doi: 10.22201/fmvz.24486760e.2025.1309
Vol. 12 | 2025

Veterinaria México OA
Publicación Digital de la Facultad de Medicina Veterinaria y Zootecnia
https://veterinariamexico.fmvz.unam.mx/

DNA vaccine targeting GnRH-receptor reduces testosterone and induces testicular atrophy in mice

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Submitted: 2024-01-19
Accepted: 2024-09-20
Published: 2025-02-21

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Abstract

Reproductive control of invasive, feral, and domesticated animals is an urgent public health issue. Immunological vaccines are employed as an emerging strategy to target different components of the reproductive system, to achieve immunocastration. Successful use of immunogens against parts of the oocyte, spermatozoon, gonadotropin-releasing hormone (GnRH), and luteinizing hormone receptor (LHr) has been reported. In this work, a recombinant DNA vaccine was prepared (P2GnRHrP30) by the fusion of the first extracellular domain of the canine GnRH receptor (GNRHr) gene to the P2 and P30 tetanus toxoid epitopes. This construction was cloned in the pCI-NEO mammalian expression vector and evaluated as an immunocontraception DNA vaccine in male mice. The developed vaccine was demonstrated to be safe, and capable of reducing serum testosterone levels and causing testicular atrophy. Our work indicates that the first extracellular domain of the GnRHr gene may act as an immune target for reproductive control and potentially be used as a non-surgical sterilization procedure in mammals.

Keywords: DNA vaccine; Canine GnRH-receptor; P2 P30 epitopes; Dog immunocontraception; Overpopulation control.

Cite this as:

Reyes-Maya S, Gayosso-Vázquez A, Pintor Ríos P, Escobar ML, Echeverría OM, Gutiérrez CG, Alonso-Morales RA. DNA vaccine targeting GnRH-receptor reduces testosterone and induces testicular atrophy in mice. Veterinaria México OA. 2025;12. doi: 10.22201/fmvz.24486760e.2024.1309.

doi: 10.22201/fmvz.24486760e.2025.1309
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Study contribution

The widespread application of an economically affordable, non-surgical method for reproductive control would provide a viable alternative to animal overpopulation. This study evaluated a vaccine against the gonadotropin-releasing hormone receptor (GnRHr) coupled to the tetanic toxin epitopes P2 and P30 in male mice, that was demonstrated to induce an immune response against one of the main key components of the reproductive process, providing a potential tool for animal reproductive control.

Introduction

Animal overpopulation and zoonoses are public health problems with an important financial impact worldwide. A cost-efficient, lasting, and broadly available non-surgical method for reproductive control would provide a viable solution for the overpopulation of diverse invasive, feral, or domestic animal species.

In recent decades, alternatives for reproductive control have been sought by developing novel contraceptive approaches. A promising strategy is immunological manipulation by employing vaccines aimed to block physiological components of the reproductive system. Indeed, immunogens targeting the zona pellucida (ZP), oocytes, spermatozoa, gonadotropin-releasing hormone (GnRH), and luteinizing hormone receptor (LHr) have been shown to effectively reduce reproduction in various animal species.[1]

Gonadotropin-releasing hormone (GnRH) is a master switch driving the reproductive axis in mammals because suppressing GnRH inhibits gonadotroph and gonadal function.[2] GnRH is a small neuropeptide produced by the hypothalamus, that is released within the pituitary portal system to reach the hypophysis, where it binds to its receptor (GnRHr). When bound, GnRH stimulates the production and release of LH and FSH hormones, which are needed for follicular development in the ovary and sperm production in the testicle.[3]

Vaccination against GnRH has been evaluated in pigs, cattle, horses, sheep, mice, camelids, cats, chickens, and dogs. It has been explored as a viable option for mass application for population control.[4] Indeed, immunocastrated pigs with Improvac® (Zoetis) showed suppression of aggressive and sexual behavior in males with a two-dose protocol. Changes associated with vaccination remained until slaughter.[5] The effect of a recombinant GnRH-DNA vaccine coupled to chitosan (GnRXG/Q) was studied for dog immunocastration. It showed that the DNA vaccine induced specific antibodies, reduced serum testosterone, and diminished sexual agonistic marking in immunized males.[4] Moreover, an immunocontraceptive GnRH vaccine conjugated to the hemocyanin protein (GonaCon™, USDA, APHIS, Wildlife Services National Wildlife Research Center -NWRC-, Fort Collins, CO, USA) was evaluated showing significant progesterone suppression in bitches.[6]

Vaccines against other targets of the reproductive axis have also been assessed. The recombinant protein GVACo8, which includes GnRH, GnRHr, and ZP3 along with canine distemper virus epitopes and tetanus toxoid, has been shown to induce a high antibody titer against the target genes and reduce litter size in immunized mice.[7] Moreover, when a DNA vaccine constructed with the feline GnRHr gene, using two plasmids, three promoters, and two routes of administration was

