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# Effectiveness of an immunocastration vaccine formulation to reduce the gonadal function in female and male mice by Th1/Th2 immune response

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ABSTRACT

Immunocastration has emerged as an alternative to surgical castration in different animal species. This study examined the effectiveness of a new vaccine formulation for immunocastration using the biopolymer chitosan as adjuvant. First, female and male mice (n = 4), in three subsequent experiments were vaccinated at Days 1 and 30 of the study, to determine the immune response profile and gonadal alterations due to immunization. The results demonstrated that the vaccine was able to elicit strong antibody responses against native GnRH hormone ($P < 0.01$), with a T helper (Th) 1/Th2 immune response profile. Along with this, a suppression of gonadal activity with a decrease of luteal bodies (1.08 ± 0.22 and 4.08 ± 0.39) and antral follicles (1.17 ± 0.32 and 4.5 ± 0.38) in the ovaries of immunized females and control, respectively, and a reduction of seminiferous tubules size (142.3 ± 5.58 mm and 198.0 ± 6.11 mm) and germinal cellular layers (3.58 ± 0.26 and 5.08 ± 0.29) of immunized males and control animals, respectively, were observed ($P < 0.01$). Then, in a study of long-term immune response due to vaccination in female and male mice (n = 4) from two subsequent experiments, a suppression of gonadal function and an induction of a Th1/Th2 immune response was also observed, determined by both, immunoglobulin and cytokine profiles, which lasted until the end of the study (7 months; $P < 0.01$). The findings of this study have demonstrated that vaccination with a new immunocastration vaccine inducing a Th1/Th2 immune response against GnRH ($P < 0.01$) elicit a decrease of gonadal function in male and female mice ($P < 0.01$). Owing to long-term duration of the antibody levels generated, this vaccine formulation appears as a promising alternative for immunocastration of several animal species where long-lasting reproductive block is needed.


## 1. Introduction

Nonsurgical sterilization methods are highly desirable in domestic animals' population control [1] and sexual behavior control of male animals reared for meat production [2,3].

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Vaccines are an alternative for these issues, inducing immunologic block of GnRH. Gonadotropin-releasing hormone is a hypothalamic decapeptide that provides a primary drive for the reproductive axis, being present in male and female mammals. Active immunization against GnRH creates an immunologic barrier between the hypothalamus and the anterior pituitary gland, which prevents GnRH receptor-binding on pituitary gonadotropes, resulting in the suppression of gonadotropin secretion, inhibiting gametogenesis, and steroids production, as well as reproductive behavior. This strategy is commonly called immunocastration [4].

In male dogs immunized with fusion proteins including canine GnRH conjugated to tetanus toxoid [5] or T-helper cell epitope p35 originated from canine distemper virus F protein [6], elevated levels of GnRH-specific antibody in blood and reduced spermatogenesis were observed. In adult male cats, a single dose of GnRH vaccine based on keyhole limpet hemocyanin as carrier protein in a mycobacterial and oil emulsion as adjuvant, effectively blocked testosterone production and spermatogenesis in a short-term study [7]. In pigs, the use of a synthetic analogue of GnRH coupled to a tetanus toxin fragment as carrier protein, reduced boar taint without affecting other meat quality variables [8] and avoided unwanted aggressive and sexual behaviors, compared with unvaccinated control male pigs [9]. Immunocastration has also been investigated in wildlife species. In female bison, it was described that a single dose of GnRH vaccine, including keyhole limpet hemocyanin as carrier protein and a mycobacterial oil adjuvant, was effective preventing pregnancy for at least 1 year [10]. Although these immunocastration vaccines have been effective blocking reproductive activity in different models, most of them induced a short-lived effect [1] and in some, adverse effects associated to formulation components, such as persistent granulomas on the inoculation site, have been reported [11]. In addition, most of the developed vaccines against GnRH were based on the incorporation of a carrier protein in their formulation, and it has been described that the use of carrier proteins induces hapten immunosuppression [12,13]. For these reasons, study and development of new immunocastration vaccine formulations become necessary.

A recombinant peptide with a tandem repeat primary structure has been designed, expressed, and purified in the Laboratory of Veterinary Vaccines of the University of Chile incorporating the aminoacidic sequence of GnRH hormone fused to a nonpathogen-associated linker sequence, which does not need a carrier protein but an adequate adjuvant to enhance its immunogenicity. The immune response against an immunogen is determined by several factors, including adjuvant, antigen dose, route of administration, and host genetic factors [14]. One way of studying the response profile induced by any adjuvant is to determine the antibody subclasses and relate them to T helper (Th) involvement [15–17]. Biopolymer chitosan is one of the promising adjuvants used in recent years, which has demonstrated to be effective and able to induce a Th1/Th2 response in studies conducted by different research groups, including our own [18–20]. In a previous study developed in our laboratory, it was confirmed that chitosan is effective when it is used as an adjuvant with an immunocastration antigen in male rats [21].

The present study has determined the effectiveness of a new recombinant immunocastration vaccine against GnRH using low-molecular-weight chitosan as adjuvant to induce specific Th1 and Th2 immunoglobulin G (IgG) and cytokine immune response (Th1/Th2) associated with histological gonadal alterations in mice.

