

University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

USDA National Wildlife Research Center - Staff
Publications

U.S. Department of Agriculture: Animal and Plant
Health Inspection Service

2014

Parenteral administration of GnRH constructs and adjuvants: Immuneresponses and effects on reproductive tissues of male mice

Sameer Sharma
University of Canberra

Ian McDonald
University of Canberra

Lowell Martin
University of Canberra

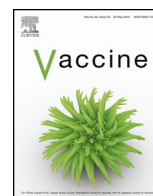
Lyn A. Hinds
National Wildlife Research Center, Lyn.Hinds@csiro.au

Follow this and additional works at: https://digitalcommons.unl.edu/icwdm_usdanwrc

 Part of the [Life Sciences Commons](#)

Sharma, Sameer; McDonald, Ian; Martin, Lowell; and Hinds, Lyn A., "Parenteral administration of GnRH constructs and adjuvants: Immuneresponses and effects on reproductive tissues of male mice" (2014). *USDA National Wildlife Research Center - Staff Publications*. 1540.
https://digitalcommons.unl.edu/icwdm_usdanwrc/1540

This Article is brought to you for free and open access by the U.S. Department of Agriculture: Animal and Plant Health Inspection Service at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in USDA National Wildlife Research Center - Staff Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.



Parenteral administration of GnRH constructs and adjuvants: Immune responses and effects on reproductive tissues of male mice



Sameer Sharma^{a,b}, Ian McDonald^{a,b,c}, Lowell Miller^d, Lyn A. Hinds^{a,b,*}

^a Commonwealth Scientific and Industrial Research Organisation (CSIRO), Biosecurity Flagship, GPO Box 1700, Canberra, ACT, Australia

^b Invasive Animals Cooperative Research Centre (IA CRC), University of Canberra, Canberra, ACT, Australia

^c School of Agriculture and Food Science, University of Queensland, Brisbane, Queensland, Australia

^d National Wildlife Research Center, USDA, Fort Collins, CO, USA

ARTICLE INFO

Article history:

Received 14 May 2014

Received in revised form 8 July 2014

Accepted 21 July 2014

Available online 15 August 2014

Keywords:

Vaccine

Endogenous peptide

Immunostimulating complexes

Cytosine-phosphate-guanosine

oligodeoxynucleotides

Testis

Epididymis

ABSTRACT

Two gonadotrophin releasing hormone (GnRH) constructs prepared by either chemical conjugation to keyhole limpet hemocyanin (GnRH-KLH) or as an expressed recombinant fusion protein (Multimer) were evaluated with or without adjuvants (immunostimulating complexes, ISCOMs, or cytosine-phosphate-guanosine oligodeoxynucleotides, CpG ODNs). After subcutaneous administration to Balb/c male mice at Weeks 0, 2 and 4, these preparations were assessed for induction of immune responses and effects on reproductive organs. GnRH-KLH plus ISCOMs formulation induced strong IgG immune responses from Week 4 through Week 12 resulting in consistent reproductive organ atrophy by Week 12 after subcutaneous administration. GnRH-KLH plus CpG ODNs generated immune responses but no atrophy of reproductive tissues by Week 12. Multimer plus ISCOMs induced poor immune responses and no effects on reproductive tissues by Week 12. In the absence of additional adjuvant, none of the GnRH constructs induced reproductive organ atrophy. GnRH-KLH induced stronger immune responses when formulated with ISCOMs or CpG ODN compared to Multimer. GnRH-KLH with ISCOMs could be an effective colloidal alternative for emulsion GnRH vaccine formulations.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Reproductive function in mammalian species is regulated by the hypothalamic-pituitary-gonadal axis [1]. GnRH (also known as LHRH, luteinizing hormone releasing hormone) is a decapeptide synthesised in neuronal cell bodies in the preoptic area of the anterior hypothalamus. Once secreted, GnRH is transported to the anterior pituitary via the hypothalamic-pituitary portal system [1]. It then stimulates production and release of the two pituitary hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), which play a critical role in the development and regulation of gonadal functions, including the production of gametes and sex steroid hormones. Targeting GnRH for contraception or sterilisation can be effective in both males and females

[2–5], and GnRH neutralisation has been reported as a treatment for hormone dependent malignancies such as prostate cancer [6].

One of the major challenges in developing and formulating an effective contraceptive vaccine is achieving strong systemic immunity against the selected reproductive antigen. The immunogenicity of small endogenous non-immunogenic peptides (such as GnRH), can be enhanced by chemically conjugating the peptide to a larger immunogenic protein or peptide (e.g. tetanus toxoid, KLH or ovalbumin) [6,7]. This can also be achieved by recombinant expression of a fusion protein prepared by combination of multiple protein(s) and/or peptide(s) [8–11]. For example, Talwar et al. [8] developed a GnRH construct in which 5 GnRH peptides were interspersed with 4 immunogenic T cell epitopes from a range of known infectious microbes.

Even when GnRH is conjugated to a larger protein or peptide(s), additional adjuvants are required in the formulation for a strong induction of immune responses [12,13]. Therefore it is hypothesised that the use of additional adjuvants in a formulation should increase overall immunogenicity and in turn inhibit fertility. Many of the conventional immunological adjuvants, such as Freund's complete adjuvant (FCA), bacterial toxins and non-purified crude agents (e.g. lipid A), induce strong stimulant effects but also frequently induce adverse effects [14,15]. The critical importance

Abbreviations: GnRH, gonadotrophin releasing hormone; KLH, keyhole limpet hemocyanin; ISCOMs, immunostimulating complexes; CpG ODNs, cytosine-phosphate-guanosine oligodeoxynucleotides.

* Corresponding author at: Commonwealth Scientific and Industrial Research Organisation (CSIRO), Biosecurity Flagship, GPO Box 1700, Canberra, ACT 2601, Australia. Tel.: +61 2 62464092.

E-mail address: Lyn.Hinds@csiro.au (L.A. Hinds).

<http://dx.doi.org/10.1016/j.vaccine.2014.07.075>

0264-410X/© 2014 Elsevier Ltd. All rights reserved.

of developing a formulation that is both efficacious and free of side effects for an animal reproductive vaccine was demonstrated by the Vaxstrate[®] vaccine, which comprised a LHRH–ovalbumin conjugate formulated with an oil emulsion adjuvant system [16]. Vaxstrate[®] was used in Australia during the 1990s but was withdrawn due to side effects (e.g. 40% animals with abscesses) and poor efficacy in the field [17].

