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Chitosan formulations improve the immunogenicity of a GnRH-I peptide-based vaccine

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ABSTRACT

Peptide vaccines using specific antigens with poor immunogenicity like GnRH-I are unable to develop an effective adaptive immune response and require the presence of adjuvants, essential to lymphocytic activation.

Three chitosan formulations were evaluated for their ability as adjuvant of a poor immunogenic peptide vaccine against GnRH-I. Male Sprague–Dawley rats were immunized subcutaneously with recombinant His-GnRH-tandem-repeat peptide in high, low and phosphorylated high molecular weight chitosan solution at 0.5% (w/v). Freund's complete adjuvant was used as a positive control of immune response. Our results suggest that different chitosan formulations as adjuvant, with high or low viscosity degree allow inducing a high and persistent immune response against a poor immunogenic recombinant peptide. We found that the immune response was mediated by a increasing of IgG isotype 1, which were significantly greater than levels presented by the animals immunized with Freund's complete adjuvant. Nevertheless, chitosan with low molecular weight and highest acetylation degree was able to induce an immune response mediated by IgG isotype 2a. Additionally, high molecular weight phosphorylated chitosan, in which the phosphate groups were linked to N-acetyl-D-glucosamine unit, the immune response was reduced. All the immune responses obtained with chitosan as adjuvant were able to neutralize effectively the GnRH hormone proves by reducing of animal steroidogenesis and spermatogenesis demonstrating its capacity to improve immunogenicity in peptide vaccine.

1. Introduction

The gonadotrophin releasing hormone GnRH-I, is a decapeptide secreted from the mediobasal portion of the hypothalamus and is responsible for inducing the release of luteinizing hormone (LH) and follicular stimulant hormone (FSH) from the hypophysis (Wojcik-Gladysz et al., 2006). For several years, scientists have tried to immunoneutralize the GnRH-I hormone, blocking its entrance into the hypophysis, and thereby controlling steroidogenesis, oogenesis, and spermatogenesis (Aissat et al., 2002; Ferro et al., 2004; Robbins et al., 2004; Elhay et al., 2007; Walker et al., 2007).

Effective vaccines against GnRH-I have been reported in a large number of animal species (Ferro et al., 2004; Geary et al., 2006). The majority of the vaccination strategies are based on the hapten bonding of GnRH-I to a highly immunogenic molecule like bovine albumin (Aissat et al., 2002), ovalbumin (Geary et al., 2006), tetanic toxin (Ferro et al., 2004) or keyhole limpet hemocyanin (Jung et al., 2005). Recombinant DNA technology has been used to create GnRH-I in tandem repeated linked to different pathogen protein sequences as immunogen for T Helper lymphocytes (Miller et al., 2004; Robbins et al., 2004; Xu et al., 2006). However, an antigenic dominance phenomenon has been described in which carrier proteins suppress the epitopes on the molecule of interest (Renjifo et al., 1998). The exclusion of epitopes with high antigenicity could not only reduce the risk of antigenic suppression, but also reduce ability to mount a robust immune response to the antigen.

A key aspect in peptide vaccine development is the absence of pathogen-associated molecular patterns (PAMP), which are responsible for stimulating antigen-presenting cells to make the

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necessary changes associated with maturation and co-stimulation that trigger initiation, amplification and orientation for an adequate adaptive immune response of sufficient magnitude and duration (Takeda et al., 2003). This aspect could be determined co-administrating adjuvants that improve antigen availability through antigenic release; but fundamentally should be able to generate co-stimulating signals to the innate immune system, which is crucial for the posterior lymphocytary activation (Sun et al., 2003).

