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Evaluation of two GnRH-I based vaccine formulations on the testes function of entire Suffolk cross ram lambs

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Abstract

A modified GnRH peptide (CHWSYGLRPG-NH_2) was conjugated to tetanus toxoid (TT) or diphtheria toxoid (DT) and formulated with Quil A saponin or a sustained release injectible PLGA (poly(lactide-*co*-glycolide)/triacetin). For the Quil A formulations, two administrations of TT conjugate at 3-weekly intervals were followed by two booster injections with the DT conjugate in entire ram lambs. With the PLGA formulations, only two injections were administered; the first containing TT and the second DT at 6-weekly intervals. Evaluation was carried out by comparing the specific antibody levels produced in relationship to hormone profiles and testicular changes. The Quil A formulation was considered the most effective, as it caused significant reduction in testosterone and follicle stimulating hormone levels, resulting in marked suppression of spermatogenesis.

Keywords: GnRH; Anti-fertility; Vaccine

1. Introduction

Surgical castration and scrotal ligation of male animals are common farming techniques that are used world-wide to control fertility, increase growth and metabolism, improve the quality of meat and prevent unsocial behaviour [1]. A practical and non-invasive alternative is the use of vaccination against the fertility controlling decapeptide, gonadotrophin releasing hormone (GnRH) [2]. An examination of the literature covering the last decade has shown that there are a diverse number of developmental GnRH vaccines, which have been examined primarily in cattle and pigs, and to a lesser extent in sheep. Native or modified GnRH molecules have been conjugated to a wide range of carrier molecules, including polyamino acids, proteins and lipids (summarised in Table 1). Adjuvants used in combination with the immunogens have ranged from mineral oils, aluminium compounds, and lipid vesicles. A limited number of commercial veterinary GnRH vaccines have been developed [3], including, Vaxtrate® (Arthur Webster Pty Ltd./Peptide Technology Ltd., Australia), for control of fertility in heifers [4], which is no longer on the market and Improvac™ (CSL Ltd., Australia) for treatment of boar taint [5].

In this study we examined two adjuvants, which have been tested successfully in a rodent model [6]; Quil A saponin and a sustained release injectable formed in situ based on 85/15-poly(lactide-*co*-glycolide) and triacetin (PLGA). The immunogens evaluated consisted of a modified GnRH peptide (CHWSYGLRPG-NH_2) [7], conjugated to

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either diphtheria toxoid (DT) or tetanus toxoid (TT). In order to avoid epitope suppression [8], caused by the large toxoid carrier molecules, the formulations were alternated. The alternation of these two particular carrier molecules has been shown to be successful in human anti-fertility vaccine trials in overcoming epitope suppression [9]. Therefore, with Quil A, two administrations of the TT conjugate, were followed by two booster injections with the DT conjugate, at 3-weekly intervals. With the sustained release PLGA, only two injections were given at a 6-weekly interval; the first containing TT and the second DT. Evaluation was carried out by comparing the specific antibody levels produced in relationship to hormone profiles and testicular changes.

2. Materials and methods

2.1. Immunogen preparation and vaccine formulation

The peptide sequence CHWSYGLRPG-NH_2 (2 μmol, 90% purity, synthesised by Immune Systems Ltd., UK) was conjugated to either 0.1 μmol tetanus toxoid (TT, a generous gift from Mark Lavery, Intervet Ltd., UK) or diphtheria toxoid (DT, Connaught Ltd., Canada) using sulpho-maleimido benzoic succinimide (S-MBS, Perbio Science Ltd., UK) as described previously [7].

Vaccine 001 (Quil A), consisted of the modified GnRH peptide conjugated to tetanus toxoid (TT-GnRH-I) administered for the first two injections, followed by two administrations of the peptide conjugated to diphtheria toxoid (DT-GnRH-I), both equivalent to 500 μg of CHWSYGLRPG-NH_2 per dose. A 10% (v/v) solution of Quil A (supplied as a 1% (w/v) solution by Novartis Animal Vaccines Ltd., UK) was made up fresh in distilled water together with an equal volume of a single dose of the immunogen and used when required.

Vaccine 002 (PLGA) consisted of TT-GnRH-I, equivalent to 1500 μg of CHWSYGLRPG-NH_2, for the first administration. This was followed by immunisation with DT-GnRH-I, equivalent to 1500 μg of CHWSYGLRPG-NH_2. The PLGA was made up by dissolving 85/15-poly(lactide-*co*-glycolide), purchased from Birmingham Polymers Inc. (Birmingham, AL, USA) in triacetin (Fluka Chemie AG, Buchs, Switzerland) at 300 rpm for approximately 24 h (magnetic stirrer, Ikamag RET S8^{TM}, Ikawerke, Stauffen) to obtain a 10% (w/v) solution. The PLGA solution was mixed with an equal volume of immunogen (equivalent to three single Quil A doses).

2.2. Immunisation schedule

Fifteen entire male Suffolk cross lambs, approximately 10 months of age, were identified by uniquely numbered ear tags. The animals were allowed to acclimatise to experimental conditions for a week prior to treatment. The lambs were located in one field and grazed on permanent pasture. They were supplemented with hay and concentrates (Pearl Finisher, Carrs Agriculture Ltd., Carlisle, UK) at a rate of 0.6 kg/head/day. Mains water was available ad libitum. Vaccination was by subcutaneous injection in the neck, using a 21 gauge × 1 in. needle. The animals were randomly allocated into three groups: group A were immunised with

Table 1
Key references comparing different developmental and commercial veterinary anti-GnRH vaccines