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administered in a single 100 µg dose to male mice, a decrease in the number of fetuses was observed after immunized animals were mated with fertile females.[8]

Nonsurgical fertility control for animal population management focusing on GnRH is accepted in companion animals.[1] The GnRH receptor (GnRHr) is an attractive target for animal contraception for several reasons: first, GnRH binding to its receptor is essential for prompting the hypothalamus-pituitary-gonadal cascade of reproductive events, that when disrupted can lead to infertility, second, the location of the pituitary gland outside of the brain facilitates systemic access to GnRHr without crossing the blood-brain barrier (unlike the hypothalamic contraceptive targets), and third, disruption of GnRH–GnRHr interaction can stop reproduction and sexual behavior in both male and female animals.[7]

To be immunogenic, a vaccine should induce specific antibodies that recognize and block a fixed target.[8, 9] DNA vaccines activate humoral and cell-mediated immune responses.[10–12] When constructed in a bacterial vector-engineered plasmid containing a target gene under promoters with tag signals, DNA vaccines can be produced at a large scale and an affordable cost. For example, Oncept™ (Merial, Lyon, France), an anti-melanoma DNA plasmid vaccine, has been proven successful for immunotherapy in dogs.[9]

When developing a vaccine against endogenous proteins, immune tolerance and thus a weak immunogenic effect can be expected. In this study, we are working on a strategy to increase the immune response toward the GnRH receptor by fusing two immune-enhancer epitopes (the tetanus toxin peptides P2 and P30) into the endogenous protein.[10–13] In the present work, a recombinant DNA vaccine against the first extracellular domain (33 aa; NH^+ MASASPEQNQNHCSAVNNSNMLMQGNLPTLTLS COO^-) of the canine GnRH receptor linked to the tetanic toxin P2 and P30 epitopes was evaluated in reproductive male mice's reproductive parameters.

Materials and methods

Ethical statement

All animal work was sanctioned by Subcomité Interno para el Cuidado y Uso de los Animales de Experimentación (SICUAE) of the Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México (Code: SICUAE. DC-2019 / 2-5).

Animal model and housing conditions

Eight-week-old CD21 male mice were housed in polypropylene cages (three cages, seven animals per cage) at the Unidad de Constatación de Productos Químico-Biológicos y Bioterio, FMVZ UNAM, in environmentally controlled rooms with 12 h daily light and a 22 °C ± 2 °C temperature with 60 % ± 5 % humidity. Mice were fed commercial Rodent Laboratory Chow® and given water *ad libitum*. Animals were sampled and euthanized on day 60 after the first immunization.

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Recombinant DNA plasmid vaccine construction

A genetic construction consisting of the first canine GnRHr gene exon (1-99 bp 3´-atg gca agc gcc tct cct gaa cag aat caa aat cac tgc tca gct gta aac aac agc aac atg ctg atg cag ggc aac ctc ccc acc ctg acc tta tct-5´; GenBank NM_00103121.1) flanked by the tetanus toxoid P2 and P30 epitopes was created as previously described by Guevara.[14] The genetic construction was subsequently cloned into a pCI-NEO mammalian expression vector (Promega, Wisconsin, USA) obtaining the P2GnRHrP30 plasmid (Figure 1A and 1B). Finally, the endofree DNA of the plasmid P2GnRHrP30 was obtained (GF-1 Plasmid DNA Extractor Vivantis®, USA) to be used potentially as a DNA vaccine.