## 2. Materials and methods

### 2.1. Recombinant antigen purification through inclusion bodies

The recombinant antigen GnRXG/Q was designed as a tandem repeat of GnRH amino sequence flanked by a spacer region of 14 aminoacids to improve the immunogenicity of GnRH sequence in the whole peptide. Nucleotide sequence of 765 pb which encodes GnRXG/Q peptide GPPFSGGGGPPFSAQHWSYGLRPG repeated 10 times was synthesized with codon optimization of *Escherichia coli*, and subcloned in pJEXPRESS expression vector by DNA 2.0, which had a T5 promoter, kanamycin resistance, and a histidine tag for subsequent identification of the protein. The vector was transformed in *E coli* BL21 (DE3)plysS (Stratagen) for its expression in inclusion bodies.

Bacteria were grown in Luria broth enriched with yeast extract medium (tryptone 1%, NaCl 1%, and yeast extract 2%) supplemented with antibiotics, at 37 °C until A600 = 2. The expression of the recombinant peptide was initiated with 1-mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and orbital agitation for 18 hours at 37 °C. Bacteria were collected from the culture by centrifugation at 3000 × *g* for 10 minutes at 4 °C and resuspended in "inclusion bodies wash buffer I" (20-mM imidazole, 20-mM sodium phosphate, pH 7.4, 500-mM NaCl) for 1 hour at 37 °C. Then, cells were lyzed by two freezing cycles at −80 °C and thawing at 37 °C. DNA was sheared by a brief sonication, and inclusion bodies were recovered by centrifugation at 2000 × *g* for 20 minutes at 4 °C. Pellet was resuspended in "inclusion bodies wash buffer II" (0.5% [v:v] Triton X-100, 20-mM imidazole, 20-mM sodium phosphate, pH 7.4, and 500-mM NaCl). After another sonication and centrifugation, the pellet was solubilized in inclusion bodies solubilization buffer (8-M urea, 20-mM imidazole, 20-mM sodium phosphate, pH 7.4, 500-mM NaCl) and sonicated at 200 W for 3 minutes in 10-second pulses.

Soluble proteins were recovered from the supernatant after centrifugation (13,000 × *g* for 30 minutes at 4 °C), and the supernatant was filtered with a 0.45-μm filter, and then with a 0.22 μm. Subsequently, proteins were identified by protein polyacrylamide-SDS gel (12.5%) and transferred to a nitrocellulose membrane for immunoblot analysis by HisDetector Nickel-HRP kit (ThermoScientific). The protein concentration was estimated by bicinchoninic acid method (Calbiochem, Merk), according to the manufacturer instructions.

### 2.2. Chitosan as adjuvant

Low-molecular-weight chitosan (MW = 70 KDa, ≥75% deacetylation, 20,000 cps viscosity) (Sigma–Aldrich Corp)

was prepared as described in previous studies with some modifications [18]. Then, briefly, chitosan 1% was dissolved in acetic acid 0.5% and put to orbital stirring at 200 rpm room temperature (18°C–24 °C) for 18 hours. Subsequently, chitosan was filtered through a sterile method, using cellulose acetate filters (Merck) of 0.80 μm and 0.45 μm, to finally carry out a last filtration using polyethersulphone filters of 0.22 μm, to use it afterward in the vaccine formulation.

### 2.3. Histologic analysis at the site of inoculation

To determine inflammatory changes at the inoculation site due to the use of chitosan, subcutaneous injection of 50 μg of the vaccine antigen was performed in a total volume of 200 μL using soluble chitosan or PBS as control, in the lower back of three male mice. At Days 3, 7, and 14 after inoculation, histologic changes were determined by surgical extraction of the inoculation site skin and posterior fixation using buffered formalin solution (10%, v:v). Afterward, obtained tissue was stained with hematoxylin–eosin (H-E) and observed under an optical microscope (Nikon Eclipse E400). Images were obtained using a Nikon Digital Sight DS-SMC camera and analyzed with Nis-Elements version 3.0 software.

### 2.4. Immunization of animals

Sixteen-week-old male and female BALB/c mice were randomly distributed. Mice were obtained from the Faculty of Veterinary Sciences of the University of Chile. Animals were kept with ad libitum food and water in a temperature and light-controlled environment. Immunization of mice was performed at Days 1 and 30, with 200 μL of a subcutaneous injection containing a 1:1 mixture of chitosan as adjuvant and 50 μg of the antigen.

Two studies, an initial study to evaluate the effect of vaccination on IgG-antibody levels and gonadal function and a second study to determine the effectiveness of vaccination to induce a long-lasting immune response in vaccinated animals, were performed.

For the first study, three consecutive experiments with duration of 75 days were conducted. Each experiment was developed using four groups (n = 4 mice/group). In a second study, to determine a long-term effect of the vaccine, two consecutive experiments were performed also generating four groups (n = 4 mice/group). For all experiments, of both studies, the groups were organized as follows: group I, female mice immunized with recombinant antigen GnRXG/Q in chitosan as adjuvant; group II, female mice inoculated with chitosan in absence of antigen as the female control group; group III, male mice immunized with recombinant antigen GnRXG/Q in chitosan as adjuvant; and group IV, male mice inoculated with chitosan in absence of antigen, as the male control group.