Developmental vaccines	Adjuvant	Target animal	Key references
GnRH-α-globulin	Mineral oil	Male pigs	[20]
GnRH-keyhole limpet haemocyanin (KLH)	Freund's, ISA	Ram lambs	[4,21]
Recombinant multi-fusion protein linked to ovalbumin (OVA) or thioredoxin	Z-Max	Heifers and bulls	[22,23]
Recombinant fusion protein–GnRH linked to T helper epitopes: canine distemper virus epitope, goat rotavirus VP6 protein	Iscomatrix adjuvant	Dogs	[24]
Alanine replacements of tandem GnRH peptide linked to OVA	Specol	Male piglets	[25]
Tandem GnRH peptide linked to branched polylysine or lipothioesters or lipo-amides or KLH	Freund's, ISCOM	Pigs	[26]
Cys-*gly*-GnRH conjugated to horse serum albumin	DEAE-dextran	Heifers	[27,28]
Des 1 Cys-GnRH-TT	Alum	Sheep, dogs	[10]
D-Lys6-GnRH tandem peptide conjugated to OVA	Specol, CoVaccine^{TM}, Carbopol 934	Female and male pigs, ponies	[29–31]
Commercial vaccines	**Adjuvant**	**Target animal**	**Key references**
Vaxtrate®	–	Adult and pre-pubertal bulls, neonatal sheep	[11,32,33]
Improvac^{TM}	–	Boars, ram lambs gilts	[5,19,34]

vaccine 001 on days 0, 21, 42 and 63 (1 ml/lamb at each vaccination point); group B were treated with vaccine 002 on days 0 and 42 (1 ml on the first administration and 3 ml on the booster); group C consisted of untreated controls.

2.3. Body weight and assessment of testicular size

Body weights were measured on days 0, 28, 56 and 84. Testicular measurements (scrotal circumference) were taken at 2-weekly intervals. Scrotal circumference was measured with the sheep standing, using a piece of string placed at the widest diameter of the scrotum and ensuring both testicles were present. The piece of string was then measured against a ruler. Statistical analysis was carried out using an unpaired *t*-test (Statview®, v5.1 software).

2.4. Blood sampling

The lambs were blood sampled at 2-weekly intervals on days 0, 14, 28, 42, 56, 70 and 84. At each sample point, four duplicate collections were taken at 1.5 h intervals, into 8 ml lithium heparin separator Vacuettes (Greiner Bio-One Ltd., Gloucestershire, UK). The blood samples were centrifuged at 1000 g for 10 min, the plasma decanted and stored at −20 °C until analysed. Terminal samples were also taken on day 132.

2.5. Antibody estimations by specific ELISA

Peptide-BSA conjugate (equivalent to 1 μg peptide/well in 100 μl phosphate buffered saline, PBS, pH 7.4) was coated onto tissue culture grade 96-well plates for 1 h at 37 °C. The plates were washed twice with wash buffer (PBS, containing 0.01% (v/v) Tween 20) and blocked with 3% (w/v) Marvel non-fat milk powder (Premier Brands, UK) in PBS-Tween, for 1 h at 37 °C. The plates were washed three times with wash buffer. The sheep plasma was diluted 1:1000 in blocking solution and left standing for 30 min at room temperature to decrease high background levels, prior to incubating 0.1 ml/well for 1 h at 37 °C (carried out in triplicate for each sample). The plates were washed three times with PBS-Tween. Horse radish peroxidase labelled rabbit-anti-sheep IgG (H + L chain specific, Perbio Science Ltd., UK) was diluted 1:5000 in PBS and 100 μl/well incubated for 45 min at 37 °C. The plates were washed three times with PBS (without Tween) and developed with 100 μl TMB substrate/well (250 μl of stock 6 mg/ml 3,3′,5,5′-tetramethyl benzidine in dimethylsulphoxide, added to 25 ml 0.1 M sodium acetate buffer, pH 5.5 with 4 μl 30% (v/v) hydrogen peroxide). The reaction was stopped with 50 μl/well 10% (v/v) sulphuric acid after 15 min and the A_{450} read.

2.6. Testosterone and gonadotrophin determination

Testosterone concentrations in the plasma were determined using a direct competitive radioimmunoassay (Coat-a-Count Total Testosterone; Euro/DPC Ltd., UK), according to the manufacturers' instructions. Quality control samples were included in all runs and they plus the sheep samples were assayed in duplicate.

The follicle stimulating hormone (FSH) concentrations were measured using an in-house homologous ovine FSH assay, the components for which were supplied by Dr. A.F. Parlow of Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, California, USA (FSH for I^{125} iodination = AFP-4117A and rabbit anti-ovine FSH serum = AFPC5288113). Samples were run in quadruplicate. A similar in-house assay was used to measure the LH plasma concentrations (LH for iodination = AFP-8614B and rabbit anti-ovine LH serum = AFP192279). Samples and quality controls were run in quadruplicate. One way Analysis of Variance (ANOVA) was used and the null hypothesis was rejected if a p-value of <0.05 was achieved.

2.7. Histology and staining of reproductive organs

Histology was carried out on the testicles of two animals chosen at random from the control group and compared with samples from the rams treated with Vaccine 001. The sheep were slaughtered by captive bolt and exsanguination. Duplicate samples (1 cm cubes) were taken from each testicle and placed in 10% (v/v) neutral buffered formalin. The tissues were dehydrated in alcohols and embedded in paraffin. The sections were cut at 5 μm and stained with haematoxylin and eosin and Masson's trichrome as described previously [10]. Morphological examination was carried out under 20× and 40× objectives on a Zeiss photomicroscope. For each sample, 50 randomly selected cross sections of seminiferous tubules were scored with respect to the stage of spermatogenesis present [10] (criteria described in detail in Table 2). A score of 10 was assigned to the most active tubules with spermatogenesis and spermatozoa in the lumen, the lowest score of 1 was assigned to tubules with no spermatogenic activity.

Table 2
Criteria used to quantify testicular spermatogenesis

Scores	Criteria to quantify the level of spermatogenesis in the tubules
10	Complete spermatogenesis with 50–80% of the lumen containing spermatozoa
9	Greater than 10 spermatozoa or spermatids present
8	Only a few spermatozoa (<5–10) present in the tubules
7	No spermatozoa but many spermatids (10–25) present (lowest standard score) with no visible lumen
6	No spermatozoa and only a few spermatids (<5–10) present
5	No spermatozoa and spermatids in the tubules, but several or many spermatocytes present (>10)
4	Only a few spermatocytes (<5–10) and no spermatids or spermatozoa present
3	Spermatogonia are the only germ cells present
2	No germ cells but only Sertoli cells present
1	No cells in the tubules

3. Results

3.1. Body weights

All the sheep showed an increase in body weight over the study period (data not shown). However, the treatments had no effect on daily live weight gain.

