou et al., 2003) or LHRH analogue treated animals (Chandolia et al., 1997a) were reported. Histological examination of the testis in LHRH immunized bulls (Robertson et al., 1982) or rams (Kiyma et al., 2000, Ulker et al., 2001) showed partial atrophy and distorted shape of tubules, a reduction in seminiferous tubule diameter and spermatogonia numbers. Nevertheless, to the best of our knowledge, there is no report on ultrasonographic appearance of testis in LHRH immunized ram lambs with a comparative approach using histological structure. Thus, the objectives of the present study were as follows: (1) to evaluate the effectiveness of recombinant fusion proteins in suppressing testicular development, sexual activities and testosterone concentrations; and (2) to compare the ultrasonographic and histological appearance of testis in ram lambs immunized against LHRH using recombinant fusion proteins at an earlier age.

2. Materials and methods

2.1. Animals and treatments

Sixteen native ram lambs weaned at 10 wk of age were divided into two groups: control (n = 8) and immunized (n = 8). Immunized animals received a cocktail of both ovalbumin–LHRH-7 and thioredoxin–LHRH-7 recombinant proteins as a primary injection and two booster injections at 10, 14 and 22 wk of age, respectively. One lamb in the immunization group died 3 wks after the primary immunization. Animals in control group were not treated. Additionally, testes of five native ram lambs (10 wk of age) were scanned using 5.0 MHz linear ultrasound transducer and biopsied to attain testicular tissue for histology. These animals were then excluded from the study without any further data collection. All procedures were performed using procedures approved by Yüzüncü Yıl University Animal Care and Use Committee (Etik Kurul) (2002/02–07).

2.2. Preparation of antigens and immunization

Ovalbumin–LHRH-7 and thioredoxin–LHRH-7 were prepared by recombinant DNA techniques previously described (Zhang et al., 1999; Quesnell et al., 2000). Briefly, the ovalbumin and thioredoxin genes were modified to contain seven LHRH inserts and different sites. Each construct was designed to contain six histidine sequences at the carboxyl-terminal (His-Tag®; pET System Manual, Novagen®, 1994) to facilitate purification by affinity chromatography. Recombinant ovalbumin–LHRH-7 and thioredoxin–LHRH-7 genes were over expressed in E. coli. His-bind affinity chromatography using a Ni2+ column allowed for purification of the proteins. Equimolar amounts of each LHRH fusion protein (10 nM) totalling 1.0 mg of protein were suspended in 6 M urea and emulsified in 0.5 ml of modified complete Freund’s adjuvant (Sigma, St. Louis, MO, USA) for the primary immunization and modified incomplete Freund’s adjuvant was used for the subsequent two booster injections. Immunizations were distributed over four subcutaneous sites on the inside surface of the legs.
2.3. Data collection

Five ram lambs (10 wk of age) were subjected to testicular ultrasound examination and biopsy to determine ultrasonographic and histological appearance of the testes at this age. For ultrasound measurements scrotal wool was cleaned and coupling gel was applied to the scrotum. Each testis was scanned in longitudinal (lateral) and transverse (caudal) planes using a 5.0 MHz linear array transducer connected to a B-mode ultrasound scanner (Concept\MCV, Dynamic Imaging Ltd., Scotland, UK) to examine testicular structure. Testicular tissue samples were collected via biopsy from each animal under local anaesthesia. Tissue samples were fixed in 10% neutral buffered Helly solution and processed for histological examination. Scrotal circumference measurements were collected every 2 wks for each animal.

All animals were bled via jugular venipuncture at 2-week intervals throughout the experiment. Blood samples were refrigerated overnight at 4°C, and then centrifuged at 2500 × g for 15 min. Serum was harvested and stored at −20°C until subsequent analyses.