### 2.5. Measurement of immunoglobulins against recombinant antigen GnRXG/Q and GnRH hormone

From the beginning of the study and every 15 days until Day 75, approximately, 200 microliters of blood were collected from external saphenous vein of animals, to obtain about 40 microliters of serum. The procedure for determining IgG isotypes was developed by an in-house ELISA but based on what was previously described by other authors [22] and also previously published by our group [21]. An indirect ELISA assay was performed on 96 well Maxisorp plates (Nunc, Thermo Scientific) to detect level of antibodies against the antigen or endogenous hormone in serum of vaccinated animals. Two micrograms of GnRXG/Q or luteinizing hormone–releasing hormone (Sigma–Aldrich Corp.) were coated in each well with 50 μL of coating buffer (150-mM $Na_2CO_3$, 350-mM $NaHCO_3$, pH 9.6) overnight at 4 °C. Then, the plates were washed with washing buffer (0.05% Tween-20 v:v in PBS) and blocked with 200 μL of blocking buffer (1% w:v BSA in PBS) for 2 hours at room temperature (RT). Subsequently, the plates were incubated with 100 μL of diluted 1:250 serum in diluent buffer (0.1% BSA w:v, 0.05% Tween-20 v:v) for 1 hour at RT. After that, plates were washed and incubated with 100 μL of diluted 1:10000 rabbit anti-mouse IgG, IgG1, or IgG2a peroxidase-conjugated antibodies (Jackson Immunoresearch Laboratories) for 1 hour at RT. Finally, the plates were washed and revealed with 1-Step, Slow TMB-ELISA (Pierce, Chemical Company) for 15 minutes at RT. The reaction was stopped with Stop solution (1.5-M $H_2SO_4$), and the absorbance was measured at 450 nm.

Moreover, to determine if the GnRH sequence was recognized into the recombinant antigen GnRXG/Q, an indirect ELISA was performed, coating GnRXG/Q on plate, and using a synthetic polyclonal antibody against GnRH (Santa Cruz Biotechnology) as primary antibody.

### 2.6. Determination of long-term specific immune response

To determine the efficacy of this vaccine inducing a long-term immune response, serum of immunized female and male mice was obtained for specific IgG, IgG1, and IgG2a determination using indirect ELISA. At Day 220 after first immunization, animals were sacrificed and splenectomized. A splenocyte-cell suspension was prepared by homogenization in 4 mL of balanced saline solution (0.01% D-glucosa w:v, 50-mM $CaCl_2$, 10-mM $MgCl_2$, 500-μM KCl, 140-mM NaCl, 15-mM Tris-HCl, pH 7,6), and erythrocytes were lyzed using erythrocyte lysis solution ($NH_4CL$ 0.15 M, $KHCO_3$ 10 mM, and EDTA 0.1 mM) obtaining the leukocyte-cell population. Leukocytes were cultivated on triplicate as primary cellular culture in 12 well-plates (Nunc) using a complete Roswell Park Memorial Institute supplemented medium (10% FBS, 2-nM L-glutamine, 1-mM sodium pyruvate, and 2.5 g/L sodium bicarbonate) in 5% $CO_2$. Then, lymphocytes were stimulated during 3 days with 30 μg/mL of recombinant GnRXG/Q antigen in PBS. For positive controls, cells were stimulated with 1-μg/mL Concanavalin A, and for negative controls, cells were treated only with PBS. The culture medium with produced cytokines was kept at −80 °C for later ELISA analysis. A sandwich ELISA assay (OptiEA, BDBiosciences) on 96 wells Maxisorp plates (Nunc, Thermo Scientific) to detect levels of interferon gamma (γ-IFN) and interleukin 4 (IL-4) cytokines, synthesized by *in vitro* stimulated lymphocytes, was performed according to the manufacturer's instructions.

### 2.7. Gonadal histologic analysis

At Days 75 (first assay) and 220 (second assay) after the first immunization, animals were sacrificed by $CO_2$ inhalation. Ovaries and testicles were surgically extracted and fixed using buffered formalin (10% v:v) solution. Tissue issue slices of 5 μm were stained with H-E and observed under optical microscope. To measure seminiferous tubules size and to count germinal cells layers in males, and follicles and luteal bodies number in females, three sections of testis or ovaries parenchyma from each animal were analyzed. Same previously described microscope, camera, and software were used.

### 2.8. Ethical concern

All experimental protocols were approved by the institutional animal bioethics committee.

### 2.9. Statistical analysis

The analysis of immunoglobulin titers was performed by a two-way ANOVA with Bonferroni post test. For analysis of cytokines and gonadal structures, one-way ANOVA with Tukey post test was used. All analyses were performed using GraphPad Prism version 5 for Windows (GraphPad Software, USA) software. For all statistical analyses, differences were considered significant at $P < 0.05$.

## 3. Results

A fragment of 765 pb, the antigen nucleotide sequence, was subcloned in pJEXPRESS vector (Fig. 1A). The recombinant peptide GnRXG/Q of approximately 25 kDa was expressed and purified from *E coli* BL21 (DE3) plysS transformed with the expression plasmid (Fig. 1B, C). Then, a synthetic antibody against GnRH, using two different dilutions, was able to recognize *in vitro* the GnRH sequence on the antigen GnRXG/Q by indirect ELISA, demonstrating cross-reactivity between both proteins (Fig. 1D).

For the purpose of determining inflammatory reactions at the inoculation site due to the vaccination with the antigen and chitosan as adjuvant, a histologic study of the inoculation site was performed. The antigen diluted in PBS triggered a moderate inflammatory reaction from Day 3, which became minimal at Day 14 (Supplementary Fig. 1A).

In the case of the antigen and chitosan as adjuvant, a strong inflammatory reaction grew from Day 3 decreasing by Day 14 after inoculation, on subcutis and deep dermis (Supplementary Fig. 1B). At Day 3, there was a dense acute inflammatory reaction (Supplementary Fig. 1B, Day 3). At Day 7, a chronic inflammatory reaction surrounded by abundant granulation tissue was observed. Finally, at Day 14, the chronic inflammation decreased considerably (Supplementary Fig. 1B, Day 14). Despite this, macroscopically, animals only showed a slight volume increase at the inoculation site (2–5 mm), without pain, wounds, or any other adverse reaction.