3.2. Antibody determination

A comparison of the GnRH specific antibody levels for the animals immunised against Vaccines 001 and 002 are shown in Fig. 1. Only the rams treated with Vaccine 001 (2 × TT-GnRH-I and 2 × DT-GnRH-I in Quil A) showed substantially increased anti-GnRH levels after the first two doses. Mean A450 greater than 1.5 were observed during the remainder of the immunisation period. Examination of individual antibody profiles showed that there was little significant inter-group variation. At the termination of the study on day 132, the mean A450 ± S.D. was 1.287 ± 0.548. The mean A450 in the animals treated with Vaccine 002 (1 × TT-GnRH-I and 1 × DT-GnRH-I in PLGA) remained below 0.2 throughout the immunisation period, with little significant inter-group variation.

3.3. Scrotal circumference

All the animals treated with Vaccine 001 showed an overall decrease in scrotal circumference ranging between 1.3 and 5.9 cm, mean 3.7 cm between days 0 and 70 (Fig. 2). This decrease was significant on days 42 ($p < 0.05$), 56 ($p < 0.001$) and 70 ($p < 0.001$). At day 84, all the sheep showed an increase in circumference compared with the day 70 measurement (ranging from 0.4 to 2.8 cm), however compared to the start of the study, this remained a significant decrease ($p < 0.05$). Only one sheep treated with Vaccine 002 showed a decrease in scrotal circumference (1 cm) over the same period, and overall there was no significant change in the group, between days 0 and 74. However, on day 84, all the sheep showed a significant ($p < 0.0001$) increase in scrotal circumference from the day 70 measurement (range 1.3–4.2 cm). In comparison, the untreated controls, showed slight but insignificant variations between days 0 and 70. Two sheep showed an overall decrease in scrotal circumference (0.5 and 1.1 cm), whilst three sheep showed an overall increase (0.7, 2.0 and 2.2 cm). However, at day 84 all the sheep showed a significant ($p < 0.0001$) increase in scrotal circumference from the day 70 measurement, which indicated that an external influence, namely season and not vaccination, was responsible for the observation in all the groups.

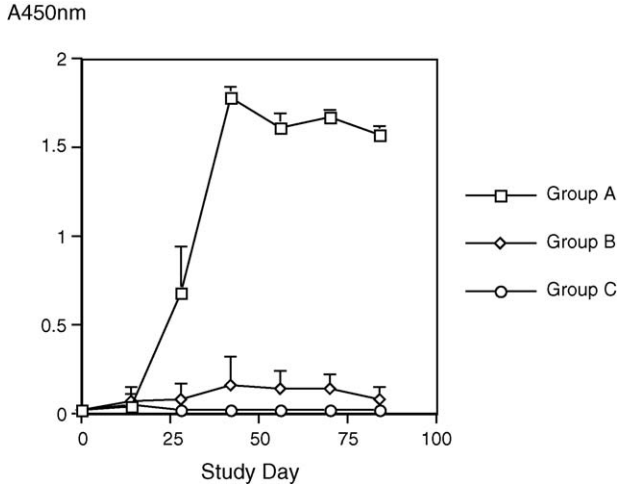

Fig. 1. The IgG antibody response of animals immunised with Vaccine 001 (group A), 002 (group B) and untreated controls (group C). The A450 were measured from specific ELISAs carried out on BSA-GnRH-I peptide coated plates. Immunisations were carried out on days 0, 21, 42 and 63 (group A) and days 0 and 42 (group B). $n = 5$.

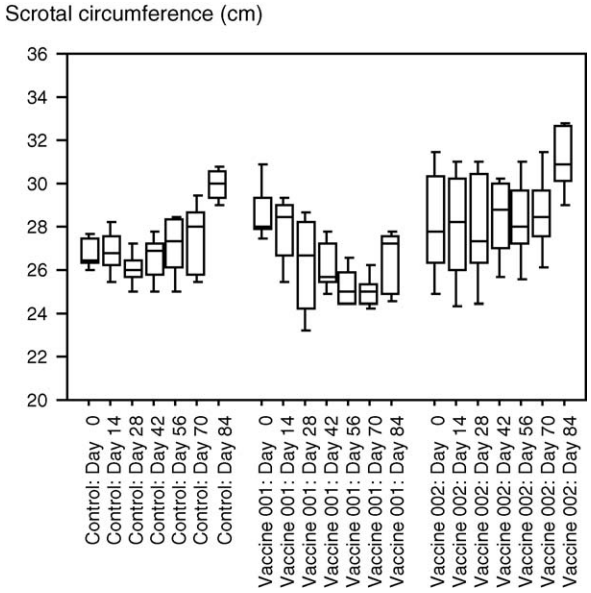

Fig. 2. Scrotal circumferences were measured in ram lambs treated with untreated controls, Vaccine 001 in Quil A and Vaccine 002 in slow release formulation PLGA. The box plots show the intra-variation between the animals in each group at each time point. Significant decrease in scrotal circumference was observed in the Vaccine 001 group on days 42 ($p < 0.05$), 56 ($p < 0.001$) and 70 ($p < 0.001$).

3.4. Testicular histology

Untreated control animals showed testicular histology of well vascularised interstitial tissue, containing many specific interstitial cells and a fine, single layer peritubular lamina propria (Fig. 3a and b). A clearly defined spermatogenic cycle was observed in the seminiferous epithelium, the spermatogenic series begins at the same level in a tubule, all the stem cells develop synchronously with successive cell generations extending from the basal lamina towards the centre of the tubule. This cycle is represented by 10 and the late release of spermatozoa and the initiation of the spermatogenic wave by 7. The control rams were all within the 7–10 range. The