On the other hand, biodegradable polymer chitosan (poly-β-(1→4)-2-amino-2-deoxy-D-glucose), which is obtained by partial deacetylation of chitin (poly-β-(1→4)-2-acetamino-2-deoxy-D-glucose) (Khor, 2001) has demonstrated adjuvant properties when administered along with an antigen in different formulations (Seferian and Martinez, 2001; Moschos et al., 2005; Zaharoff et al., 2007a, b). When chitosan formulations are administered intranasally its mucoadhesive properties allow it to induce release of macromolecules through the mucosa (Illum, 1998; Moschos et al., 2005). In parenteral administration, chitosan can generate a type Th1/Th2 adaptive immune response, principally due to the high viscosity of chitosan, which can generate cellular expansion in the lymphatic nodes and retain the antigen in the site of injection for a prolonged period (Zaharoff et al., 2007a, b). Additionally, it has been observed that the administration of chitosan without antigen is capable of stimulating secretion of cytokines, up regulation of MHC class II and mannose receptors in dendritic cells and macrophages (Porporatto et al., 2005). The immune response is optimized by simultaneous stimulation of antigen-presenting cells and lymphocytes by the adjuvant inmunostimulator and the antigen, generating a specific microenvironment for the immune response in the site of inoculation (Bendelac and Medzhitov, 2002).

In the present article we determined the effectiveness of three chitosan formulations as adjuvant in blocking the steroidogenesis and spermatogenesis *in vivo*, using a model of anti-GnRH-I peptide vaccine that includes an antigen with poor immunogenicity.

2. Materials and methods

2.1. Recombinant antigen

The nucleotide sequence encoding the recombinant GnRH-tandem-repeat peptide was synthesized by IDT (Integrated DNA Technologies, Inc.). The nucleotide sequence of the amino acid residues 24–33 of the rat GnRH-I protein (GenBank accession PO7490) was repeated 20 times for annealing and ligation of concatomers with an **atg** 3′ cohesive-end. The annealing of sequences was made as follow, the forward sequence 5′-*cagcactggtcctacggtctgcgtccgggt**atg***-3′ and the reverse sequence 5′-*acccggacgcagaccgtaggaccagtgctg**cat***-3′ were annealed in buffer A (100 mM NaCl and 50 mM HEPES pH 7.4) were heated at 90 °C for 4 min, and then cooled at 70 °C for 10 min, 37 °C for 15 min and finally 10 °C. The annealed sequences were ligated overnight at 4 °C and then inserted into the MCS of pQE 80L expression vector (Qiagen, Inc., USA) throughout SphI and SmaI restriction site. The 3′ overhang of concatomer was previously removed with klenow fragment (New England BioLabs Inc.).

Transformed *E. coli* BL21(DE3)*plysS* (Stratagene, La Jolla, USA) with the pQ80L-GnRH-tandem-repeat (pQ80L-GnRH-TR) vector were grown in Luria broth until OD_{595} of 0.6. Expression of the recombinant peptide was initiated with 0.1 mM IPTG and orbital agitation for 18 h at 37 °C.

The bacteria were collected from the culture by centrifugation at 3000 × *g* for 10 min and resuspended in lysis buffer (8 M Urea, 10 mM Tris, 100 mM NaH_2PO_4, 1% Triton X-100, pH 8.0) for 1 h at 37 °C.

The soluble proteins were recovered by centrifugation at 16,000 × *g* and separated in Ni-NTA columns (Ni-NTA Purification System, Invitrogen Co.). The recombinant his-tag peptide His-GnRH-TR was separated by electrophoresis in SDS-PAGE gels for visualization.

Finally the purified peptide was dialyzed against phosphate-buffered saline (PBS, pH 7.4).

2.2. Preparation of chitosan formulations

One hundred grams of chitosan (Chi) samples of high molecular weight (MW = 350 kDa, ≥83% deacetylation, 800,000 cps viscosity) from Aldrich and low molecular weight (MW = 70 kDa, ≥75% deacetylation, 20,000 cps viscosity) from Fluka were washed with acetone and methanol and dried to constant weight. Each stock solution of Chi was formulated by dissolving 1 g of purified Chi in 100 ml of 0.5% acetic acid (pH 4.0) and sterile filtered to be used for immunization.