A radioactive binding assay was used to evaluate the percentage of 125I-LHRH that would bind to the anti-LHRH antibody present in the serum at a 1:1000 dilution (Johnson et al., 1988). Mouse anti-sheep globulin was used as the second antibody at 1:20 dilution. Iodination of LHRH was performed using LHRH (2.5 µg/25 µl H2O) with 0.5 mCi (5 µl) 125I, 30 µl of 0.5 M PBS (pH 7.5) and 10 µl Chloramine-T (600 ng/10 µl of 0.5 M PBS, pH 7.5). I-LHRH was separated on a QAE-Sephadex column using column buffer (10 mM Tris, 1 mM CaCl2; 0.1% BSA, pH 7.2). The percentage of 125I-LHRH bound to serum diluted 1:1000 was used as the index for LHRH antibody titres. Serum testosterone concentrations were determined by RIA (DSL-4000, Diagnostic Systems Laboratories Inc., Webster, TX). All samples were done in a single assay and intra-assay coefficient of variation was 2.05%.

Mounting behaviour was evaluated weekly beginning at 25 wk of age by introducing the ram lambs to one or two estrus ewes. Estrus was induced by 600 IU PMSG (Synchroject) injection after removal of medroxyprogesteron acetate (MAP) pessaries (Vetimex, Bladel, Netherlands) that were placed 14 days prior. When a ram lamb mounted completely over the ewe several times consecutively it was considered a complete mounting behaviour. Sperm was collected using an electro-ejaculator. The collected sperm was evaluated under phase-contrast microscope equipped with heating plate. By 35 wk of age all ram lambs in the control group had exhibited mounting behaviour for 3 consecutive weeks. At this time all animals in both groups were subjected to ultrasound scanning of testes and accessory sex glands to determine differences in ultrasonographic appearance between groups. Scanning of the prostate and vesicular gland were conducted using a 7.5 MHz linear array transducer that was inserted into the rectum of each ram lamb. Ultrasonographic measurements were made using electronic caliper built in scanner. Gain settings were set to a predetermined standard before each ultrasound examination.

Since ram lambs of this breed were prepubertal at this age, 2 wks were allowed before determining any changes in testicular appearance. All animals were slaughtered at 37 wk of age. Before slaughter, testes of animals in both groups were scanned to determine latest ultrasonographic image and these images were recorded. Immediately after slaughter, a small testicular tissue was taken and fixed in 10% neutral buffered Hyaline solution. Tissue samples were then processed for histological examination. Six micrometer-thick
sections from paraffin embedded samples were stained with Mallory triple techniques. Mounted slides were examined and pictures were taken under light microscope (Nikon optiphot2).

Data analysis was performed using GLM procedure of SAS (SAS Inst. Inc., Cary, NC) for repeated measures to determine main effects of treatment, time and treatment × time for each of response variables (serum anti-LHRH antibody percentage bound, testosterone and scrotal circumference). Prostate width and accessory glands weight were analyzed for the effect of treatment using GLM analysis. Data are presented as means ± S.E.M.

3. Results

Anti-LHRH antibodies were detected first at 14 wk of age in immunized ram lambs ($P < 0.01$, Fig. 1). The LHRH binding activity increased steadily from 14 wk of age, and reached a plateau between 18 and 26 wk of age, and then increased steadily until the end of trial with 26.40% bound at a 1:1000 dilution. One immunized ram lamb did not generate any anti-LHRH antibodies throughout the study, although its scrotal circumferences were small as in other immunized animals and testosterone concentrations were below low standard. Control group did not have detectable amounts of LHRH antibodies at any time during the study.

Except 14 wk of age, serum testosterone concentrations in immunized animals were lower throughout the study ($P < 0.01$, Fig. 2).

Scrotal circumference (Fig. 3) for the control animals showed a steady increase throughout the study. However, scrotal circumference for animals in the immunization group differed from controls beginning at 14 wk of age and remained smaller for the duration of the study ($P < 0.01$).