Alternative reagents such as purified and/or receptor-specific adjuvants [9,10] are being investigated to enhance efficacy and reduce side effects. ISCOMs are a saponin containing lipophilic cage-like structured immunostimulant with regulatory approval for veterinary vaccines in various countries [18,19]. ISCOMs are also of interest due to their cage-like structure which could incorporate a soluble antigen [20,21]. Another group of adjuvants are CpG ODNs which have toll-like receptor (TLR-9) specific immunostimulant properties due to the presence of the CpG motif [22,23]. In this study, acellular mono-phasic aqueous adjuvants (ISCOMs and CpG ODNs) have been selected for in vivo assessment with two GnRH-constructs GnRH-KLH and Multimer in male mice. Immune responses over time and effects on reproductive tissues have been determined.

2. Materials and methods

2.1. Animals

Male Balb/c mice aged between 5 and 6 weeks were purchased from Animal Resource Centre, Perth, Western Australia. All animal studies were approved by the CSIRO Ecosystem Sciences Animal Ethics Committee (approval numbers 10-01 and 13-04).

2.2. Experimental design

2.2.1. Trial 1: assessment of GnRH-KLH formulations

Five groups of 5 male mice received subcutaneous injections of their respective treatment at Weeks 0, 2 and 4. The treatments were: GnRH-KLH (50 µg) with or without an adjuvant (either ISCOMs (10 µg), CpG ODN-1826 (25 µg) or CpG ODN-2006 (25 µg)) and a negative control group received subcutaneous buffer injections (100 µl). A sixth group (positive control) received a single subcutaneous injection of Gonacon[™] (GnRH conjugated to Blue Mollusc protein ~100 µg) at Week 0. For all subcutaneous injections, 100 µl of formulation volume or buffer (phosphate buffer saline (PBS)) was administered.

Animals were weighed weekly using a Persola balance (±0.5 g). Blood samples were collected (see Section 2.3) between 0830 and 1100 h from each mouse at Weeks 0, 2 and 4 before subcutaneous treatments of formulations, and at Weeks 6, 9 and 12.

2.2.2. Trial 2: assessment of Multimer formulations

Five groups of 5 male mice received subcutaneous injections of their respective treatment at Weeks 0, 2 and 4. These treatments were: Multimer (50 µg) with or without an adjuvant (either ISCOM (10 µg) or CpG ODN-2006 (25 µg)); GnRH-KLH with added ISCOMs as in Trial 1, and a negative control group received subcutaneous buffer injections (100 µl). CpG ODN-2006 was selected over CpG ODN-1826 based on both reproductive tissue and immune responses for treated animals in Trial 1. A sixth group (positive control) received a single subcutaneous injection of Gonacon[™] (~100 µg) at Week 0. For all the subcutaneous injections, 100 µl of formulation volume or buffer (PBS) was administered. Animals were weighed and blood samples were collected as explained above trial 1.

2.3. Sample collection

Peripheral blood (75–100 µL) was collected from the saphenous vein into a heparinised capillary tube (1.1–1.2 mm width; Chase, Scientific Glass Inc., USA). The blood sample was transferred to a 400 µL micro tube (Sarstedt Pty Ltd., Australia) and placed on ice. Samples were then spun in an Eppendorf 5415R microcentrifuge set at 9000 rpm (7500 × g) and 4 °C for 20 min. The plasma was collected into aliquots and placed at –20 °C until needed.

At Week 12, mice were euthanased using 4% isoflurane in oxygen (Laser Animal Health, Australia). When the mouse was under deep anaesthesia, heart puncture was used to collect approximately 1 mL of blood into a heparinised tube. The mouse was then killed by cervical dislocation and reproductive tissues dissected, weighed and placed in Bouin's fixative for histological examination.

2.4. Preparation of formulations

The GnRH-KLH construct was synthesised by Biomatik laboratories, Canada. It was prepared by chemically conjugating keyhole limpet haemocyanin (KLH) to the modified GnRH peptide (amino acid sequence: pEHWSYGLRPGGC) at the C terminus using a sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) coupling agent at a 1:1 ratio. GnRH-KLH was initially dissolved in a small volume of phosphate buffer containing 8 M urea followed by 10-fold dilution with PBS. ISCOMs (Pfizer Animal Health, Australia) or CpG ODN (Sapphire Biosciences, Australia) solution was added to the GnRH conjugate solution to achieve specified concentrations for subcutaneous treatments (see Section 2.2).

The Multimer construct comprised five oligonucleotide strands coding for the GnRH decapeptide interspersed with four oligonucleotide sequences coding for promiscuous T helper non B-cell epitopes. These T_h cell epitopes were from the circumsporozoite protein of *Plasmodium falciparum* (T₁), *Mycobacterium tetani* Tetanus toxoid (T₂), respiratory syncytial virus (T₃) and measles virus (T₄). Multimer structure is GnRH-T₁-GnRH-T₂-GnRH-T₃-GnRH-T₄-GnRH [8]. Multimer was expressed and purified at National Wildlife Research Center (NWRC), Fort Collins, CO, USA. Freeze dried Multimer was dissolved in sterile water to obtain a construct solution in sodium phosphate buffer pH 5.0.

GonaCon[™] vaccine, developed as a single-shot treatment, was used as a positive control vaccine. It contains GnRH conjugated to a blue mollusc protein emulsified in AdjuVac[™] adjuvant [24,25]. Adjuvac[™] adjuvant has been developed by NWRC and contains small quantities of killed *Mycobacterium avium* in a stable water in oil (w/o) emulsion. Adjuvac[™] is similar to FCA in that it is a w/o emulsion formulation but FCA contains *Mycobacterium tuberculosis*.

2.5. Anti-GnRH plasma antibodies

Plasma samples were assayed for IgG antibodies against GnRH using an ELISA. The following protocol was used in order:

Coating buffer (50 µL, 0.1 M carbonate bicarbonate buffer pH 9.6; Sigma Aldrich, Australia) containing 200 ng of GnRH conjugated to bovine serum albumin (GnRH-BSA) (Genscript, Australia) was added per well of flat-bottom Nunc F96 MicroWell[™] plates (In vitro Technologies, Australia) in duplicate and incubated overnight at 4 °C. Blocking buffer (200 µL), sterile PBS with 0.5% Fraction V BSA (Sigma Aldrich, Australia) was added per well and incubated at 37 °C for 2 h. Plasma (50 µL of 1:50 dilution in PBS) was added per well, in duplicate, and incubated at 37 °C for 1 h. Goat anti mouse IgG HRP-conjugated secondary antibody (Sigma Aldrich, Australia) (50 µL of 1:8000 dilution in PBS containing 0.5% BSA) was added per well and incubated at 37 °C for 1 h. All wells were washed 3 times with wash buffer (PBS with 0.05% (v/v) Tween 20

(Sigma Aldrich, Australia)) between each incubation step. 50 μ L of 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) substrate (Sigma Aldrich, Australia) was added per well. The plates were placed on a shaker (150 rpm) at room temperature and left to develop for 10 min. The reaction was stopped by the addition of 50 μ L of 2 M sulphuric acid solution to each well. The absorbance of each well was measured at 450 nm in a Thermo Labsystems Multi-skan Ascent photometric plate reader (ThermoScientific, Australia) and analysed with Ascent Software (v.2.4.2). Results are presented as optical density (OD) values minus the OD readings for the blank wells (containing PBS only) from the same plate. A result was considered positive if it was above the average Week 0 readings + 3 SD (0.512 for Trial 1 or 0.557 for Trial 2).