The phosphorylation reaction of Chi was carried out by a procedure reported elsewhere (Varma et al., 1999). Briefly, 1 g of purified Chi of high molecular weight (Chi-HMW), 3 g of 98% orthophosphoric acid, 30 g urea and 30 ml of 85% dimethylformamide were mixed together in a three neck flask fitted with a condenser, thermometer and argon gas inlet tube for 3 h at 70 °C. Without cooling the contents, the phosphorylated Chi (P-Chi-HMW) were removed and thoroughly rinsed with distilled water. The average yield of the phosphorylation reaction was 70% (Neira-Carrillo et al., 2005).

2.3. Immunization of animals

Fifteen male Sprague–Dawley rats of 8-week-old were obtained from the Faculty of Veterinary Sciences of the University of Chile. The animals were randomly distributed in groups of three rats to be inoculated and they were kept with food and water *ad libitum* in a temperature and light-controlled environment. This protocol was approved by the institutional animal bioethics committee.

The animals were anesthetized with a mixture of isoflurane/O_2 for the blood extraction and vaccination. The immunization of rats was performed with the same formulation at days 0 and 30 of experimentation, with 200 μl subcutaneous injection that contained a 1:1 mixture of the adjuvant and 100 μg of the recombinant His-GnRH-TR peptide diluted in PBS, pH 7.4.

The experimental groups (n = 3 rats/group) were composed as follows; group 1: The recombinant His-GnRH-TR peptide in PBS; group 2: His-GnRH-TR peptide in high molecular weight chitosan (Chi-HMW) at 0.5% (w/v); group 3: His-GnRH-TR peptide in phosphorylated high molecular weight chitosan (P-Chi-HMW) at 0.5% (w/v); group 4: His-GnRH-TR in low molecular weight chitosan (Chi-LMW) at 0.5% (w/v); group 5: His-GnRH-TR in Complete Freund's Adjuvant (CFA).

2.4. Measurement of IgG against the His-GnRH-TR recombinant protein and GnRH

Fifteen microliters of blood were extracted from the external iliac vein of the anesthetized animals at weeks 0, 2, 4, 6, 8 and 10. The serum was separated by centrifugation and stored for analysis at −20 °C.

An indirect ELISA assay was performed on 96 well plates (MaxiSorp, Nunc) to detect the level of antibodies against the recombinant peptide in the serum of the inoculated animals. Two micrograms of His-GnRH-TR or Luteinizing Hormone Releasing Hormone (LHRH, 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) for 2 h, then the plates were washed five times with wash-