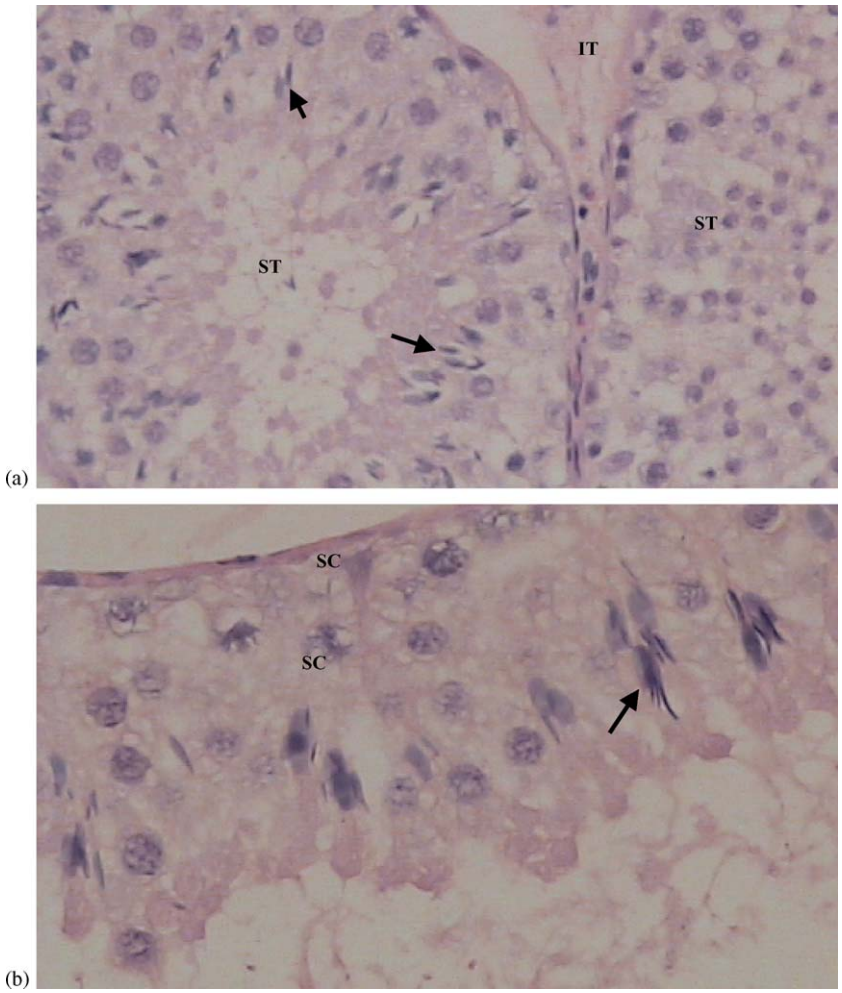

Fig. 3. (a) Light micrograph (H & E stained) of an untreated ram testis showing a portion of two adjacent seminiferous tubules (ST) separated by loose interstitial tissue (IT). The seminiferous tubules are lined by a stratified epithelium of spermatogenic cells. Late stage spermatozoa are arrowed. 20× magnification. (b) Light micrograph (H & E stained) of an untreated ram testis illustrating late stage spermatogenesis with developing spermatozoa (arrowed) embedded in Sertoli cells (SC). 40× magnification.

animals treated with Vaccine 001, showed distorted tubules with thickened peritubular connective tissue and lamina propria, small blood vessels and poorly defined interstitial cells in the dense, fluid filled interstitial tissue (Fig. 4a and b). Preliminary examination of the tubules for evidence of spermatogenesis showed that the Vaccine 001 caused spermatogenic arrest at the primary spermatocyte stage, with no evidence of later stages of spermatocytes, spermatids or spermatozoa. In some tubules, only Sustentacular cells were present. There was a clear shift in scoring from 7 to 10 in the untreated animals to 2–4, following treatment (Table 3).

3.5. Endocrine analysis

Mean group hormone profiles (Fig. 5a) show that overall, compared with the pre-treatment concentrations (1.36 ± 1.51 ng/ml), the decrease in testosterone concentrations in the Vaccine 001 treated animals was significant on days 28 ($p < 0.01$, 0.37 ± 0.48 ng/ml) and 56 ($p < 0.05$, 0.52 ± 0.72 ng/ml). Compared with the untreated controls, the decrease showed a similar pattern on days 28 ($p < 0.05$) and 56 ($p < 0.05$). This alteration was almost certainly attributed to vaccination. In the Vaccine 002 treated animals there was a significant increase in testosterone concentrations on days 28 ($p < 0.05$, 1.88 ± 1.23 ng/ml), 70 ($p < 0.05$, 2.29 ± 2.12 ng/ml) and 84 ($p < 0.01$, 2.52 ± 2.2 ng/ml) compared with pre-treatment levels (1.09 ± 1.02 ng/ml). However, only day 28 was significant ($p < 0.05$) compared with untreated controls. Detailed analysis of individual hormone profiles (Fig. 5b) shows that there was a significant difference in testosterone concentrations within the Vaccine 001 treated group. Two animals had basal concentrations from days 28 to 84 in every bleed, which was significantly ($p < 0.05$) decreased compared with pre-treatment concentrations and those in the untreated controls. However, by day 132, the concentrations were 1.05 and 4.74 ng/ml, respectively, this being very strong evidence that the testosterone suppression