This result matches what has been observed in subsequent studies carried out for 75 and 220 days, where after 2 weeks, animals did not show macroscopic signs of swelling on inoculation site. Moreover, none of the animals belonging to the different groups of study showed granulomas, wounds, or any other adverse reaction.

To characterize the profile of immunoglobulins induced because of vaccination by indirect ELISA, the production of IgG, IgG1, and IgG2a was evaluated. As shown in Figure 2, vaccination allowed increasing anti GnRXG/Q IgG levels from first immunization in female mice ($P < 0.01$), unlike what has been seen in males, occurring after the second immunization ($P < 0.01$; Fig. 2A). The same rise pattern was observed with the immunoglobulins IgG1 ($P < 0.01$), antibody associated with Th2 response (Fig. 2B) and IgG2a ($P < 0.01$), associated with Th1 immune response (Fig. 2C), inducing a Th1/Th2 immune response. These immunoglobulin levels remained high until the end of the study in both sexes. Furthermore, the immunization induced cross-immunogenicity, with high levels of specific IgG against the native hormone GnRH in immunized animals after second immunization ($P < 0.01$) (Fig. 2D).

To determine the effect of vaccination on gonadal function, a histologic analysis of ovaries and testicles was carried out. In immunized females, a small number of follicles in advanced stages and predominant presence of follicles in early stages of development were observed (Table 1; Fig. 3A and B), and a decrease of clearly defined luteal tissue in the ovaries of immunized animals was found ($P < 0.01$; Table 1; Fig. 3A and B). In immunized males, a spermatogenesis alteration, visualized by the decrease of spermatozoa, marked atrophy and a reduction of seminiferous tubules size ($P < 0.01$) and germinal cellular layers were observed (Table 1, Fig. 3C and D).

With the aim of determining the efficacy of this formulation vaccine inducing a long-term immune response, a second assay evaluating specific immunoglobulins and cytokines profile was developed. Vaccinated female mice showed high level of IgG (Fig. 4A), IgG1 (Fig. 4B), and IgG2a (Fig. 4C) against GnRH, from second immunization to the end of the study (Day 220; $P < 0.01$). Also, high levels of IL-4 and γ-IFN produced by *in vitro* antigen-stimulated lymphocytes from immunized mice were detected (Fig. 4D), with significant differences against the control group ($P < 0.05$) and without significant differences between males and females (data not shown). Regarding the gonadal function, similar to that was observed at Day 75, alterations of gonadal activity in vaccinated males and females were observed (Table 1).

## 4. Discussion

In the present study, the effectiveness of a new formulation vaccine to induce a long-lasting specific and Th1/Th2 immune response against GnRH and to reduce gametogenesis in female and male mice was demonstrated.

Most of the vaccines against GnRH are based on incorporation of a carrier protein in their formulation [6,23]; however, it is reported that the presence of a carrier in the formulation, induces suppression of the antibody response to a self hapten, such as GnRH [13] and that this effect could be avoided by generating recombinant proteins for immunocastration, where carrier proteins are replaced by epitopes T-not B [24]. Previously, we have described the