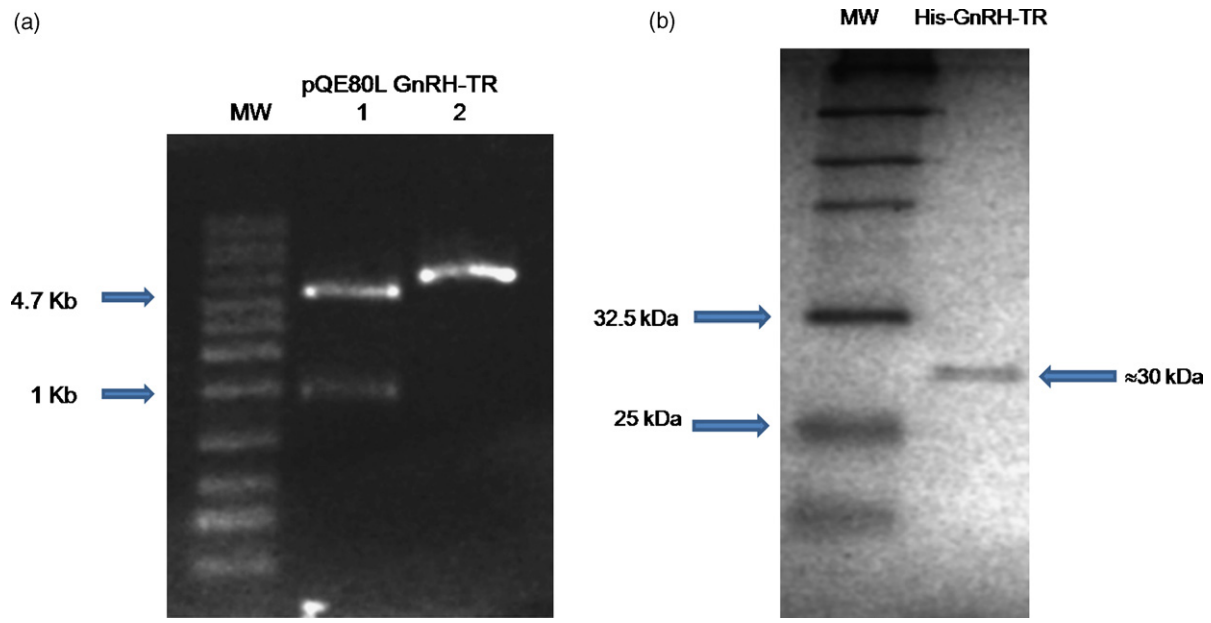

Fig. 1. Visualization of recombinant GnRH-TR molecule. 1% TAE agarose gel stained with ethidium bromide, the GnRH-TR sequence of approximately 1000 bp (line 1) was inserted in the pQE80L bacterial expression vector. pQE80L-GnRH-TR vector (line 2) (a). 12% SDS-PAGE with comassie blue; the recombinant His-GnRH-TR protein of approximately 30 kDa purified with Ni-NTA (b).

ing buffer (0.05% Tween-20, v/v in PBS) and blocked with 200 μl of blocking buffer (1%, w/v bovine serum albumin in PBS) overnight at 4 °C. Subsequently, the plates were washed five times with washing buffer and incubated with 150 μl of each serum diluted 1:250 in diluent buffer (0.1% BSA, w/v, 0.05% Tween-20, v/v) for 1 h at 37 °C. After that, the plates were washed five times with washing buffer and incubated with 150 μl of Rabbit anti-Rat IgG, IgG1 or IgG2a peroxidase-conjugated antibodies (Jackson Immunoresearch, Laboratories), diluted 1:5000 in diluent buffer for 1 h at 37 °C. Finally the plates were washed and developed with 150 μl of 1-Step™, Slow TMB-ELISA (Pierce, Chemical Company) for 15 min at room temperature. The reaction was stopped with 150 μl of 1.5 M H_2SO_4 and the absorbance was read at 450 nm.

2.5. Detection of testosterone levels in serum

To evaluate the concentration of testosterone in the serum of control and the immunized animals with the recombinant peptide, a Competitive Enzyme Immunoassay (ACE™ Cayman Chemical Company) was performed. 96 well pre-blocked and pre-coated plates with mouse monoclonal anti-rabbit IgG were incubated with the serum of experimental animals (1:50) along with acetylcholinesterase linked to testosterone and testosterone rabbit antiserum in EIA buffer (Cayman Chemical Company) for 2 h at room temperature, permitting the competition between the free testosterone in the serum and the testosterone linked to acetylcholinesterase, which was visualized after incubation with Ellman's Reagent (Cayman Chemical Company) for 1 h and read at 405 nm.

2.6. Testicular histological analysis

Ten weeks after first immunization, the animals were sacrificed by exposure to CO_2 and both testicles were surgically extracted, and fixed in buffered formalin (10%, v/v).

Five micrometer thick slices of testicular tissue were stained with Hematoxilin–Eosin (H–E) and observed under an optical microscope (Olympus BX 41). The presence of multiple layers of germ cells in the seminiferous tubule was recorded for one histological section from each testis. The images were obtained with MicroPublisher 3.3 RTV (QImaging Corporation) and analyzed with QCapture Pro version 5.1.1.14.