Fig. 1. Mean (±S.E.M.) antibody binding to LHRH expressed as a percentage bound $^{125}$I-LHRH at 1:1000 dilution in control and immunized ram lambs (S.E.M. ± 1.29 and 1.49, respectively). Arrows represent age of immunizations.
All animals in the control group exhibited mounting activity at 30 wk of age, however, mounting activity was not observed in immunized animals until 35 wk of age (Table 1). Six of eight (75%) animals in the control group had ejaculates containing mature spermatozoa at 35 wk of age. Conversely, none of the immunized ram lambs produced mature spermatozoa at any time throughout the study (Table 2). Prostate width taken from recorded

Table 1

<table>
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<th>Group</th>
<th>Age (wk)</th>
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<th>25</th>
<th>26</th>
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<th>28</th>
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<th>32</th>
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<th>34</th>
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<tr>
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<td>0</td>
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<td>7</td>
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</table>
Table 2
Ram lamb numbers produced mature spermatozoa containing ejaculate after electroejaculation

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>Immunization</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3
Mean (±S.E.M.) prostate width (cm) taken from recorded ultrasonographic images at 35 wk of age, and prostate-vesicular gland weight (g) measured at slaughter in control and immunized ram lambs

<table>
<thead>
<tr>
<th>Group</th>
<th>Prostate width (cm)</th>
<th>Prostate + vesicular gland weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.31 ± 0.09</td>
<td>0.019 ± 0.002</td>
</tr>
<tr>
<td>Immunization</td>
<td>0.50 ± 0.09***</td>
<td>0.004 ± 0.002**</td>
</tr>
</tbody>
</table>

Within columns asterisk donates a significant difference (***P < 0.01) from control.

ultrasonographic images at 35 wk of age and prostate and vesicular gland weights measured at slaughter were smaller in the immunized group (P < 0.01) (Table 3).

The testicular parenchyma in 10 wk of ram lambs was homogeneously hypoechoic (Fig. 4). There were some moderately echogenic area around mediastinum seen as slight echogenic lines. At 37 wk of age testicular parenchyma of the control animals was homogeneous with a coarse medium echo-pattern (Fig. 5). The mediastinum appeared as a thin highly echogenic central structure. Tunica vaginalis, spermatic fascia and skin area appeared as hyperechoic covering over parenchyma in this group. The testicular parenchyma in the immunization group at the same age appeared as hypoechoic structure with moderately echogenic area around mediastinum seen as slight echogenic lines (Fig. 6). The ultrasonographic appearance of testicular parenchyma of 10 wk of age ram lambs (Fig. 4) appeared to be similar to that of 37 wk of age (Fig. 6), nevertheless, echogeneity of parenchyma was slightly better in these animals.

Fig. 4. Ultrasonographic appearance of testis of ram lambs at 10 wk of age (arrow heads show the borders of testicular parenchyma). Tp: testicular parenchyma; mt: mediastinum testis.
Fig. 5. Ultrasonographic appearance of testis of control ram lambs at 37 wk of age (arrow heads show the borders of testicular parenchyma). Tp: testicular parenchyma; mt: mediastinum testis.

Fig. 6. Ultrasonographic appearance of testis of immunised ram lambs at 37 wk of age (arrow heads show the borders of testicular parenchyma). Tp: testicular parenchyma; mt: mediastinum testis.
Histological examination of 10-week-old ram lambs showed seminiferous tubules in regular shape, narrow lymphatic sinusoids and few Leydig cells in the soft interstitial tissue. The wall of the seminiferous tubules were layered with Sertoli cells. Some non-mitotically active spermatogonia were observed between Sertoli cells (Fig. 7). Control animals had regular seminiferous tubules in diameter and shape at 37 wk of age. There were different mitotic figures of spermatogenesis in the tubules and mature spermatozoa in the lumen (Fig. 8). Testes of immunized rams appeared as have a severe hypoplasia. Additionally, diameters of seminiferous tubules were significantly smaller than those of testes from control ram lambs at both 10 and 37 wk of age. Lymphatic sinusoids were disappeared at a large scale.

Immunized animals showed an increase in Leydig cell numbers around the blood vessels in interstitial tissue and had hyalinization and thickening of the basal membrane. There was an increase in peritubular connective tissue. A considerable amount of the seminiferous tubules’ walls were layered with Sertoli cells and a few non-mitotically active spermatogonia were between Sertoli cells. No proliferating spermatogonia or mature spermatozoa were present in the tubules (Fig. 9).

No differences were noted in the ultrasonographic appearance of prostate and vesicular gland between control and immunization groups (data not shown).