2.6. Histology of reproductive tissues

After fixation in Bouin's fluid for 24 h, a portion of the right testis and of the caput (head) and cauda (tail) of the right epididymis was processed by an automatic Medite Tissue Processing Centre and embedded using a Medite TBS 88 Paraffin Embedding System in a single paraffin wax block. Sections (5 μ m) were taken using a Leica RM2255 Microtome and mounted onto premium glass slides (Livingstone International Pty Ltd., Australia) and left to dry on a heat mat at 35 °C for 48–72 h. Slide sections were deparaffinised and rehydrated through a graded series of ethanol baths and stained using a Gill's haematoxylin and eosin stain. After slides were mounted and cover slipped, they were left to dry on a heat mat set at 35 °C for a further 4–8 h and stored in a covered slide box.

Testis sections (three per animal) were observed under a Leica Axioimager microscope and three to four representative photos were taken. The diameter of thirty circular seminiferous tubules was measured as described by Predes et al. [26] using AxioVision Release software V.2.8.2. The caput and cauda sections of the epididymis were observed for cell structure and presence of sperm. The examinations were carried out without knowledge of the treatment by randomly coding the samples and not revealing the codes until after analysis was completed.

2.7. Statistical analysis

Data from the control and treatment groups were compared by a one way analysis of variance (ANOVA) followed by Dunnett's post hoc test using Sigma Plot Version 12.0. For body weight, a two way repeated measures ANOVA (using Graphpad Prism 6 software) was performed followed by Dunnett's post hoc test.

3. Results

3.1. Trial 1

3.1.1. Body weight

Mice gained or maintained their weight during the 12-week trial. Animals in the GnRH-KLH plus ISCOMs group remained healthy and alert but were significantly lighter ($P < 0.05$; range: 20–23 g) compared to the negative control group (range: 24–26 g), from Week 8 to Week 12. All other treatment groups continued to gain body mass between Week 6 and Week 12 (Fig. 1). One mouse given GnRH-KLH with CpG ODN 1826 died from unknown causes during Week 9.

3.1.2. Immune responses

All mice given a single injection of the Gonacon™ vaccine had GnRH-specific antibodies by Week 2, and four mice maintained this response through to Week 12 (Fig. 2b). A similar immune response was observed in mice given GnRH-KLH with ISCOMs (Fig. 2d). All

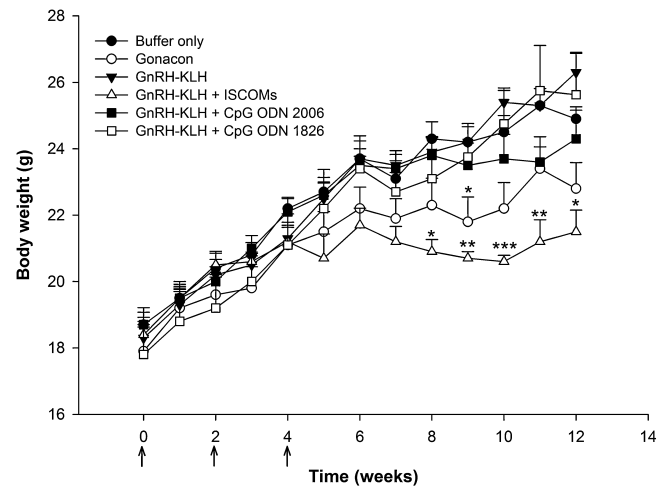


Fig. 1. Changes in body weight (mean \pm SEM) of mice in the different treatment groups in Trial 1. Solid arrows on x-axis indicate treatment times for all groups except Gonacon™ group which received a single treatment at Week 0 only. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two way repeated measures ANOVA, followed by Dunnett's post hoc analysis).

mice treated with GnRH-KLH and CpG ODN 1826, showed an antibody response by Week 4 (Fig. 2f). Two mice given GnRH-KLH with CpG ODN 2006 showed an antibody response by Week 4 through to Week 12, with two others showing variable responses from Week 6 onwards (Fig. 2e). Mice given GnRH-KLH without an adjuvant had measurable antibody responses by Week 6 (Fig. 2c). No GnRH-specific immune responses were detected in any of the mice given the negative control treatment (Fig. 2a).

3.1.3. Tissue weights

Three of five mice given Gonacon™ showed decreases in testis, epididymis and seminal vesicle/prostate gland weights; for the overall group, however, only the average weight of the testes showed a significant decrease ($P < 0.05$) (Table 1). All mice receiving the GnRH-KLH with ISCOMs treatment had significantly smaller testes, epididymides and seminal vesicles/prostate gland weights ($P < 0.001$) and heavier spleen weights ($P < 0.01$) compared with the negative control mice. No other treatment group showed consistent decreases in reproductive tissues (Table 1). However, some individuals showed reproductive effects by Week 12 after the start of their respective treatments. In one mouse given GnRH-KLH with CpG ODN 2006 there was a reduction in all reproductive tissue weights compared with the control mice. Its combined testes, combined epididymides, and total seminal vesicle and prostate weights were 70 mg, 18 mg and 18 mg, respectively.

3.1.4. Histology of reproductive tissues

In mice which showed no changes in reproductive tissues weights, all normal stages of spermatogenesis were observed within the tubules of the testis and epididymis, and their seminal vesicles were fluid filled (Fig. 3a, b, e, and f). By contrast at Week 12, in all mice given GnRH-KLH with ISCOMs the majority of seminiferous tubules lacked a defined lumen and few to no spermatozoa were present (Fig. 3c, d, g, and h). Spermatids were absent, although spermatocytes and Sertoli cells were observed (Fig. 3c). As atrophy occurred, the seminiferous tubules also became significantly ($P < 0.001$) smaller in diameter (control = $199 \pm 16 \mu$ m vs. GnRH-KLH plus ISCOMs = $121 \pm 27 \mu$ m) (Fig. 3b and d).