2.7. Statistical analysis

Significances and statistical differences with control group were determined by unpaired Student tests with a value of $p \leq 0.05$.

3. Results

3.1. Cloning and expression of the recombinant peptide

A fragment of approximately 1000 bp corresponding to the GnRH-TR insertion sequence was extracted from the pQ80L-GnRH-TR vector by BamHI and HindIII enzymatic digestion (Fig. 1a). The fragment was sequenced to verify the success of the cloning. A recombinant peptide of approximately 30 kDa, called His-GnRH-TR was expressed and efficiently purified from *E. coli* BL21(DE3)*plysS* transformed with the expression vector (Fig. 1b).

3.2. Chitosan as adjuvant permits immunoneutralization of GnRH

Animals immunized with the recombinant His-GnRH-TR peptide at days 0 and 30 using Chi-HMW as adjuvant at a final concentration of 0.5% (w/v) (group 2), presented a significant increase and constant levels of specific IgG against the recombinant protein, compared with the animals immunized with the recombinant His-GnRH-TR peptide mixed only with PBS (group 1) (Fig. 2a) ($p \leq 0.01$). The recombinant peptide *per se* was incapable of stimulating the formation of specific antibodies, however with Chi-HMW as adjuvant allowed increased levels of IgG from 1:10,000 dilutions (Fig. 2b) ($p \leq 0.01$). The antibodies generated against the His-GnRH-TR peptide were capable of binding to the unmodified luteinizing hormone releasing hormone (LHRH) ($p \leq 0.05$) (Fig. 2c).