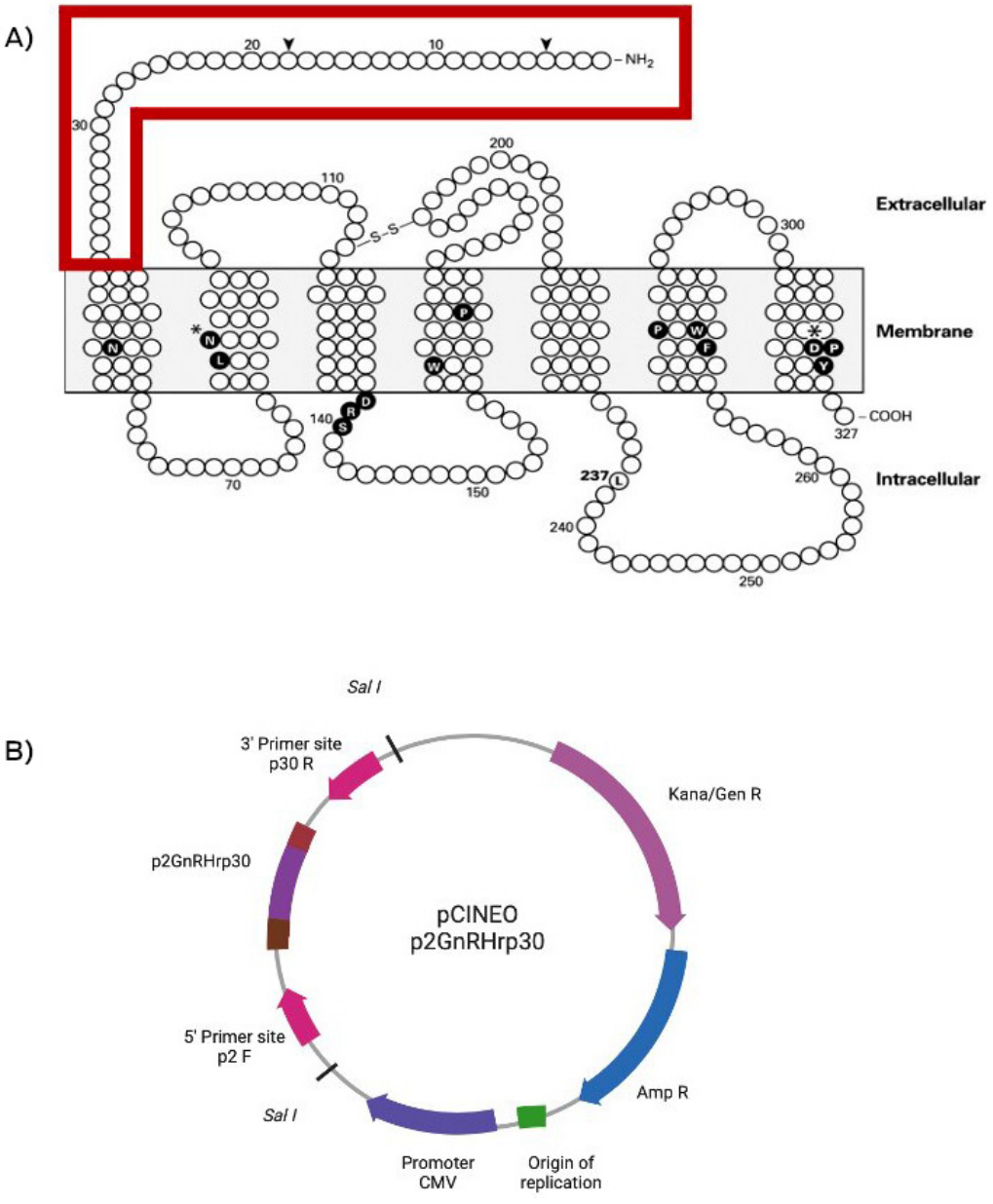

Figure 1. Schematic representation A) GnRHr first extracellular domain in the red box (From Chung et al.).[15] B) Plasmid pCI-NEO P2GnRHrP30 construction used for the DNA vaccine.

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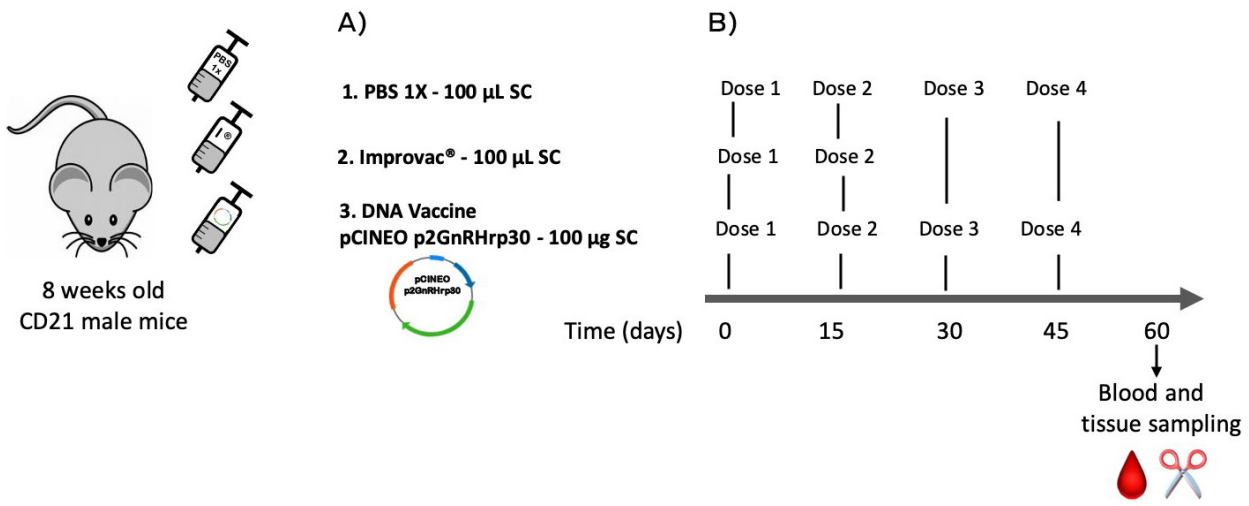

Figure 2. Schematic representation of the immunization protocol using PBS 1× as a negative control, Improvac® as a positive control, and the DNA vaccine P2GnRHrP30 as the experimental group. A) Treatment groups are identified as PBS 1× (Group NC), Improvac® (Group Improvac), and DNA vaccine (Group DNAv). B) The timing of vaccination, as well as blood and tissue sampling, is indicated below the horizontal arrow.