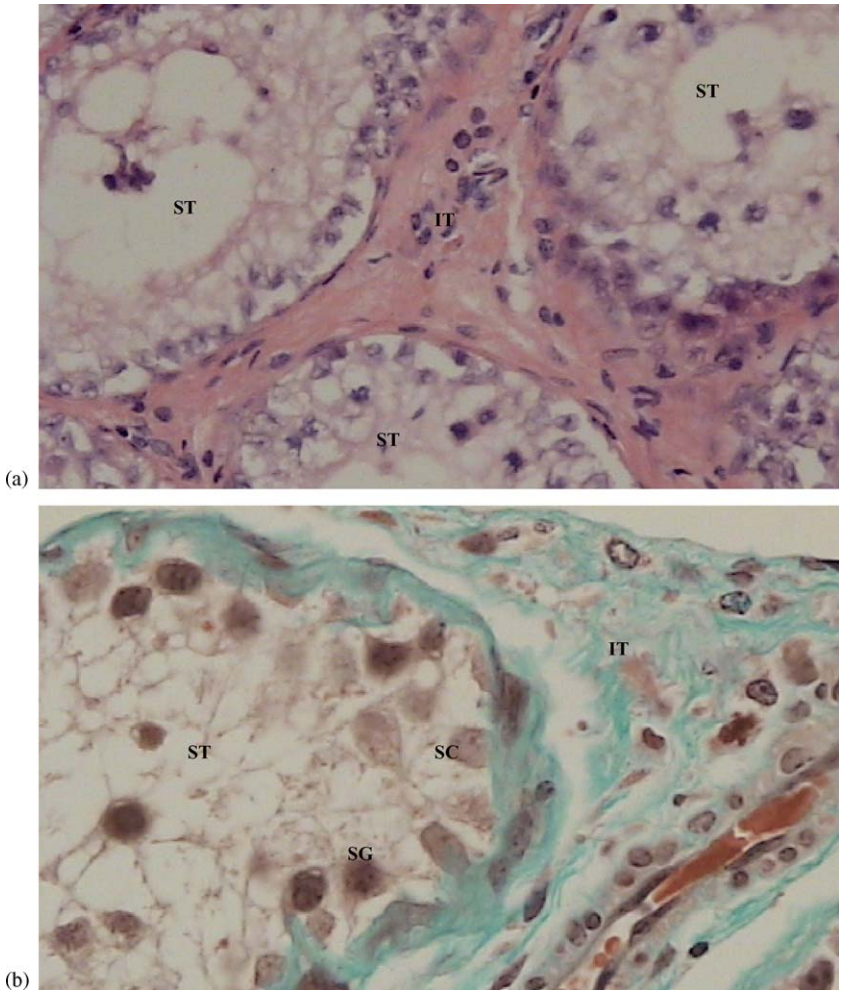

Fig. 4. (a) Light micrograph (H & E stained) of a vaccinated ram testis showing a portion of three seminiferous tubules (ST) separated by dense interstitial tissue (IT). The seminiferous tubules are lined by an inactive epithelium; there are no late stage spermatozoa. 20× magnification. (b) Light micrograph (Masson's Trichrome stained) of a vaccinated ram testis showing a seminiferous tubule lined by Sertoli cells (SC) and spermatogonia (SG). There is no evidence of spermatogenesis, the basement membrane (BM) is thickened and uneven. 40× magnification.

was of a temporary nature, lasting longer than 14 weeks, but no more than 19 weeks.

The mean group follicle stimulating hormone (FSH) profiles (Fig. 6a) showed a significant reduction ($p < 0.01$, below 0.41 ng/ml) in the Vaccine 001 treated animals compared with their own pre-treatment concentrations (0.5 ± 0.12 ng/ml) between days 14 and 56. Compared with the untreated controls (0.58–0.95 ng/ml), the concentrations were markedly reduced ($p < 0.001$, 0.4–0.47 ng/ml) between days 56 and 84. On the other hand, the Vaccine 002 treated animals showed significant increase in FSH levels on days 70 ($p < 0.05$, 0.75 ± 0.43 ng/ml) and 84 ($p < 0.001$, 0.99 ± 0.59 ng/ml), compared with their own pre-treatment concentrations (0.49 ± 0.16 ng/ml). The untreated controls showed an increased response ($p < 0.001$) from day 56 onwards, this being indicative of a normal seasonal increase in this gonadotrophin. The individual hormone profiles (Fig. 6b) showed that the two animals with basal testosterone levels also showed a significant decrease

Table 3
Quantification of the level of spermatogenesis (mean ± S.D.) in two untreated control and two immunocastrated animals (treated with Vaccine 001)

Score	Control ram 1	Control ram 2	Treated ram 1	Treated ram 2
% Number of views				
10	36	32	0	0
9	44	38	0	0
8	10	6	0	2
7	10	24	0	0
6	0	0	0	6
5	0	0	0	0
4	0	0	48	34
3	0	0	48	46
2	0	0	4	12
1	0	0	0	0

Randomly selected 50 tubular cross-sections per animal were studied under low (25×) magnification. All tubular sections in one field of vision were given a score of 1–10 (Table 2) and the results were plotted as the number of similar scores for each animal.