The absence of spermatogenesis in the testis was paralleled by atrophy in the epididymis and seminal vesicles. The volume of epithelial cells lining the epididymis was reduced, with almost no sperm present in the fluid filled tubules (Fig. 3g). These effects were

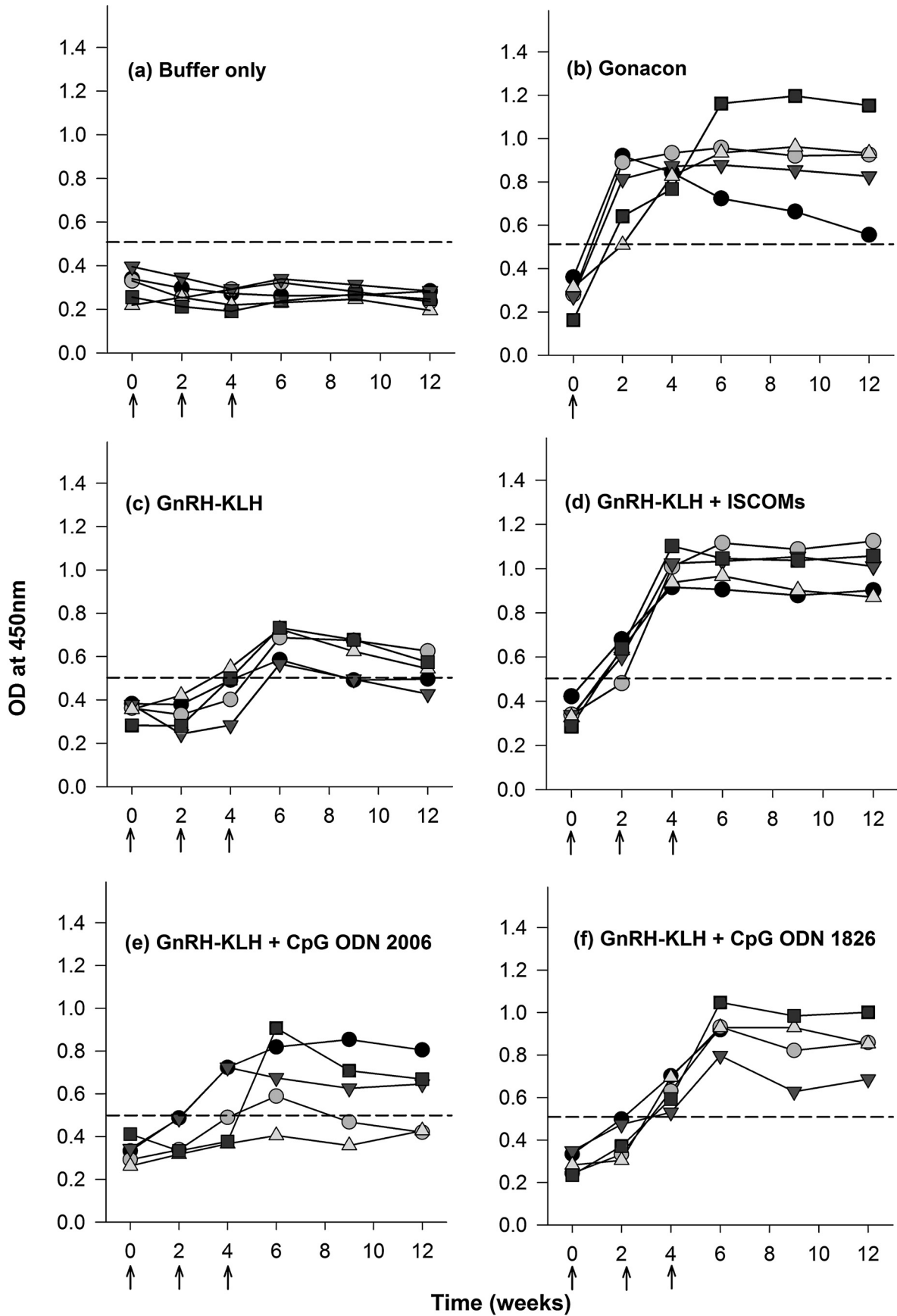


Fig. 2. Anti-GnRH IgG antibody response profile (1:50 plasma dilution) for respective treatments in Trial 1. Arrows on x-axis indicate treatment times. Dashed line in the graphs represents the assay cut-off point.