Experimental design

Animals were randomly placed in three experimental groups (Figures 2A and 2B):

- NC. (N = 6) negative control group: four 100 µL injections of sterile PBS 1x were subcutaneously applied to mice in the interscapular region on days one, 15, 30, and 45.
- Improvac. (N = 4) Improvac® positive control group: two 100 µL (20 µg) doses of Improvac® —a Gonadotropin-Releasing Factor (GnRF) analog protein conjugate (synthetic GnRF analog peptide conjugated to diphtheria toxoid, Zoetis®)— was subcutaneously administered to rodents in the interscapular region on days one and 15 as indicated by the manufacturer's protocol.
- DNAv. (N = 8) DNA vaccine group: four 100 µg doses of the constructed vaccine were injected *sq*. to mice at the interscapular region (100 µL of the total volume of DNA vaccine p2GnRHrp30 per injection), on days one, 15, 30, and 45 (Figure 2A and 2B). The 100 µg dose used for this treatment was selected from previous research done by Samoylov et al.[8] and Ramírez et al.,[11] where DNA contraceptive vaccines applied at this dosage were shown to be effective in reducing testosterone concentrations and other reproductive parameters in male mice.

Blood sample collection

Blood samples were obtained on day 60 post-immunization, using BD Microtainer™ tubes with gel for separation. Serum was recovered and kept at -70 °C until quantification.

Testosterone quantification

Serum testosterone levels were determined by a commercial ELISA kit (Testosterone EIA 1559; DRG Instruments GmbH) according to the manufacturer's protocol.

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Histological and microscopical analyses of the testes

Testicles were removed at day 60 for histological processing and morphological evaluation. Testicular tissue was fixed at 4 °C for one hour using a 4 % paraformaldehyde solution in phosphate buffer at 0.2 M (7.3 pH). Testicles were subsequently sectioned in half using a scalpel and then left in the fixative solution for 30 min. Later, sectioned testes were washed thrice using phosphate buffer for 10 min under constant agitation. Tissue dehydration was performed by gradual passages in ethanol solutions at increasing concentrations, starting with 60 %, then 70 %, 80 %, 96 %, and finally twice in absolute ethanol. Tissue remained submerged in each of these solutions for 30 min. After dehydration, samples were placed on a grid and successively immersed into 1:1 xylol alcohol (30 min), 1:1 xylol cedar oil (60 min), xylol (15 min), 1:1 xylol paraffin (60 min), and twice in paraffin (60 min) to obtain paraffin cubes for sectioning.

Paraffin cubes were sectioned at five microns for light microscopy analysis. Mounted slides were deparaffinated and rehydrated. Samples were stained with hematoxylin and eosin. Histological sections of all testicles were observed using a Leica® DM500 microscope.

As numbers and characteristics of cells in different stages of the spermatogenic line can potentially be used as indicators of testicular function,[16] spermatogonia, spermatocytes, and spermatids were visually recognized and counted in 30 transversally sectioned seminiferous tubules by treatment group. A decrease in their numbers, compared to the control group, was regarded as an indicator of testicular atrophy. Cell number data were obtained by the Analyze Particles application with the Software ImageJ 1.54g (Wayne Rasband and contributors National Institutes of Health, USA http://imagej.org). In addition, distinctive histological findings that were repeatedly observed in seminiferous tubules of treatment groups are mentioned. For this purpose, images were recorded with LAS EZ Leica® Application Suite Version 3.4.0 Leica Microsystems (Switzerland).

Statistical methods

We compared serum testosterone level medians using the Kruskal-Wallis test, followed by Dunn's post hoc test to determine differences between groups. To compare sperm precursor cell (spermatogonia, spermatocytes, and spermatids) numbers, an ANOVA for repeated measures was performed with the seminiferous tubule nested within the individual. A Tukey post hoc test was used to identify differences between groups. All analyses were executed with the GenStat for Windows (23rd ed. VSN International) 2023.

Results

Testosterone serum levels in male CD21 mice in response to the DNA vaccine application

A significant reduction in serum testosterone levels was observed in both the DNA vaccinated group and the Improvac® treated animals, when compared to the negative control group (PBS 1x) ($P < 0.05$). The testosterone serum levels were not different between the Improvac® and the DNA groups (Figure 3).