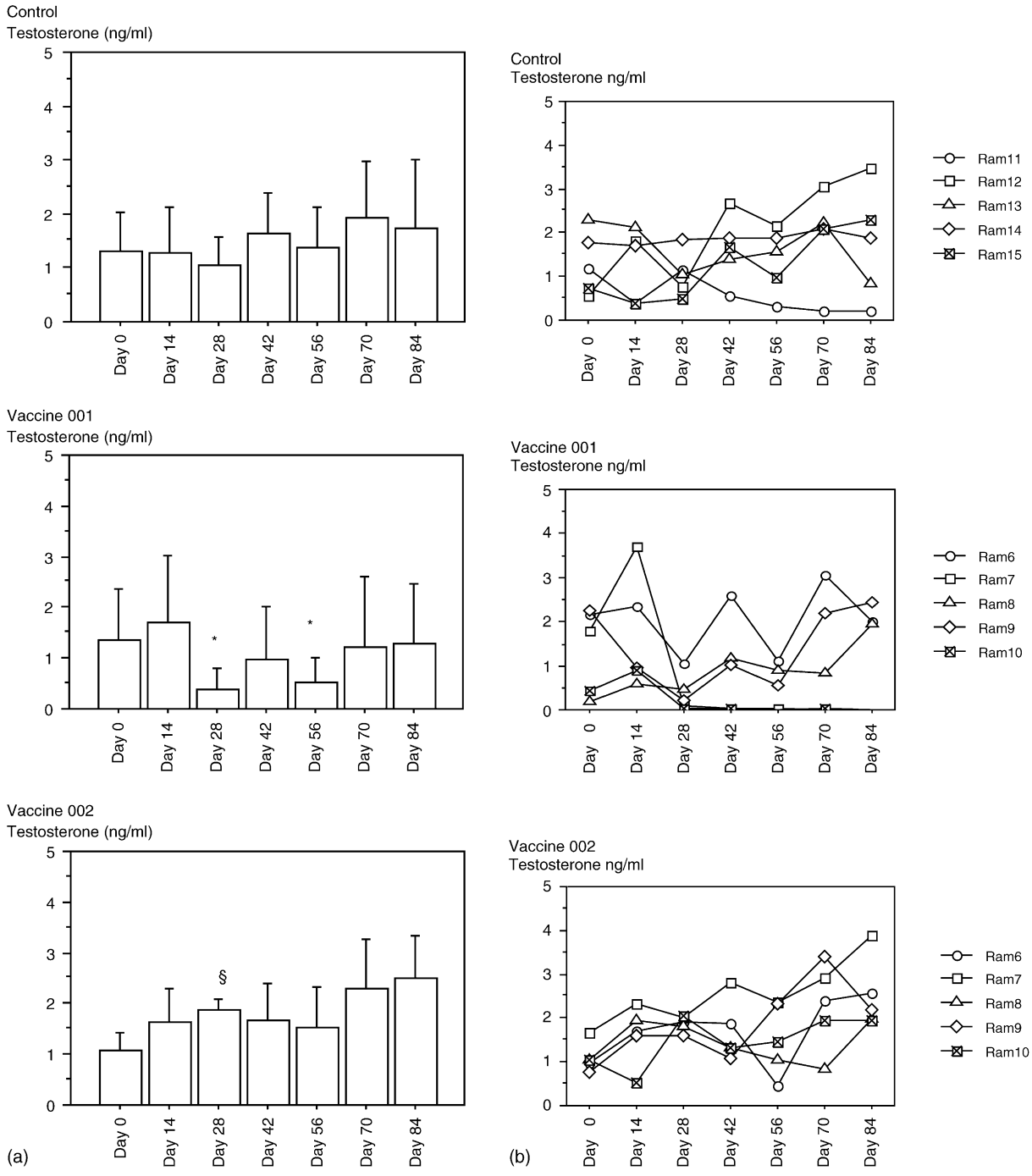

Fig. 5. (a) Group testosterone profiles following immunisation with Vaccine 001 (days 0, 21, 42 and 63) or Vaccine 002 (days 0 and 42), compared with untreated controls. Each point represents the mean ± S.D. * Significant decrease compared with untreated controls. § Significant increase compared with untreated controls. (b) Individual animal testosterone profiles following immunisation with Vaccine 001 (days 0, 21, 42 and 63) or Vaccine 002 (days 0 and 42), compared with untreated controls.

in FSH on days 70 ($p < 0.05$, 0.31 ± 0.04 ng/ml) and 84 ($p < 0.01$, 0.26 ± 0.07 ng/ml) compared with their own pre-treatment concentrations (0.4 ± 0.1 ng/ml). Compared with the untreated controls significant decrease in FSH was also observed on days 42 ($p < 0.05$) and 56–84 ($p < 0.001$). However, unlike the testosterone levels the remaining animals also showed significant decrease in FSH at varying times, compared at pre-treatment and with the untreated controls. The level of significant difference is summarised in Table 4.

Luteinizing hormone (LH) levels in the Vaccine 001 treated animals (Fig. 7a) was not significantly altered from the untreated controls or compared with the pre-treatment levels (0.68 ± 0.44 ng/ml). The Vaccine 002 treated animals

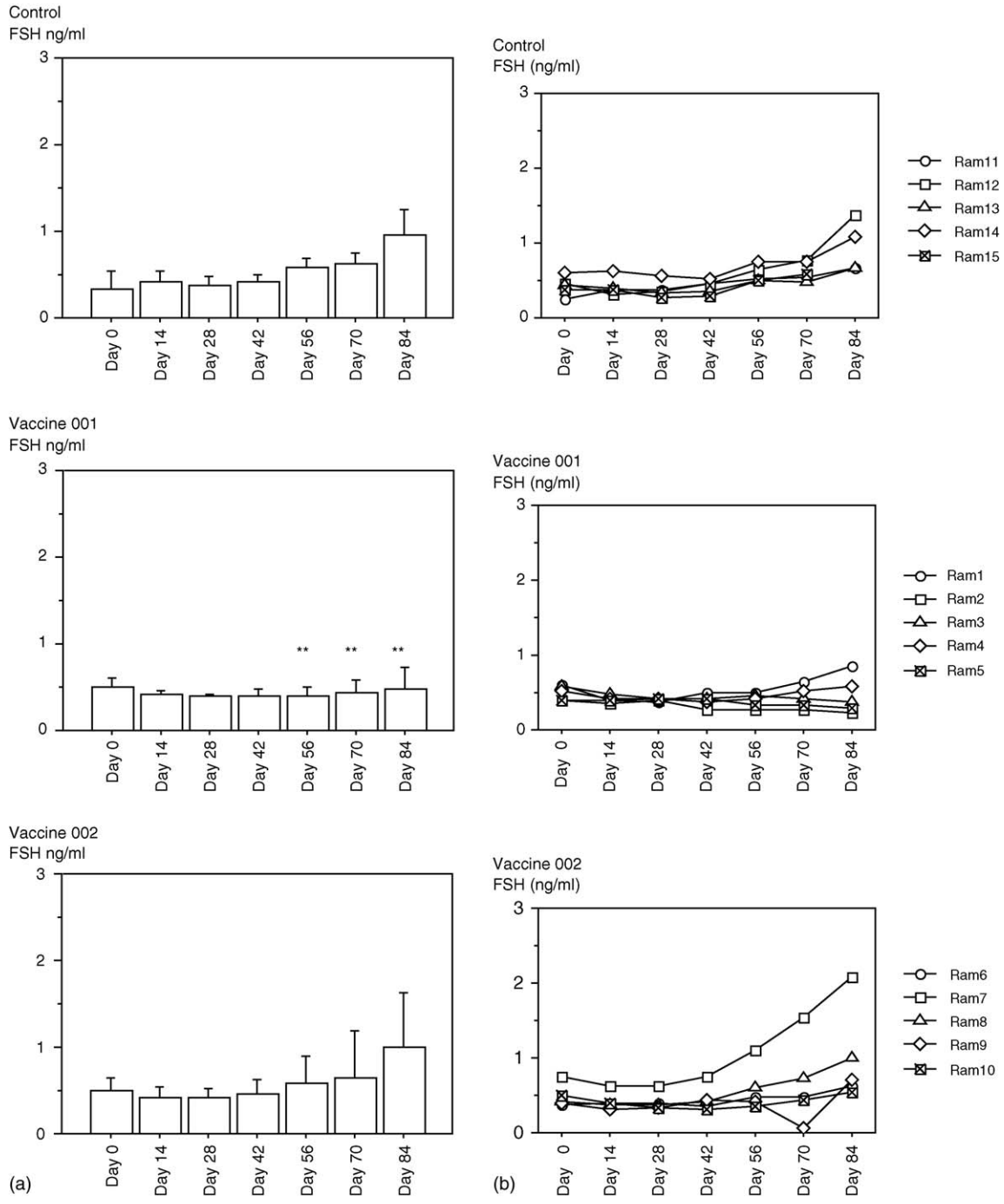

Fig. 6. (a) Follicle stimulating hormone profiles following immunisation with Vaccine 001 (days 0, 21, 42 and 63), (b) or Vaccine 002 (days 0 and 42), compared with untreated controls. Each point represents the mean ± S.D. $^{**}p < 0.001$, significant decrease compared with untreated controls. (b) Individual animal follicle stimulating hormone profiles following immunisation with Vaccine 001 (days 0, 21, 42 and 63) or Vaccine 002 (days 0 and 42), compared with untreated controls.