4. Discussion

The early response to immunization in terms of antibody titre and low testosterone concentration in the present study support the success of two LHRH fusion proteins in
combination developing antibody-binding activity and biological response in sheep. The use of two carrier proteins simultaneously might help to overcome carrier-induced immune suppression, which is a result of an overwhelming secondary response to the carrier protein, which inhibits the response to the hapten portion of the vaccine (Sad et al., 1991). Thus,
immunized animals produced antibodies which peaked and then remained elevated after the last booster. Regardless of the LHRH antibody concentrations, testosterone concentrations and scrotal circumferences were uniformly suppressed in all immunized ram lambs. The observation that one immunized ram lamb did not generate any anti-LHRH antibodies throughout the study but its scrotal circumferences were small as in other immunized animals and testosterone concentrations were below low standard could be attributed to the antibody affinity to LHRH as observed in the previous study (Quesnell et al., 2000). Antibodies although low in titer may have had a high affinity to LHRH.

Active immunization against LHRH at earlier ages such as 3–4 wks (Brown et al., 1994; Daley et al., 1995), 15 wk of age (Kiyma et al., 2000) or 20–21 wks (Brown et al., 1994) all suppressed testicular development and gonadotropic hormone concentrations in ram lambs. Similar results were obtained in a previous study in which ram lambs were immunized against LHRH at 18 wk of age using same recombinant fusion proteins as used in the present study (Ulker et al., 2001). Although testicular development was suppressed in all mentioned studies, an increase in scrotal circumference or testicular mass remained. Immunization was performed at 10 wk of age in the presented study. By the 6th week after the first immunization scrotal circumferences differed in immunized animals and, testicular and scrotal sizes remained at a comparable size as at the time of the first immunization (Fig. 3). Brown et al., (1994) obtained better testicular suppression in ram lambs immunized at 20–21 wk of age than those of immunized at 3–4 wk of age. In contrast, in the present study immunization was performed at 10 wk of age and a better suppression was obtained compared to the previous study in which immunization was performed 18 wk of age. This better suppression could be attributed to the adjuvant used in the immunization protocol. While an adjuvant designed for use with recombinant fusion proteins that have a His-Tag had been used in the previous study, modified Freund’s complete adjuvant, the best known adjuvant combination, was utilized in the current study.

All animals in control group exhibited mounting behaviour by 30 wk of age. This was delayed to 35 wk of age in immunized animals. Mounting activity is learned under the presence of testosterone, and even when testosterone is decreased the mounting activity is still present. The findings of the present study are in agreement with previous studies that sexual behaviours decreased in physically or immunologically castrated animals (Brown et al., 1994; Finnerty et al., 1996; Godfrey et al., 1996; Pinckard et al., 2000; Parthasarathy et al., 2002). It is a well-established phenomenon that castration causes reduction in sexual behaviour due to lack of testosterone produced in the testes. The changes in castration induced sexual behaviours have been found to be highly variable, and usually a complete elimination has not been obtained. The failure of complete elimination of sexual behaviour in castrate animals regardless of castration age (pre- or post-pubertal) indicates the presence of some functional aspects of central nervous system that affects sexual behaviour independent of testosterone (Hafez, 1987; Sachs and Meisel, 1994; Senger, 1997). Although it is delayed, the presence of sexual activity in the absence or very low testosterone concentrations in immunized animals in the present study coincides with this phenomenon. Therefore, it appears that sexual behaviour alone is not a valuable sign for the reproductive status of an individual. Control animals exhibited mounting activity at an earlier age, nevertheless, only six out of eight of them produced mature spermatozoa containing ejaculate by 37 wk of age, and these animals were not considered to have reached sexual maturity (puberty). This
could be attributed to fact that these native animals reach puberty at later ages. In any cases, the present study demonstrated that recombinant LHRH fusion proteins caused a delay in expressing sexual behaviour and inhibited sperm production.