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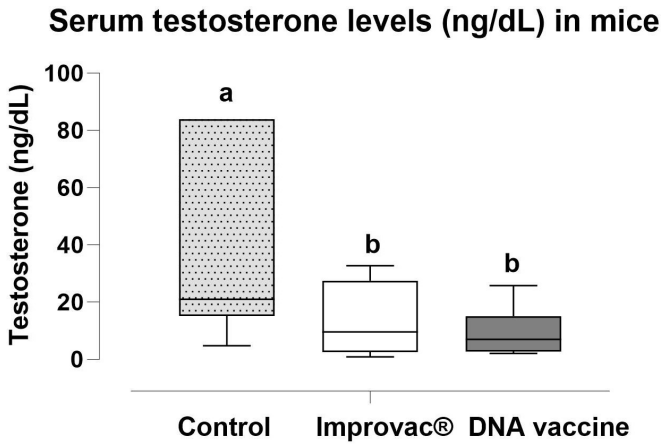

Figure 3. Median serum testosterone (ng/dL) in male mice immunized with PBS 1x (dotted light grey), Improvac® (white), or the DNA vaccine (dark grey). Different superscripts show statistical differences $P < 0.05$

Spermatogonia, spermatocyte, and spermatid cell count

A number of spermatogonia were similar between groups ($P > 0.05$). Nonetheless, the numbers of spermatocytes and spermatids were decreased both in the Improvac® ($P = 0.0002$) and the DNAv ($P = 0.01$) groups, when compared to the NC group. Spermatocyte and spermatid numbers did not differ between the Improvac® and DNAv groups (Figures 4 and 5, Table 1).

Histological findings of testes

Seminiferous tubules were lined by spermatogenic epithelium and basal sustentacular cells in all groups, and spermatozoa were observed in the tubular lumina. In addition, Leydig cells were seen to be arranged in cohesive clusters resting on interstitial fibrovascular tissue trabeculae (Figure 6). However, in the DNA vaccine group, cytoplasmic vacuoles were observed in Leydig cells, and eosinophilic protein material was observed in the interstitial spaces (Figure 6 f).

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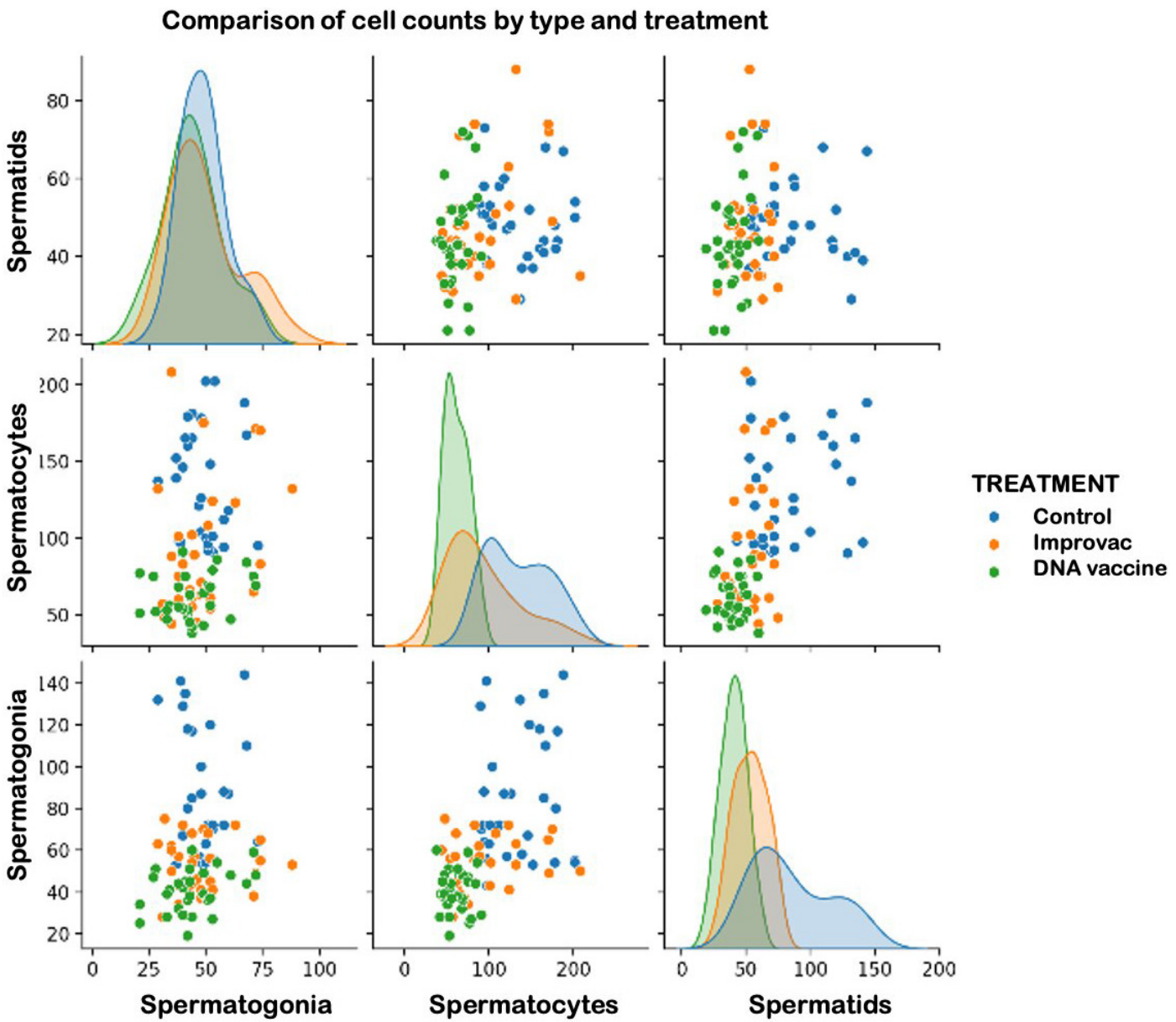

Figure 4. Comparison of cell counts by type (spermatogonia, spermatocytes, and spermatids) and treatment (Control, Improvac®, and DNA vaccine) in mice.