showed elevated concentrations of LH on day 84 ($p < 0.04$, 1.11 ± 0.65 ng/ml), compared with the pre-treatment levels (0.76 ± 0.4 ng/ml). The individual animal profiles (Fig. 7b) showed that the two animals with basal testosterone levels, showed a significant increase ($p < 0.02$, 0.64 ± 0.03 ng/ml) in LH on day 70 compared with their own pre-treatment levels (0.52 ± 0.12 ng/ml).

4. Discussion

Vaccines against GnRH have the potential to play an important role in the humane control of fertility in farm animals [3,5,11]. However, there is considerable variation in response between vaccines tested in laboratory animal models and their subsequent efficacy in field trials. The aim of

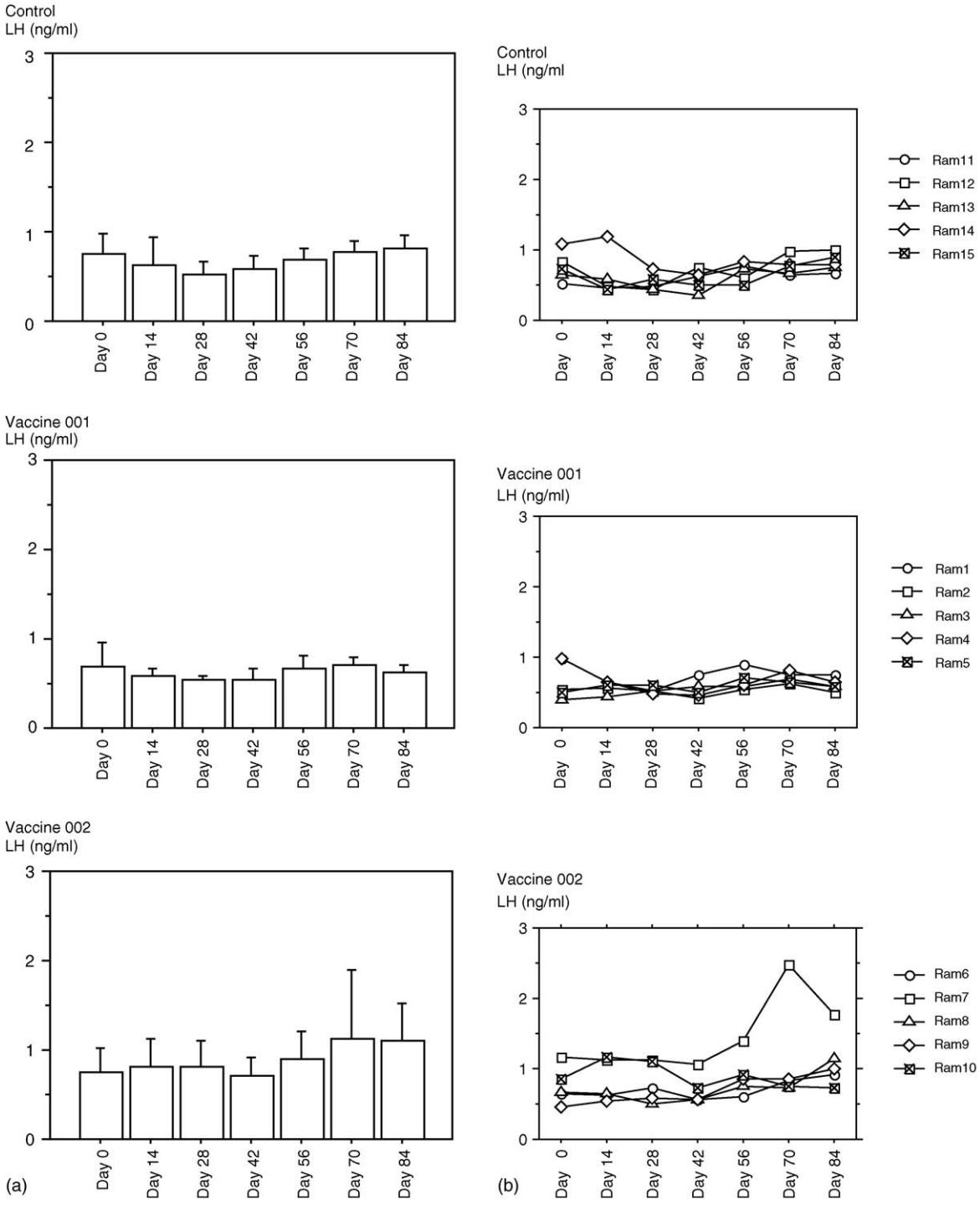

Fig. 7. (a) Luteinising stimulating hormone profiles following immunisation with Vaccine 001 (days 0, 21, 42 and 63), (b) or Vaccine 002 (days 0 and 42), compared with untreated controls. Each point represents the mean ± S.D. (b) Individual animal luteinising hormone profiles following immunisation with Vaccine 001 (days 0, 21, 42 and 63) or Vaccine 002 (days 0 and 42), compared with untreated controls.

this study was to examine the efficacy of an immunogen that has consistently ablated fertility in laboratory animals in an agricultural model.