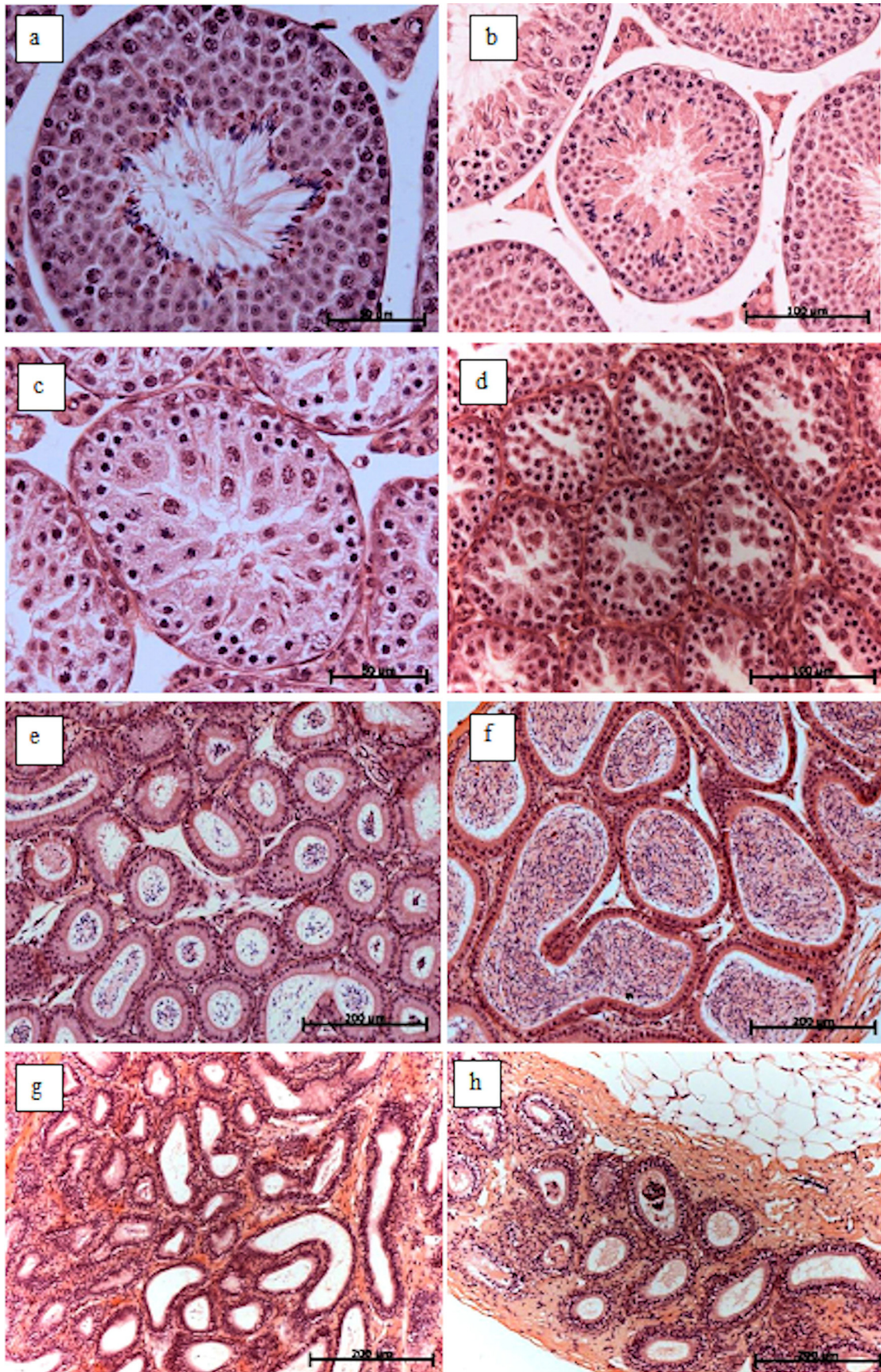


Fig. 3. Representative histological sections of mouse reproductive tissues at Week 12, from Trial 1. (a) and (b) Normal seminiferous tubules from a control animal; (c) and (d) degenerating and atrophied seminiferous tubules of a mouse treated with GnRH-KLH plus ISCOMs; (e) normal caput epididymis and (f) normal corpus epididymis from a Control mouse; and (g) atrophied caput epididymis and (h) atrophied corpus epididymis of a mouse treated with GnRH-KLH plus ISCOMs. Scale bar (μm) shown on each section.

Table 1
Weights (mean \pm SEM) of reproductive tissues and spleen at Week 12 for animals in different treatment groups in Trial 1.

Treatment	No. of animals	Spleen (mg)	Testes (mg)	Epididymides (mg)	Seminal vesicles and prostate gland (mg)
Buffer only (negative control)	5	81.8 \pm 4.6	219.0 \pm 4.9	67.6 \pm 2.4	289.8 \pm 8.3
GonaCon™ (Positive control)	5	99.0 \pm 6.9	118.2 \pm 27.6*	46.2 \pm 10.5	125.4 \pm 68.7
GnRH-KLH	5	85.2 \pm 2.4	229.4 \pm 5.2	71.4 \pm 2.1	287.8 \pm 29.8
GnRH-KLH + ISCOM	5	108.0 \pm 3.5**	67.0 \pm 11.8***	22.8 \pm 4.6***	22.8 \pm 9.3***
GnRH-KLH + CpG ODN 2006	5	84.0 \pm 7.5	171.0 \pm 27.3	58.0 \pm 10.5	178.6 \pm 46.7
GnRH-KLH + CpG ODN 1826	4	90.8 \pm 2.3	191.8 \pm 3.9	65.0 \pm 3.2	260.5 \pm 9.3

* $P < 0.05$ (one way ANOVA followed by Dunnett's post hoc analysis).

** $P < 0.01$ (one way ANOVA followed by Dunnett's post hoc analysis).

*** $P < 0.001$ (one way ANOVA followed by Dunnett's post hoc analysis).

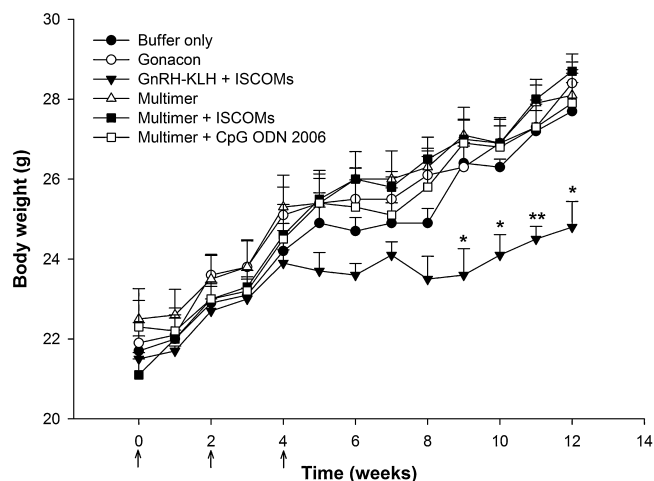


Fig. 4. Body weight (mean \pm SEM) of animals in different treatment groups during the Trial 2. Solid arrows on x-axis indicate treatment times for all groups except Gonacon™ group which received a single treatment at Week 0 only. * $P < 0.05$, ** $P < 0.01$ (Two way repeated measures ANOVA, followed by Dunnett's post hoc analysis).

also observed in 3 of 5 mice given the Gonacon™ vaccine and 1 of 5 mice given GnRH-KLH with CpG ODN 2006 treatment.

3.2. Trial 2

3.2.1. Body weights

All groups showed increased body weights with time during the trial (Fig. 4), similar to those observed in Trial 1. Animals treated with GnRH-KLH with ISCOMs had significantly ($P < 0.05$) lower mean body weights compared to negative control group from Week 9 to Week 12.

3.2.2. Immune responses

All mice given a single injection of the Gonacon™ vaccine had GnRH-specific antibodies by Week 4, and two mice maintained this response through to Week 12 (Fig. 5b). Mice given GnRH-KLH with ISCOM showed a strong immune response at Week 4 through to Week 12 (Fig. 5c). Three mice administered with Multimer plus ISCOMs showed immune responses by Week 4 but none of these animals maintained the level to Week 12 (Fig. 5e). All other animals treated with other formulations showed no anti-GnRH IgG immune response (Fig. 5a and f).

3.2.3. Tissue weights

There was a significant decrease ($P < 0.001$) in the weights of testes, epididymides and seminal vesicles/prostate gland weights in mice receiving the GnRH-KLH with ISCOMs treatment compared with the negative control mice (Table 2). All mice which received ISCOM as part of their treatment had significantly heavier ($P < 0.01$) spleen weights compared with the negative control mice (Table 2).

No other treatment group, including the positive control group treated with Gonacon™, showed consistent decreases in reproductive tissue weights (Table 2).

3.2.4. Histology of reproductive tissues

As in Trial 1, similar changes in the structure of the testis and epididymis were observed in animals treated with GnRH-KLH with ISCOMs group (data not shown).