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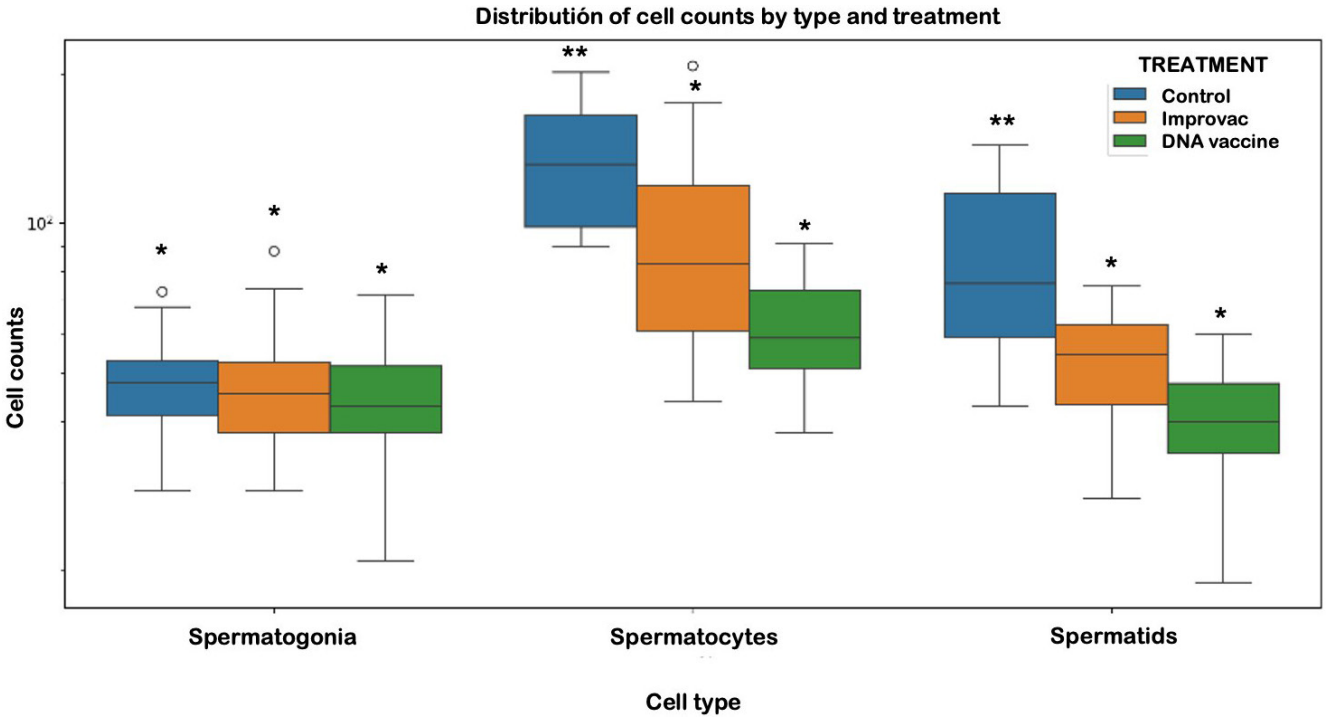

Figure 5. Distribution of cell counts by type (spermatogonia, spermatocytes, and spermatids) and treatment (control (blue), Improvac® (orange), DNA vaccine (green). **Shows the statistical difference between groups.

Table 1. Cell count means and standard deviations (Std) for spermatogonia, spermatocytes, and spermatids within treatment groups (Control, Improvac,® and DNA vaccine) in mice

Treatment	Cell type count		
	Spermatogonia	Spermatocytes	Spermatids
PBS 1 x control	*	*	*
Mean	48.73	134.80	86.1
Std	10.01	36.95	30.74
Improvac®	*	**	**
Mean	49.53	93.23	53.23
Std	15.33	43.52	12.65
DNA vaccine	*	**	**
Mean	44.27	61.53	40.40
Std	12.96	14.27	10.07
	P<0.001	P=0.0002	P=0.01