The modified GnRH peptide (CHWSYGLRPG-NH_2) was chosen as it enables highly specific antibodies to be generated, which have little cross-reactivity with other isoforms of GnRH [12]. Two different carrier proteins, tetanus and diphtheria toxoid, were used alternately with the peptide to reduce epitope suppression [10,13,14]. Both these molecules have previously caused significant castration when conjugated to GnRH peptides [15,16] and used singly. In general, Quil A is known to induce a good cellular immune response [17],

Table 4
Significant difference in hormone levels compared with own pre-treatment or untreated control levels

Study days	0	14	28	42	56	70	84
Control	N/A	–	–	–	–		–
Testosterone	N/A	N/A	N/A	N/A	N/A		N/A
Vaccine 001	N/A	–	<0.01*	–	<0.05*	–	–
Testosterone	–	–	<0.05*	–	<0.05*	–	–
Vaccine 001[a]	N/A	–	<0.05*	<0.05*	<0.05*	<0.05*	<0.05*
Testosterone	–	–	<0.05*	<0.05*	<0.05*	<0.05*	<0.05*
Vaccine 001[b]	N/A	–	–	–	–	–	–
Testosterone	–	–	–	–	–	–	–
Vaccine 002	N/A	–	<0.05§	–	–	<0.05§	<0.01§
Testosterone	–	–	<0.05§	–	–	–	–
Control	N/A	–	–	–	<0.001§	<0.001§	<0.001§
FSH	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Vaccine 001	N/A	<0.01*	<0.01*	<0.01*	<0.01*	–	–
FSH	–	–	–	–	<0.001*	<0.001*	<0.001*
Vaccine 001[a]	N/A	–	–	–	–	<0.05*	<0.01*
FSH	–	–	–	<0.05*	<0.001*	<0.001*	<0.001*
Vaccine 001[b]	N/A	<0.001*	<0.001*	<0.01*	<0.01*	–	–
FSH	<0.01*	–	–	–	<0.01*	<0.05*	<0.01*
Vaccine 002	N/A	–	–	–	–	<0.05§	<0.001§
FSH	–	–	–	–	–	–	–
Control	N/A	–	–	–	–	–	–
LH	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Vaccine 001	N/A	–	–	–	–	–	–
LH	–	–	–	–	–	–	–
Vaccine 001[a]	N/A	–	–	–	–	<0.02§	–
LH	–	–	–	–	–	–	–
Vaccine 001[b]	N/A	–	–	–	–	–	–
LH	–	–	–	–	–	–	–
Vaccine 002	N/A	–	–	–	–	–	<0.04§
LH	–	–	–	–	–	–	–

–: Not significant; N/A: not applicable.
[a] Animals with basal levels of testosterone (rams 12 and 15).
[b] Remaining animals in group (rams 11, 13, 14).
* Significant decrease in hormone.
§ Significant increase in hormone.

however when used with CHWSYGLRPG-NH_2 conjugated to tetanus toxoid in rats, we obtained a mixed cellular and humoral immune response. In comparison, the conventional adjuvant used in veterinary vaccines based on aluminium salts [18], induced a predominant humoral or antibody neutralising response for a very short period of time [10]. To date, the mechanism for effective castration using anti-GnRH vaccines has not been elucidated, however, there is growing evidence to indicate that a cellular immune response is essential [6]. In veterinary applications, a slow release vaccine has advantages in terms of welfare concerns and cost [1], therefore, PLGA was assessed as a sustained release injectable in this study. It has also been shown to cause a mixed immune response in a rodent model, similar to Quil A [10].

In terms of the specific IgG levels obtained following immunisation, only the Vaccine 001 group showed significant antibody levels. Clearly, the alternate carrier molecule combination was effective with Quil A, as has also been shown by other studies, where carrier suppression has presented a problem in a particular species [8]. However, when used in a slow release formulation, it was expected that a bolus of TT-GnRH-I, followed by one consisting of DT-GnRH-I with a 6-weekly interval, would also act in a similar way. This did not occur and the specific antibody levels raised were not significant. It is highly likely that four injections (two of the TT-conjugate, followed by two DT-conjugate) administered over a longer period of time would have provided the necessary hormone ablation without incurring epitope suppression. Despite this failure, an indication

of the importance of multiple administration of the carrier protein in producing an antibody response to a hapten was understood.

The decrease in scrotal circumference was primarily a manifestation of gonadal atrophy, shown by changes in the structure of the tubules and significant decrease in spermatogenesis. This was consistent with previous observations in rams immunised with TT-GnRH-I adsorbed onto an aluminium-based adjuvant [10].

The pulsatile nature of testosterone and gonadotrophin secretion makes it extremely difficult to interpret individual data, taken at single time points. However, in this study four samples were taken throughout a 4.5 h period. Previous work by us (unpublished data) suggests that this protocol is not significantly different from one in which 90 min intervals were used over 7.5 h. Overall, only testosterone and FSH levels appeared to be affected in the Vaccine 001 treated animals with significant reduction after two administrations of the vaccine. The most notable feature in the study was that two of the rams vaccinated with Vaccine 001 showed basal levels of testosterone and a suppression of the normal seasonal increase in both testosterone and FSH, which were observed in the untreated controls and the Vaccine 002 treatment. However, the hormone levels could not be correlated with antibody response in the Vaccine 001 treated animals, since all the animals showed similar antibody levels. Therefore, the presence of high levels of IgG does not necessarily cause testosterone ablation to basal levels and some other mechanism has a part to play, possibly at cellular level. Histological examination of the testes from these animals clearly showed a shift from the untreated control scores between 7 and 10 with active spermatogenesis to views mainly showing suppressed spermatogenesis (scores between 2 and 4). Taken together, these observations indicate an extremely positive physiological reaction following vaccination with Vaccine 001.

A recent study [19], which evaluated the effect of a commercial GnRH vaccine (Improvac^{TM}) in ram lambs (aged 2–3 months) showed basal testosterone concentrations for at least 12 weeks, following two injections. It is interesting that our results show a very similar trend in that two of the animals treated with Vaccine 001 had complete suppression of the testosterone concentrations and reduced FSH concentrations up to day 84, but the vaccine was no longer effective by day 132. Unfortunately, for ethical reasons it was not possible to carry out blood sampling between days 84 and 132, to determine when the efficacy of the vaccine waned. The fact that only a single sample was taken at day 132 does not reduce the significance of the hormonal rebound, as the testosterone concentrations were basal at each of the four samplings of the 5 bleeding days, and the single sampling were similar for the control rams. The fact that Vaccine 001 was extremely effective in two rams suggests that this vaccine merits further investigation in terms of drug dose and immunisation regime, as it has the potential to provide a unique and highly specific anti-GnRH treatment for veterinary application.

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