4. Discussion

Over two different trials subcutaneous treatment with GnRH-KLH plus ISCOMs induced significant immune responses against GnRH within 4 weeks of treatment and a subsequent significant decrease in reproductive tissue size and weight by Week 12 after the start of treatment. By contrast, while some individual mice showed immune responses following treatment with Multimer plus ISCOMs, these were not sustained and did not result in effects at the reproductive level by Week 12 after start of treatment (Trial 2). Thus although the Multimer construct was immunogenic, it likely requires the use of higher doses, and/or the addition of a stronger adjuvant for the induction of uniform and sustained responses. The significantly larger molecular size of GnRH-KLH compared to Multimer could account for some of the differences observed in immunogenicity of the two constructs. No other treatments generated equivalent uniform immune responses or changes in reproductive tissues to that of GnRH-KLH plus ISCOMs.

Many studies have reported the immunogenicity of different GnRH constructs in various mammals, in the presence of unmodified or modified FCA and/or Incomplete Freund's adjuvant (IFA) [8,27–29]. Beekman et al. [30] reported GnRH lipo-thioester and GnRH lipo-amide formulated with FCA and/or IFA induced an anti-GnRH immune response and immunocastration in pigs. However, no significant decrease in testis weight and minimal anti-GnRH antibody response was observed in the same study when male piglets were treated with GnRH lipo-amide incorporated in ISCOMs [30]. Only a few studies [31,32] have induced an anti-GnRH immune response and gonadal regression in the absence of Freund's adjuvants. Conforti et al. [31] reported CpG ODN 2006 as an efficient replacement of FCA for a recombinant LHRH-ovalbumin fusion protein antigen. Vaccine treatments using various doses of the antigen emulsified with CpG ODN 2006 in mineral oil caused gonadal regression and estrus suppression in heifers. In our trials, uniform gonadal regression was not observed after treatment with either GnRH construct plus CpG ODN. This could be due to the use of different animal models (heifers vs. mice), and/or the duration of our study. However, it is more likely due to their use of a mineral oil emulsion base, similar to FCA, in their trial, whereas we used only phosphate buffer. The use of ISCOMs in two different trials generated stronger and more uniform adjuvant effects compared to CpG ODNs with both GnRH-KLH and Multimer in the buffer base formulations. ISCOMs have also been reported to be safe and effective as an adjuvant by mucosal routes such as for intranasal vaccination

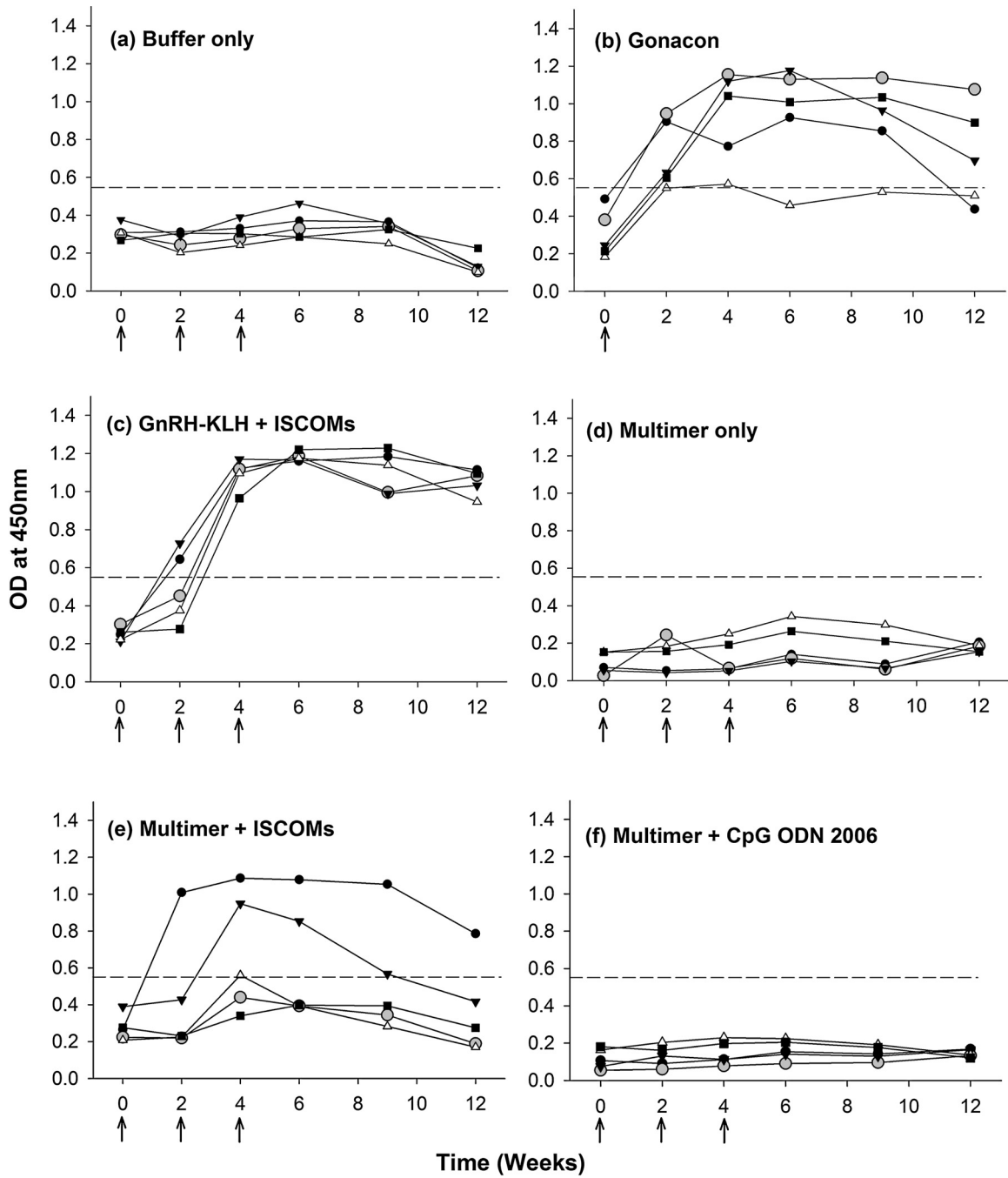


Fig. 5. Anti-GnRH IgG antibody response profile (1:50 plasma dilution) for respective treatments in Trial 2. Arrows on x-axis indicate treatment times. Dashed line in the graphs represents the assay cut-off point.

Table 2
Weights (mean ± SEM) of reproductive tissues and spleen at Week 12 for animals in different treatment groups in Trial 2.