**Shows statistical difference for cell type count between groups (columns)

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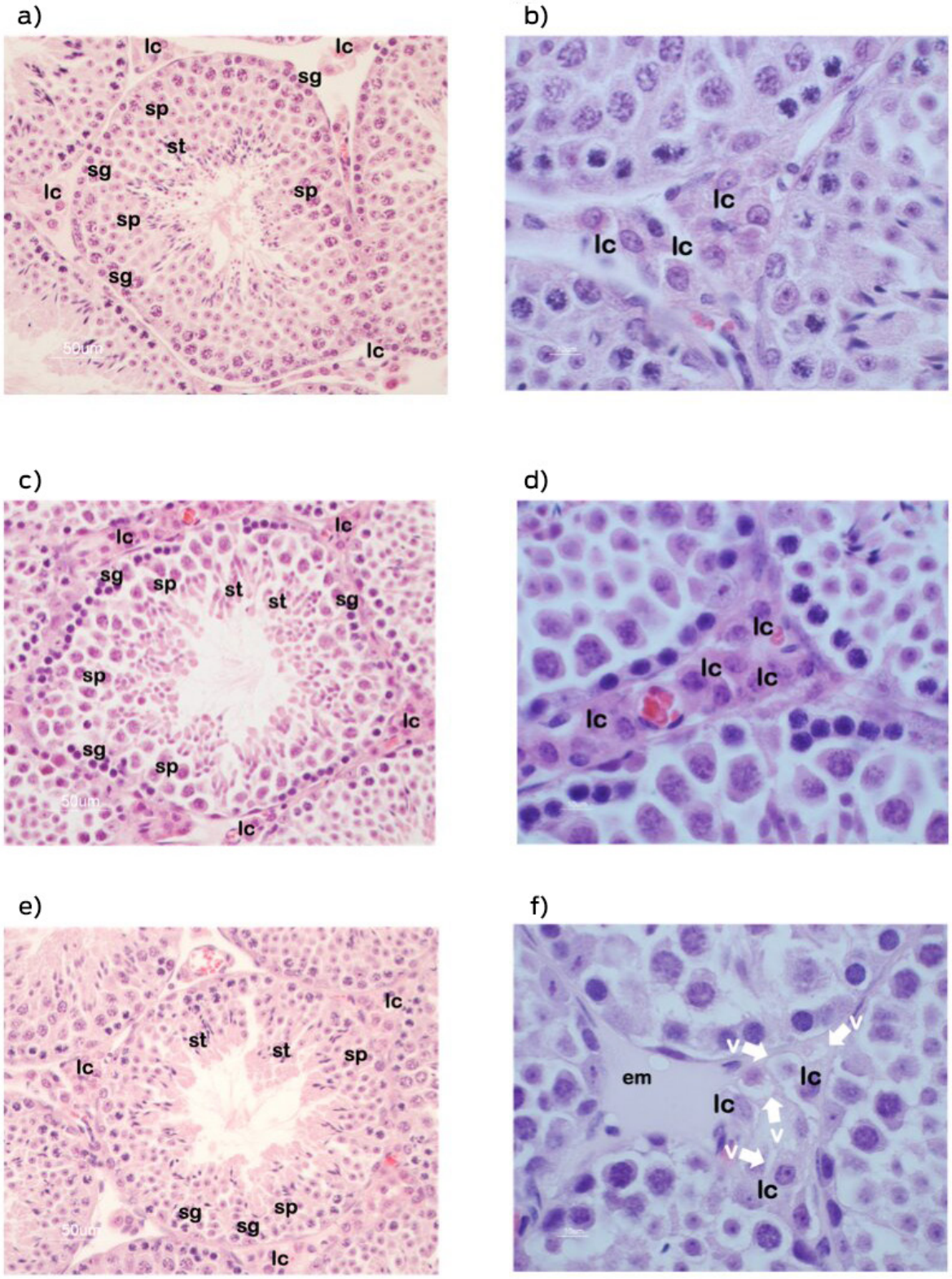

Figure 6. Histomorphological evaluation of mice testes: a) and b) PBS 1× negative control, c) and d) Improvac®, e) and f) DNA vaccine. Cross sections were stained with HE. sg: spermatogonia, sp: spermatocytes, st: spermatids, lc: Leydig cell, v: vacuolae, em: eosinophilic material.
a), c) and e) 40×; b), d) and f) 100×.

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Discussion

The control of animal overpopulation through a non-surgical method holds significant global importance as it has been pursued by numerous research groups and humanitarian animal associations for many decades. The ultimate goal is to develop an affordable, large-scale, safe, and irreversible immunocontraceptive.[1] In this work, was evaluated a DNA vaccine targeted against the first domain of the canine GnRHr, coupled to the P2 and P30 tetanic toxin epitopes. A four-dose protocol of this vaccine was demonstrated to be safe, and to induce a reduction of serum testosterone levels and testicular atrophy in mice.