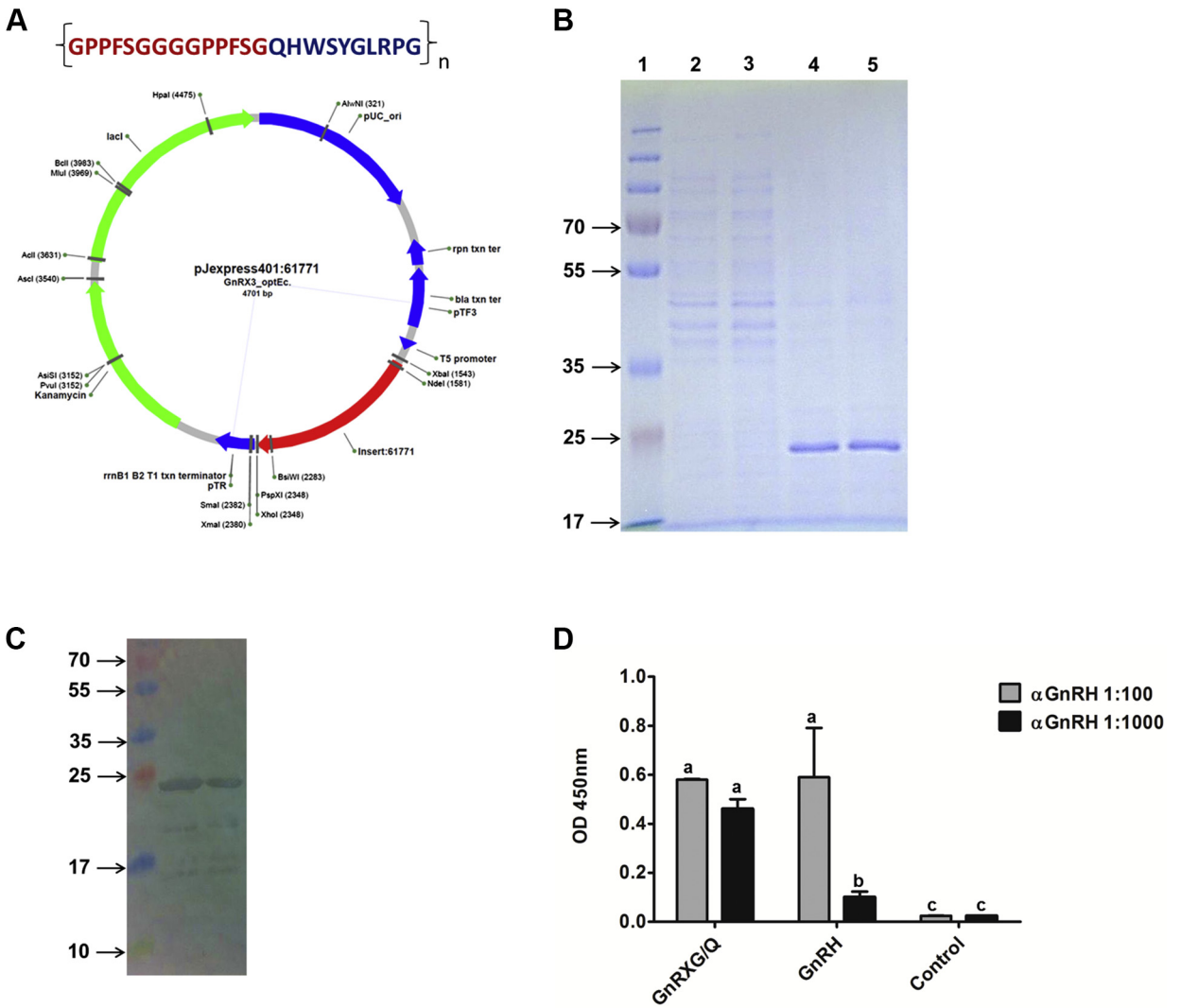


**Fig. 1.** Recombinant antigen GnRXG/Q expression. (A) 4701-bp long pJexpress401:61771 plasmid map indicates the relative position of the vaccine construct, the immediate T5 promoter. The diagram describing the engineering of the nucleotide sequence of 765 bp, which encodes the GnRXG/Q peptide GPPFSGGGGPPFSAQHWSYGLRPG (B) Protein polyacrylamide-SDS gel (12.5%) in different stages of the recombinant protein purification. Lane 1, molecular weight marker (KDa); lanes 2 and 3, sample after the first wash; and lanes 4 and 5, solubilized recombinant protein (C) Specific recombinant protein identification by Western blot using nickel covalently conjugated to a reporter enzyme peroxidase. The recombinant protein GnRXG/Q is located approximately in the 25 kDa (D) Indirect ELISA to determine the recognition of GnRH sequence into the GnRXG/Q antigen sequence, using the antigen coat on wells and a commercial antibody against GnRH at 1:100 (gray columns) or 1:1000 (black columns) dilutions as primary antibody. The antibody recognized GnRH sequence into GnRXG/Q using both dilutions. OD, optical density. Different letters indicate $P < 0.05$.

possibility of generating an effective immune response against a GnRH hormone tandem sequence, without a carrier, using chitosan as adjuvant [21]. In this study, the antigen GnRXG/Q was used not including a carrier protein in its formulation but including a new spacer sequence between GnRH. Through the use of Kolaskar and Tongaonkar method [25], it is predicted that the antigen GnRXG/Q has the amino acid segment PPFSAQHWSYG, able to act as a B-cell epitope. This fragment harbors aminoacids from the spacer (PPFSA) and from the GnRH (QHWSYG) hormone; thus, we could presume that within the total amount of antibodies produced after vaccination, there are neutralizing antibodies against the native hormone, as shown in Figure 3A–C. Furthermore, when analyzing the capability of several segments from the antigen GnRXG/Q of acting as T helper cells epitopes, i.e., to say, of being loaded into major histocompatibility complex class II (MHC-II) molecules. Bioinformatic analysis has revealed that segments including aminoacids from the spacer sequence and from GnRH hormone are able to be loaded in MHC-II. The MHC-II binding predictions were made using the Immune Epitope Database analysis resource Consensus tool [26,27].

Moreover, the tandem structure of the antigen probably plays a key role because recombinant proteins with multiple GnRH insert have shown that immunogenicity increased with the number of GnRH inserts [28]. Furthermore, a