Although there were no differences in the ultrasonographic appearance of prostate and vesicular gland between control and immunization groups, prostate width measured with ultrasonic caliper was lower in immunized group. This was confirmed with lower prostate–vesicular gland weight at slaughter in the immunization group indicating that immunization suppressed testosterone concentrations. To the best of our knowledge there is no study reporting testicular width measured by ultrasonic caliper in LHRH immunized animals and therefore we cannot make any comparison.

Ultrasonographic appearance of the testes of 10-week-old ram lambs happened to be homogeneously hypoechogenic. The appearance of infantile testis was previously reported by Chandolia et al. (1997a) who noted lower pixel units which indicate dark image in 4–20-week-old ram lambs’ testes. Similarly, Aravindakshan et al. (2000a, 2000b) determined lower pixel values in 4–34-week-old bull calves’ testes. Apparently testicular structure of the infantile testis lacks some testicular structures and therefore testicular tissue does not produce reflections for ultrasound image. As the age increased, the testes gained its normal echogenic ultrasound appearance (data not shown). Consequently, testicular parenchyma of the control animals became moderately echogenic at 37 wk of age. These data were in agreement with other studies (Chandolia et al., 1997a, 1997b; Aravindakshan et al., 2000a, 2000b; Gouletsou et al., 2003). Immunization affected ultrasonographic appearance of the testes drastically. While control animals had moderately echogenic ultrasonographic image at 37 wk of age, immunized animals had smaller, uniform hypoechogenic testicular structure (Fig. 6) as observed at 10 wk of age ram lambs (Fig. 4). To the best of our knowledge, there is no study that investigated ultrasonographic appearance of testis in LHRH immunized ram lambs. Chandolia et al. (1997a) treated ram lambs with a LHRH agonist at 3–11 wk of age to suppress LH and, consequently, testicular development. No differences in testicular appearance were reported between control and treatment group in the mentioned study. Nevertheless, that study ended when ram lambs reached at 26 wk of age and no apparent effect of agonist on testicular development was determined. Although Sidibe et al. (1992) reported no apparent changes in the ultrasonographic appearance of the testis in bulls with induced testicular degeneration, hypoechogenic ultrasound image of testes was observed in testicular atrophies resulted from trauma (Cross et al., 1999) and cryptorchidism (Fowler, 1993). Here we report hypoechogenic ultrasonographic appearance of an atrophic testis caused by gonadotropin deficiency.

The ultrasonographic appearance of 10 wk of age ram lambs (Fig. 4) and 37 wks of immunized ram lambs (Fig. 6) appeared to be similar. Testicular histology of 10 wk of age ram lambs (Fig. 7) and 37 wks of immunized ram lambs (Fig. 9) and 37 wks of control ram lambs (Fig. 8) were significantly different. Since in both normal histological appearance at 10 wk of age and abnormal appearance at 37 wk of age the ultrasonographic image in immunized group was similar, whether the hypoechogenic appearance of the testis is a result of the lack of Sertoli cells or other testicular structures is unclear. Many investigators indicated that ultrasonographic imaging of testis has considerable potential for the evaluation of the testicular function in domestic animals (Chandolia et al., 1997a, 1997b; Gabor et al., 1998; Aravindakshan et al., 2000a, Kastelic et al., 2001). The most practical
sign of immunological castration in ram is the smaller testis. This observation could be accompanied with ultrasonographic appearance of atrophic testis induced by gonadotropin deficiency as reported in the present study. As a non-invasive technique, more detailed ultrasonographical evaluations could be utilized in determining the changes in testicular histology in immunocastration studies performed in either juvenile or adult ages.

Although prostate width measured with ultrasonographic caliper was lower in immunized group (Table 1), there were no differences in the ultrasonographic appearance of prostate and vesicular gland between control and immunization groups (data not shown). This was confirmed with lower prostate–vesicular gland weight at slaughter.

5. Conclusion

The recombinant LHRH fusion proteins were effective in suppressing testosterone concentrations and testicular development in ram lambs. Furthermore, these results indicated that immunologically castrated animals could be practically assessed from ultrasonographic appearance of their testes. The changes in testes’ ultrasonographic appearance related to age and immunization time in combination with related changes in testicular histology require farther parallel ultrasonographic and histological investigations.

Acknowledgements

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References