Treatment	No. of animals	Spleen (mg)	Both testes (mg)	Epididymides (mg)	Seminal vesicles and prostate gland (mg)
Buffer only (negative control)	5	85.6 ± 3.8	239.4 ± 2.1	82.8 ± 4.6	258.6 ± 25.6
Gonacon™ (positive control)	5	88.0 ± 1.2	225.8 ± 10.6	74.4 ± 1.0	256.6 ± 14.1
GnRH-KLH + ISCOM	5	107.0 ± 4.5 ^{***}	108.2 ± 19.5 ^{***}	34.4 ± 9.3 ^{***}	61.8 ± 35.2 ^{***}
Multimer only	5	89.2 ± 3.0	226.0 ± 5.1	76.2 ± 1.0	284.2 ± 35.2
Multimer + ISCOM	5	103.2 ± 2.6 ^{**}	226.6 ± 2.5	75.4 ± 0.7	283.2 ± 15.5
Multimer + CpG ODN 2006	5	89.6 ± 1.8	241.0 ± 5.8	77.2 ± 1.6	302.6 ± 12.2

^{**} $P < 0.01$ (one way ANOVA followed by Dunnett's post hoc analysis).

^{***} $P < 0.001$ (one way ANOVA followed by Dunnett's post hoc analysis).

[33–35]. Thus GnRH-KLH plus ISCOMs formulation trialled in our study could not only be a model alternative for modified or non-modified FCA/IFA based emulsion GnRH parenteral formulations but also be a potential formulation for GnRH mucosal vaccination.

The significant difference between body weights of animals administered with GnRH-KLH with ISCOMs compared to the negative control group in both trials by Weeks 8–9 could be the result of diminished anabolic effects due to steroid deprivation which might have occurred because of reproductive organ atrophy. Similar results have been reported for GnRH immunised or castrated animals (various species) in many previous studies [10,36,37]. Similarly, mean body weight of the group administered with Gonacon™ in Trial 1 was significantly less compared to negative control group ($P < 0.05$) at Week 9, although the difference was not maintained through to Week 12 probably due to the variability in reproductive organ atrophy among animals administered with Gonacon™.

Gonacon™ is very effective in inducing contraception after a single injection in many different mammals although the effects are generally more consistent in females than males [2,3,24,38]. In our trials in male Balb/c mice, a single injection of Gonacon™ induced anti-GnRH immune responses and gonadal atrophy in some, but not all of the mice. This variable response could indicate that the administered dose was sub-optimal or that male Balb/c mice are less sensitive to this vaccine compared to other mammals in which it has been tested [38]. In our studies, animals injected with ISCOMs formulations showed an enlarged spleen, an effect which could be due to the presence of a saponin component; some saponin adjuvants are known to possess an angiogenic effect and the spleen enlargement may thus be caused by increased blood supply [39,40].

For those mice which showed atrophy of the reproductive tissues, it could be assumed they would be infertile given the absence of spermatogenesis in the testis and absence of sperm in the epididymis. The duration of infertility needs to be assessed in further trials. Mice treated with GnRH-KLH plus ISCOMs also showed significant decreases in prostate size; these effects indicate that the GnRH-KLH plus ISCOMs formulation could be used as a model system for the development of a potential immunotherapeutic treatment of androgen-responsive prostate cancer.

To conclude, our results indicate that, under the same test conditions, GnRH-KLH is more immunogenic compared to Multimer in male Balb/c mice on subcutaneous administration. GnRH-KLH induced more uniform and stronger immune responses when administered with ISCOMs or CpG ODNs as adjuvants. However, the GnRH-KLH plus ISCOMs was the only formulation which also induced consistent reproductive organ atrophy in male Balb/c mice. More studies are required to evaluate the efficacy of this formulation by mucosal and parenteral routes.

Acknowledgements

This research was funded by the Invasive Animals CRC; Ian McDonald was the recipient of a University of Queensland PhD scholarship and top-up funding from the IA CRC. Mr Steve Henry provided excellent animal care and assistance with animal experimentation.