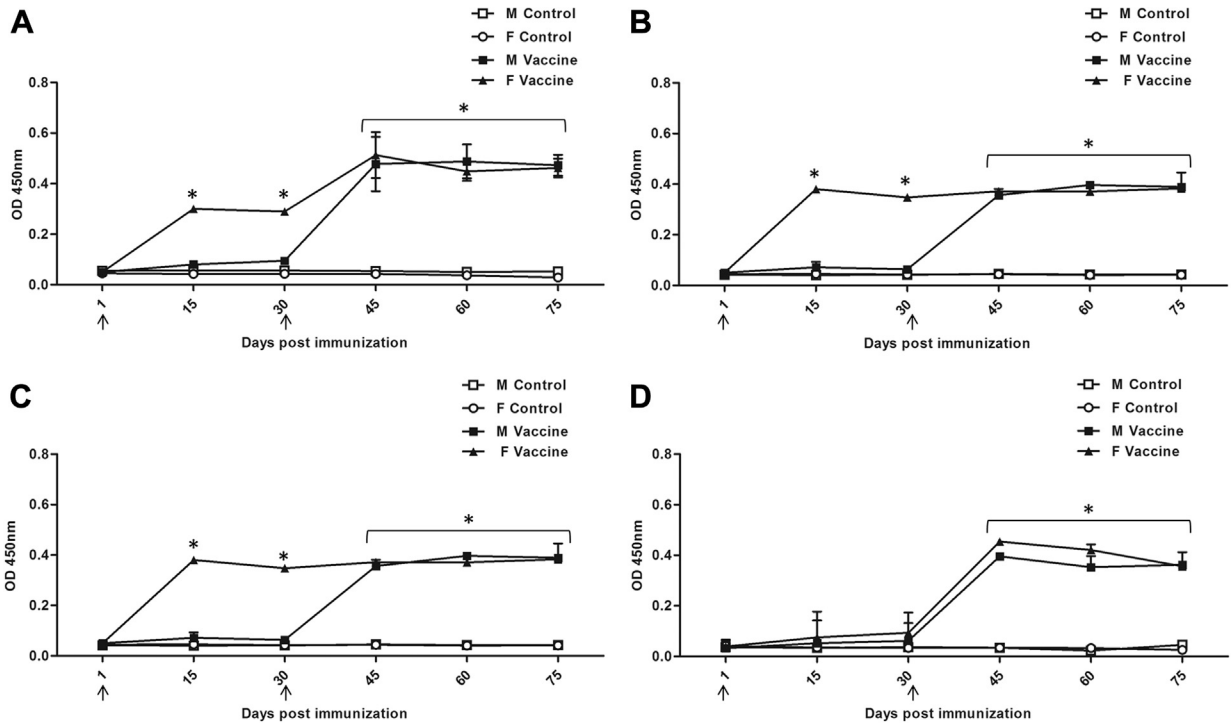


**Fig. 2.** Vaccination induces specific Th1/Th2 immune response. Female (F) and male (M) BALB/c mice were inoculated on Days 1 and 30 (arrows) with 50 μg of GnRXG/Q antigen and chitosan as adjuvant or chitosan in absence of the antigen (male and female control groups). Vaccination induces a Th1/Th2 immune response, with high levels of (A) IgG (B) IgG1, and (C) IgG2a against the recombinant antigen from Day 15 in females (F, vaccine) and Day 45 in males (M, vaccine), and cross-immunogenicity by high levels of (D) IgG against native GnRH hormone from Day 45 to Day 75 of the study. All data are represented as a mean ± SEM of three successive experiments (n = 4). IgG, immunoglobulin G; OD, optical density. Asterisks (*) indicate $P < 0.01$.

specific commercial antibody against native GnRH was able to recognize and bind to the recombinant protein in an indirect ELISA assay (Fig. 1D), recognizing the specific sequence of GnRH into the GnRXG/Q sequence.

Thus, the improvement of the antigen sequence, has demonstrated to be crucial to decrease gonadal function and to increase the immune response that can be maintained in time even over 7 months in mice, suggesting induction of memory cells, which would keep immunoglobulin levels throughout the study. There are several mechanisms that have been described associated to the immunologic memory of B-cells that could explain the duration of antibody levels for months after the last vaccination; through the existence of long-term survival plasmatic cells harbored in the bone marrow, which keep producing specific antibodies during years, and even for life; through the differentiation of memory B-cells into plasmatic cells due to the stimulus given by innate signals and/or to unspecific T-cell signals; through the differentiation of memory B-cells into plasmatic cells due to restimulation or persistence of the antigenic stimulation (reviewed in [29]), being this latter mechanism, possibly the one implicated in the duration of antibody titles throughout time regarding the framework of this study.

Successful vaccination against endogenous molecules such as GnRH requires a sufficient level of neutralizing antibodies during the full treatment period [28]. For this, chitosan, a chitin-derived biopolymer [30] was used as adjuvant. Chitosan has demonstrated effective adjuvant activity when administered parentally with various

**Table 1**
Quantitative evaluation of gonadal parenchyma structures obtained from vaccinated and control groups.

| Assay | Group | Females | | Males | |
|---|---|---|---|---|---|
| | | Antral follicles | Luteal bodies | Tubules size (mm) | Germinal cells layers |
| Day 75 | Control | 4.5 ± 0.38[a] | 4.08 ± 0.39[a] | 198.0 ± 6.11[a] | 5.08 ± 0.29[a] |
| | Vaccine | 1.17 ± 0.32[b] | 1.08 ± 0.22[b] | 142.3 ± 5.58[b] | 3.58 ± 0.26[b] |
| Day 220 | Control | 5.2 ± 0.4[a] | 4.04 ± 0.22[a] | 201.0 ± 5.21[a] | 5.26 ± 0.23[a] |
| | Vaccine | 1.09 ± 0.23[b] | 1.06 ± 0.21[b] | 122.4 ± 2.24[b] | 3.96 ± 0.38[b] |

Different letters for each parameter: $P < 0.01$.
Female and male BALB/c mice were vaccinated on Days 1 and 30 (n = 4). Data are represented as a mean ± SEM of structures number of three gonadal slices (5 μm) stained with hematoxylin–eosin per animal, 75 and 220 days after vaccination.