Mice in the DNA vaccine and the Improvac® groups had lower concentrations of serum testosterone when compared to the control group. This result indicates that GnRH function may have been impaired by induced antibodies that decreased the availability or the performance of the GnRHr in gonadotrophs, thus hindering gonadal stimulation. When a DNA vaccine targeting the gonadotropin-releasing hormone receptor was used for immunocontraception in dogs, the mRNA levels of the GnRHr were significantly reduced. Authors attributed this finding to gonadotroph lysis due to a cytotoxic effect of specific T lymphocytes or to the activation of macrophages, monocytes, and natural killer cells, all stimulated by the production of anti-GnRHr antibodies from vaccination.[8] Moreover, an increase in anti-GnRH antibodies has been directly related to a decrease in testosterone concentration when a vaccine for immunological castration was used in boars.[17]

Testicular atrophy in male mammals is influenced by hormonal factors, particularly low testosterone levels, leading to infertility due to reduced sperm production.[18] Indeed, to prompt spermatogenesis, by stimulating cell division and differentiation of the sperm cell line, testosterone needs to be present at appropriate levels.[8, 18] Comparison of sperm precursor cell numbers in this work showed that the spermatogonia cell count was not different between groups, which is to be expected considering they constitute the stem cell pool, and are present in lower numbers than other more developed cells within the seminiferous tubules.

However, spermatocytes and spermatids, which are more numerous as they progress through the stages of spermatogenesis, were diminished in both the DNA vaccine and the Improvac® groups. Low testosterone secretion, plus, changes in other factors that may have been impacted by the alteration of the GnRH axis driven by GnRHr antibody presence, negatively altered sperm cell production, causing a degree of testicular atrophy. In addition, further signs of affected testicular function were suggested by histological changes observation such as vacuolated Leydig cells that were founded in the DNA vaccine group.

It has been reported that histological alterations in Leydig cells such as different nuclear shapes, reduced cytoplasmic volume, and vacuolization may imply impaired steroidogenesis and cell dysfunction. Furthermore, according to Rebourcet et al., the presence of vacuolated Leydig cells could serve as a histological marker to indicate testicular pathologies associated with hormonal imbalances or as a response to stressors affecting the seminiferous tubule environment and cause testicular dysfunction.[18]

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Conclusions

The P2GnRHrP30 DNA vaccine constructed in this study was shown to be safe in immunized animals in a four-dose protocol. Moreover, the reduction in testosterone levels, as well as the decrease in spermatocyte and spermatid numbers observed in treated mice indicate a clear negative effect on spermatogenesis. Further work will aim to evaluate the DNA vaccine under various administration protocols, as the prime boost system. This could potentially enhance the effectiveness of GnRH receptor blockage, reducing the number of immunizations and the time to achieve biological responses.

Since the GnRhr gene has a highly conserved DNA sequence among mammals, vaccines targeting this protein could be used in species other than rodents, such as dogs and cats, as well as in both males and females. This holds significant promise in achieving crucial objectives, such as controlling animal overpopulation and preventing zoonotic diseases, thereby contributing to humanitarian and public health efforts.

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Data availability

All data and material are available from the corresponding author upon request.

Acknowledgments

The authors wish to thank Dr. Gabriel Gutiérrez Ospina from the Instituto de Investigaciones Biomédicas-UNAM for academic guidance, and Dr. René Álvaro Segura Velázquez from the Unidad de Investigación, FMVZ-UNAM, for technical support. Thanks also go to MVZ Julio César Sánchez Lemus and Biol. Ernesto Enríquez Enríquez from the Unidad de Constatación de Productos Químico-Biológicos y Bioterio, FMVZ-UNAM, for care and handling of mice, to Dr. Mario Fragoso Saavedra for *in silico* analysis support, to Dr. José Gerardo Perera Marín, MSc. Clara Murcía Mejía and Dr. Ana Delia Rodríguez Cortez from the Departamento de Reproducción, FMVZ-UNAM, for their assistance in testosterone quantification, to Dr. Laura Patricia Romero Romero, MSc. Issac Martínez Racine, and MSc. Diana Rivera Colín from the Departamento de Patología, FMVZ-UNAM, for their support with histological processing, image recording, and analysis; and to Dr. Bárbara Nova-Franco from BioNova Scientific Coaching for her valuable comments and suggestions.

Funding statement

Reyes-Maya S was fully supported by VIVENT Centro Veterinario de Esterilización para Perros y Gatos, Ciudad de México, and MSc. Rosa Elena Méndez Aguilar.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

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Formal analysis: CG Gutiérrez, S Reyes Maya.
Investigation: S Reyes Maya, RA Alonso-Morales, CG Gutiérrez.
Methodology: S Reyes Maya, RA Alonso-Morales, ML Escobar, I Muñoz-Velasco, OM Echeverría, A Gayosso-Vázquez, V Ramírez-Andoney, P Pintor Ríos.
Project administration: S Reyes Maya, RA Alonso-Morales.
Resources: S Reyes Maya, RA Alonso-Morales, A Gayosso-Vázquez.
Supervision: RA Alonso-Morales, CG Gutiérrez.
Validation: S Reyes Maya.
Visualization: S Reyes Maya.
Writing-original draft: S Reyes Maya.
Writing-review and editing: RA Alonso-Morales, CG Gutiérrez, S Reyes Maya.

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