References

- Millar RP. GnRHs and GnRH receptors. *Anim Reprod Sci* 2005;88(1–2):5–28.
- Fagerstone KA, Miller LA, Eisemann JD, O'Hare JR, Gionfriddo JP. Registration of wildlife contraceptives in the United States of America, with OvoControl and GonaCon immunocontraceptive vaccines as examples. *Wildlife Res* 2008;35:586–92.
- Fagerstone KA, Miller LA, Killian G, Yoder CA. Review of issues concerning the use of reproductive inhibitors, with particular emphasis on resolving human-wildlife conflicts in North America. *Integr Zool* 2010;1:15–30.
- Ladd A. Progress in the development of anti-Lhrh vaccine. *Am J Reprod Immunol* 1993;29(3):189–94.
- Sabeur K, Ball BA, Nett TM, Ball HH, Liu IKM. Effect of GnRH conjugated to pokeweed antiviral protein on reproductive function in adult male dogs. *Reproduction* 2003;125(6):801–6.
- Junco JA, Peschke P, Zuna I, Ehemann V, Fuentes F, Bover E, et al. Immunotherapy of prostate cancer in a murine model using a novel GnRH based vaccine candidate. *Vaccine* 2007;25(50):8460–8.
- Goubau S, Silversides DW, González A, Laarveld B, Mapletoft RJ, Murphy BD. Immunization of sheep against modified peptides of gonadotropin releasing hormone conjugated to carriers. *Domest Anim Endocrinol* 1989;6(4):339–47.
- Talwar GP, Raina K, Gupta JC, Ray R, Wadhwa S, Ali MM. A recombinant luteinising-hormone-releasing-hormone immunogen bioeffective in causing prostatic atrophy. *Vaccine* 2004;22(27–28):3713–21.
- Conforti VA, de Avila DM, Cummings NS, Wells KJ, Ulker H, Reeves JJ. The effectiveness of a CpG motif-based adjuvant (CpG ODN 2006) for LHRH immunization. *Vaccine* 2007;25(35):6537–43.
- Karakus F, Yilmaz A, Hakan B, Stormo K, Ulker H. The Effectiveness of recombinant OL fusion protein (ovalbumin-LHRH-7) in suppressing reproductive functions when injected in single-dose vaccination protocols with different adjuvants. *Anim Reprod Sci* 2013;138(3–4):228–32.
- Chua BY, Zeng WG, Lau YF, Jackson DC. Comparison of lipopeptide-based immunocontraceptive vaccines containing different lipid groups. *Vaccine* 2007;25(1):92–101.
- Goubau S, Silversides DW, Gonzalez A, Laarveld B, Mapletoft RJ, Murphy BD. Immunization of cattle against modified peptides of gonadotropin-releasing hormone conjugated to carriers – effectiveness of Freund and alternative adjuvants. *Theriogenology* 1989;32(4):557–67.
- Chua BY, Al Kobaisi M, Zeng WG, Mainwaring D, Jackson DC. Chitosan microparticles and nanoparticles as biocompatible delivery vehicles for peptide and protein-based immunocontraceptive vaccines. *Mol Pharm* 2012;9(1):81–90.
- Claassen E, Deleuw W, Degreeve P, Hendriksen C, Boersma W. Freund complete adjuvant – an effective but disagreeable formula. *Res Immunol* 1992;143(5):478–83.
- Shah NM, Mangat GK, Balakrishnan C, Buch VI, Joshi VR. Accidental self-injection with Freund's complete adjuvant. *J Assoc Physicians India* 2001;49:366–8.
- Hoskinson RM, Rigby RD, Mattner PE, Huynh VL, D'Occhio M, Neish A, et al. Vaxstrate: an anti-reproductive vaccine for cattle. *Aust J Biotechnol* 1990;4(3):166–70.
- Meeusen ENT, Walker J, Peters A, Pastoret PP, Jungersen G. Current status of veterinary vaccines. *Clin Microbiol Rev* 2007;20(3):489–510.
- Heldens JGM, Pouwels HGW, Derks CGG, Van de Zande SMA, Hoeijmakers MJH. The first safe inactivated equine influenza vaccine formulation adjuvanted with ISCOM-matrix that closes the immunity gap. *Vaccine* 2009;27(40):5530–7.
- Crouch CF, Daly J, Henley W, Hannant D, Wilkins J, Francis MJ. The use of a systemic prime/mucosal boost strategy with an equine influenza ISCOM vaccine to induce protective immunity in horses. *Vet Immunol Immunopathol* 2005;108(3–4):345–55.
- Sun HX, Xie Y, Ye YP. ISCOMs and ISCOMATRIX. *Vaccine* 2009;27(33):4388–401.
- Sjolander A, Cox JC, Barr IG. ISCOMs: an adjuvant with multiple functions. *J Leukocyte Biol* 1998;64(6):713–23.
- Ren JL, Yang L, Xu HF, Zhang YS, Wan M, Liu GY, et al. CpG oligodeoxynucleotide and montanide ISA 206 adjuvant combination augments the immune responses of a recombinant FMDV vaccine in cattle. *Vaccine* 2011;29(45):7960–5.
- Gupta K, Cooper C. A review of the role of CpG oligodeoxynucleotides as toll-like receptor 9 agonists in prophylactic and therapeutic vaccine development in infectious diseases. *Drugs R&D* 2008;9(3):137–45.
- Miller LA, Gionfriddo JP, Fagerstone KA, Rhyhan JC, Killian GJ. The single-shot GnRH immunocontraceptive vaccine (GonaCon™) in white-tailed deer: Comparison of several GnRH preparations. *Am J Reprod Immunol* 2008;60(3):214–23.
- Miller LA, Rhyhan JC, Drew M. Contraception of bison by GnRH vaccine: a possible means of decreasing transmission of brucellosis in bison. *J Wildlife Dis* 2004;40(4):725–30.
- Predes FD, Diamante MAS, Dolder H. Testis response to low doses of cadmium in Wistar rats. *Int J Exp Pathol* 2010;91(2):125–31.
- Miller LA, Johns BE, Killian GJ. Immunocontraception of white-tailed deer with GnRH vaccine. *Am J Reprod Immunol* 2000;44(5):266–74.
- Fang FG, Yang YP, Liu Y, Zhang YH, Tao Y, Wang SL, et al. Immunization of male mice with a new recombinant GnRH fusion protein reduces the testicular function. *Agr Sci China* 2009;8(3):380–5.
- Campbell TA, Garcia MR, Miller LA, Ramirez MA, Long DB, Marchand JB, et al. Immunocontraception in male feral swine treated with a recombinant gonadotropin-releasing hormone vaccine. *J Swine Health Prod* 2010;18(3):118–24.
- Beekman NJCM, Schaaper WMM, Turkstra JA, Meloen RH. Highly immunogenic and fully synthetic peptide-carrier constructs targeting GnRH. *Vaccine* 1999;17(15–16):2043–50.
- Conforti VA, De Avila DM, Curnmings NS, Zanella R, Wells KJ, Ulker H, et al. CpG motif-based adjuvant as a replacement for Freund's complete adjuvant in a recombinant LHRH vaccine. *Vaccine* 2008;26(7):907–13.
- Ferro VA, Stimson WH. Investigation into suitable carrier molecules for use in an anti-gonadotropin releasing hormone vaccine. *Vaccine* 1998;16(11–12):1095–102.

- [33] Hu KF, Lovgren-Bengtsson K, Morein B. Immunostimulating complexes (ISCOMs) for nasal vaccination. *Adv Drug Deliv Rev* 2001;51(1–3): 149–59.
- [34] Ghazi HO, Potter CW, Smith TL, Jennings R. Comparative antibody responses and protection in mice immunised by oral or parenteral routes with influenza virus subunit antigens in aqueous form or incorporated into ISCOMs. *J Med Microbiol* 1995;42(1):53–61.
- [35] Coulter A, Harris R, Davis R, Drane D, Cox J, Ryan D, et al. Intranasal vaccination with ISCOMATRIX adjuvanted influenza vaccine. *Vaccine* 2003;21(9–10):946–9.
- [36] Kiyama Z, Adams TE, Hess BW, Riley ML, Murdoch WJ, Moss GE. Gonadal function, sexual behavior, feedlot performance, and carcass traits of ram lambs actively immunized against GnRH. *J Anim Sci* 2000;78(9): 2237–43.
- [37] Schanbacher BD, Crouse JD, Ferrell CL. Testosterone influences on growth, performance, carcass characteristics and composition of young market lambs. *J Anim Sci* 1980;51(3):685–91.
- [38] Levy JK, Friary JA, Miller LA, Tucker SJ, Fagerstone KA. Long-term fertility control in female cats with GonaCon™, a GnRH immunocontraceptive. *Theriogenology* 2011;76(8):1517–25.
- [39] Liu H, Bungener L, ter Veer W, Collier BA, Wilschut J, Huckriede A. Preclinical evaluation of the saponin derivative GPI-0100 as an immunostimulating and dose-sparing adjuvant for pandemic influenza vaccines. *Vaccine* 2011;29(11):2037–43.
- [40] Morisaki N, Watanabe S, Tezuka M, Zenibayashi M, Shiina R, Koyama N, et al. Mechanism of angiogenic effects of saponin from ginseng Radix-Rubra in human umbilical vein endothelial-cells. *Brit J Pharmacol* 1995;115(7): 1188–93.