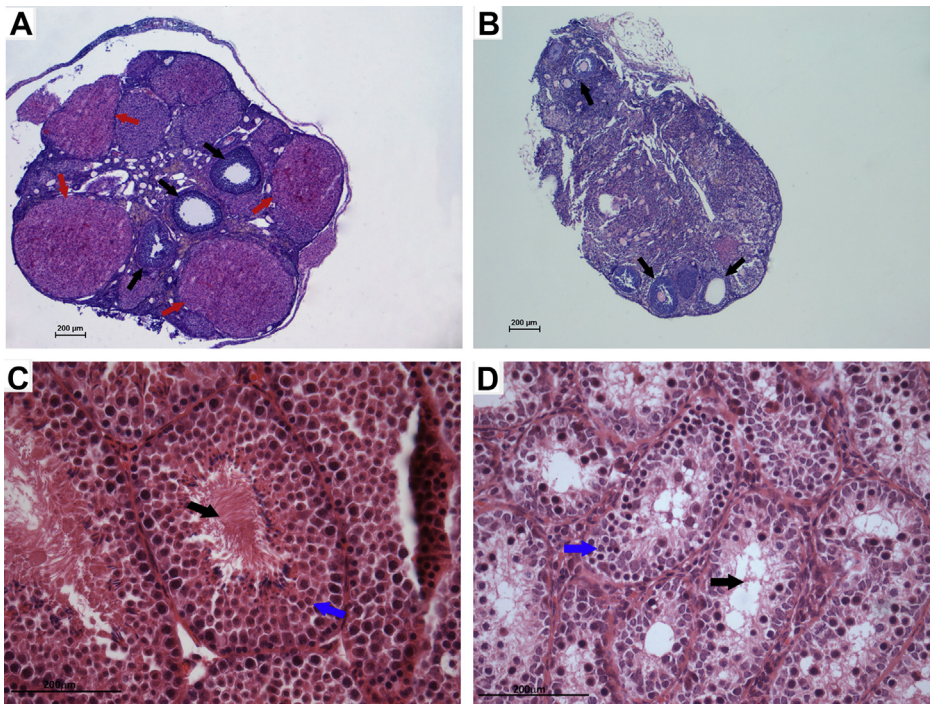


**Fig. 3.** Immunization induces changes in gonadal architecture in vaccinated animals. Females BALB/c mice were inoculated on Days 1 and 30 with (A) chitosan in the absence of the antigen (control group) or (B) with 50 µg of the GnRXG/Q and chitosan as adjuvant (vaccine group). Immunization decreased ovarian function with a predominant presence of follicles in early stages of development and a decrease of clearly defined luteal tissue at the end of the study. Black arrows show follicles, and red arrows show luteal bodies. Males BALB/c mice were inoculated with (C) chitosan in the absence of the antigen (control group) or (D) with 50 µg of the GnRXG/Q and chitosan as adjuvant (vaccine group). Immunization decreased spermatozoa in the seminiferous lumen and the number of germinal layers at the end of the study. Black arrows show spermatozoa in the lumen of seminiferous tubules, and blue arrows show germinal cellular layers. Five-micrometer slices were stained with hematoxylin–eosin to analyze ovarian parenchyma. The figure represents the results obtained from three successive experiments (n = 4). 4X. Bar = 200 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

antigens [18,20,31]. In this study, a potent depot effect of chitosan in the injection site has been demonstrated, along with an inflammatory infiltrate, which were maintained for up to 2 weeks after vaccination. This is consistent with previous data of other authors describing chitosan as an effective adjuvant, capable of inducing a depot effect with local inflammation, facilitating antigen recognition by the immune system, and enhancing an antigen-specific immune response [20,31].

About the antibody response against antigen GnRXG/Q (Fig. 3), the increase of immunoglobulins was observed from first vaccination in females, contrary to what was observed in males, in which antibody rise occurred after the second dose. This could be explained for a more potent immune response produced in females than in males, mainly due to the effect of estrogens on innate cells [32,33]. Different studies could explain the enhancer effect of the immune response given by estrogens. Similarly to other species, in murines, mature immune cells on lymphoid organs, such as dendritic cells, NK cells, B and T lymphocytes, express estrogen receptors [34–38]. It has been established that estrogen promotes production of IFN type 1 in murine splenocytes. Different studies show that genes associated to IFN signaling paths are expressed in higher levels in females [39–41]. Several investigation groups have shown that estrogen receptors promote the production of proinflammatory cytokines, as a response against macrophague and dendritic cell stimulation through Toll like receptor (TLR) pathway [42]. For example, estradiol promotes TLR-4 signaling pathway in macrophages, enhancing the production of proinflammatory cytokines such as IL-1b IL-6 [43]. Other studies carried out in mice agree with what has been observed in humans, where it has been described that women develop stronger immunitary responses and that when estrogen levels are higher, humoral responses are higher as well (reviewed in [44]).

Moreover, according to the antibody isotypes and cytokines profile (Figs. 3 and 4), the immunity induced by vaccination was a Th1/Th2 immune response. In this sense, it is known that the activation of B-cells by soluble protein antigens requires Th cells. The antigen binding to B-cell receptor does not induce an effective immune response without additional collaboration of membrane molecules and cytokines expressed by Th cells [45]. Although Th1 cells are crucial for cell-mediated immunity and are characterized by the production of the signature cytokine γ-IFN and opsonizing antibodies like IgG2a in mice, Th2 cells promote humoral immunity and secrete IL-4, IL-5, and IL-10 [16,46]. This Th1/Th2 immune response has been previously described by other authors, using chitosan associated to an antigen-protein model [20]. The induced immune profile is important in immunocastration vaccines. Apparently,

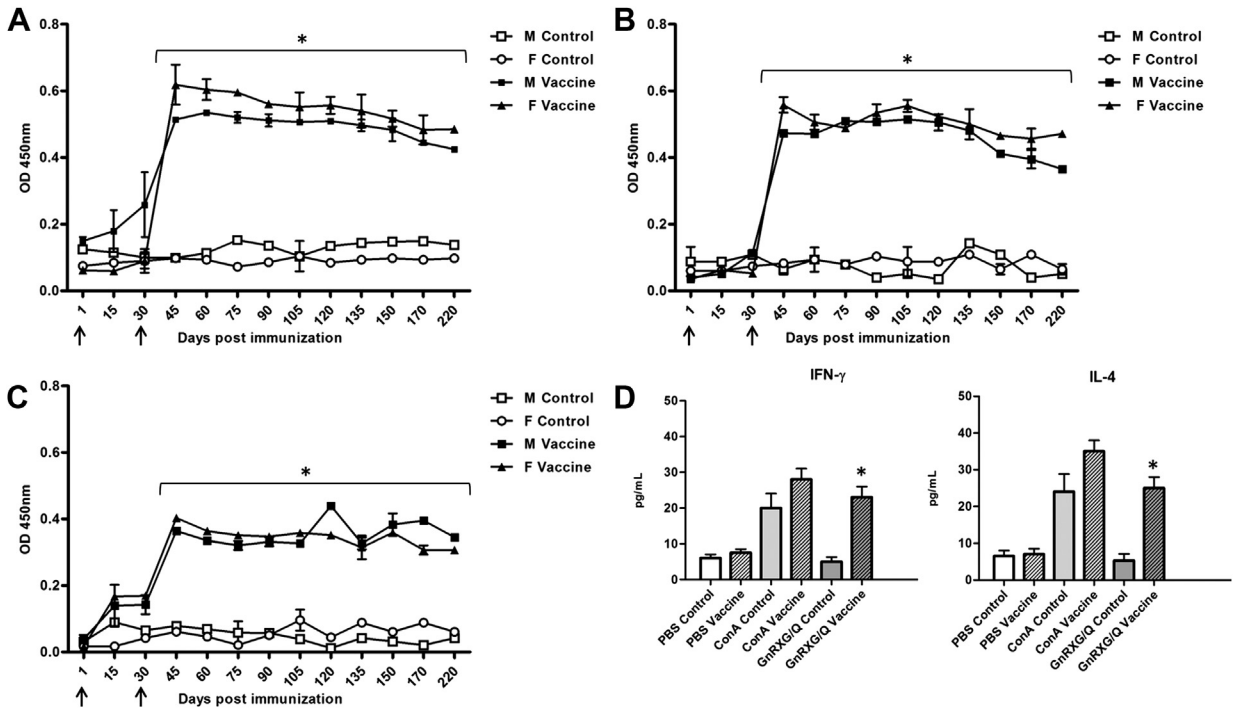


**Fig. 4.** Vaccination induces Th1/Th2 long-term specific immune response against native GnRH hormone. Male and female BALB/c mice were inoculated on Days 1 and 30 (arrows) with 50 μg of GnRXG/Q antigen and chitosan as adjuvant or chitosan in absence of the antigen (control groups). Immunized mice showed high levels of (A) IgG, (B) IgG1, and (C) IgG2a against GnRH hormone from Day 45 to Day 220 of study, measured by ELISA. (D) At Day 220, lymphocytes from vaccinated and control animals were separated and stimulated *in vitro* with the antigen for 72 hours, inducing high levels of interferon gamma and interleukin 4 cytokines (pg/mL) in vaccinated animals. Phosphate-buffered saline (PBS; negative Control), GnRXG/Q (GnRXG/Q in PBS), and Concanavalin A (positive control). The results of cytokines are the average of the results obtained in males and females (with no difference between sexes). All data are represented as a mean ± SEM of two successive experiments (n = 4). IgG, immunoglobulin G; OD, optical density. Asterisks (*) indicate $P < 0.01$.

neutralizing antibodies associated with a cell effector response would be more efficient than a Th2 response on its own. As previously described, where it has been observed that an adjuvant inducing a balanced Th1/Th2 response resulted in more consistent castration than an adjuvant inducing a unbalanced Th2 response [47].

In our long-term study, antibodies levels were observed to remain high until about 7 months after first vaccination (Fig. 4A–C) and when we *in vitro* induced lymphocytes from the spleen of vaccinated animals, lymphocytes were able to respond by a cytokine profile Th1/Th2 (Fig. 4D). These results suggest the generation of B and T memory lymphocytes, which would keep the response after 7 months, probably because of adjuvant and antigen characteristics. Long-lasting immune response is thought to occur because of a combination of persisting antigen, which is capable of B-cell differentiation into plasma cells, and the persistence of long-lasting plasma cells [48].

The specific immune response against GnRH induces functional gonadal alterations in females [20,49–51] and male animals [18,51,52]. In this study, vaccination was effective in decreasing gonadal function, inducing a decrease of antral follicles and luteal bodies in ovarian parenchyma, and in males, decreasing luminal spermatozoa and germinal layers number in the seminiferous tubules was observed. Therefore, the immunologic barrier was effective blocking the hypothalamic-pituitary-gonadal axis, inducing gonadal atrophy and inhibiting normal development of gametes in both sexes.

Thus, long-term immune response associated to gonadal atrophy at the end of the study makes this new formulation vaccine for immunocastration a promising alternative to reproductive activity control of animals where a long-term immunocastration is required, such as dogs and wildlife species.

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## Appendix A. Supplementary Data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.theriogenology.2016.05.019.

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