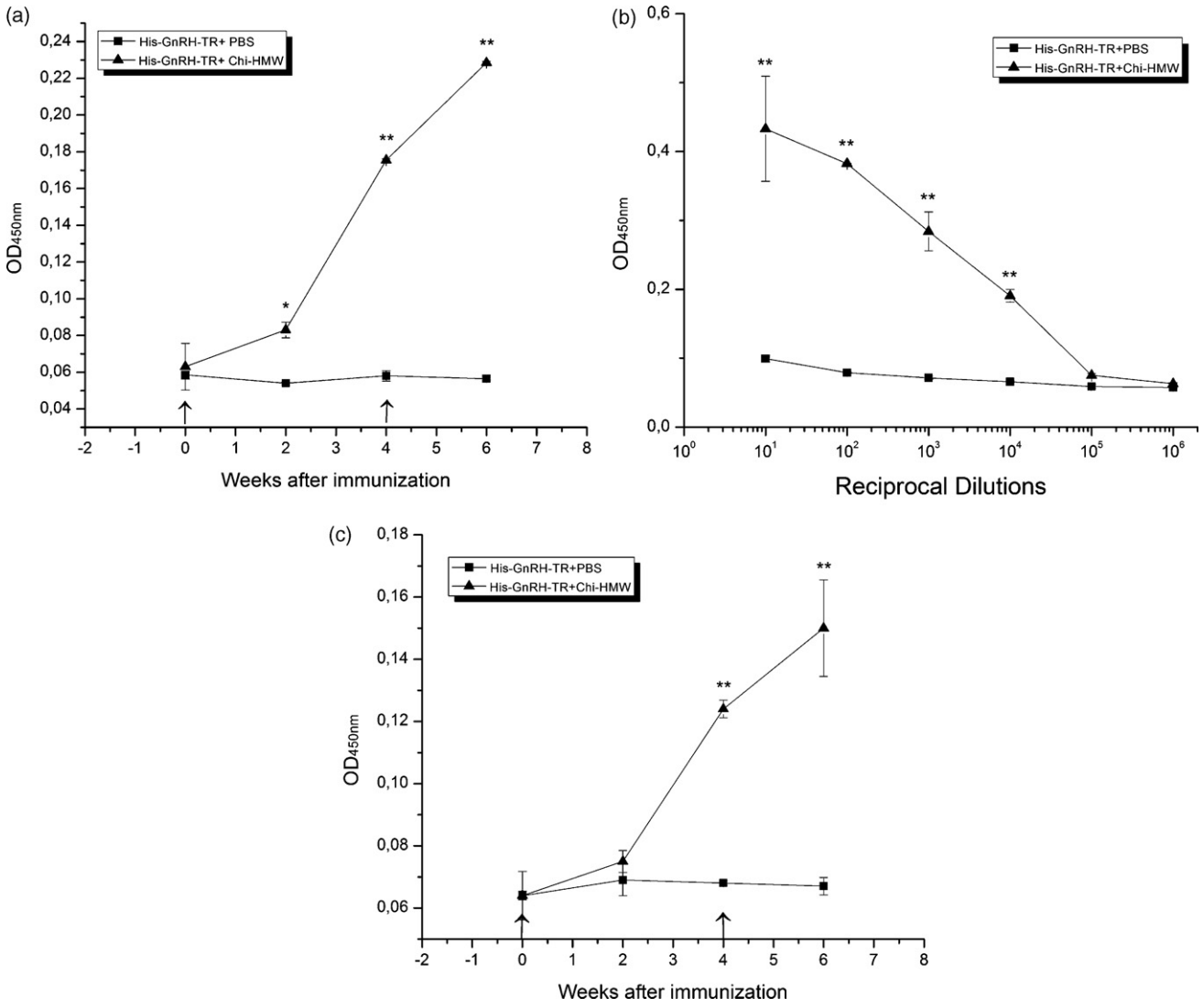

Fig. 2. Immunoneutralization of GnRH using Chi-HMW as adjuvant. Males Sprague–Dawley rats ($n = 3$) were immunized on days 0 and 30 (arrows) with 100 μg of the His-GnRH-TR peptide in 200 μl of PBS (■) (group 1) or in 200 μl of 0.5% (w/v) (▲) Chi-HMW (group 2) IgG levels on weeks 0, 2, 4 and 6 were measured by ELISA. IgG levels against the His-GnRH-TR peptide (a). Reciprocal dilutions at week 6 (b). IgG levels against the unmodified LHRH (c). All data are represented as a mean ± S.E.M. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$

3.3. Immune response against His-GnRH-TR with different formulations of chitosan

Other two solutions of chitosan were tested for their effectiveness as adjuvant; a P-Chi-HMW with the same viscosity and acetylation degree (≥83% deacetylation, 800,000 cps viscosity) and Chi-LMW with low viscosity and a great degree of acetylation (≥75% deacetylation, 20,000 cps viscosity). Animals immunized at days 0 and 30 with the recombinant His-GnRH-TR peptide and a 0.5% (w/v) P-Chi-HMW solution as adjuvant (group 3), were capable to increase specific IgG levels against the recombinant peptide, with less potency than the animals immunized with Chi-HMW (group 2) at week 6 ($p \leq 0.005$), equivalent at week 10 ($p \leq 0.005$) and greater than that generated by the CFA (group 5) ($p \leq 0.01$) (Fig. 3a). In addition, animals immunized with Chi-LMW at 0.5% (w/v) (group 4) also showed an increase of specific IgG levels against the peptide with a very similar pattern that group 3 ($p \leq 0.005$).

The presence of IgG1 and IgG2a specific immunoglobulin isotypes can be taken as a guide in order to know the type of immune response generated against the antigen. Fig. 3b shows that all chitosan formulations used as adjuvant were able to significantly elevate specific IgG1 levels ($p \leq 0.0005$) at week 10 greater than that generated by the CFA ($p \leq 0.005$). The animals of group 2 displayed a significant increase in IgG1 levels from week 2 ($p \leq 0.005$) unlike the groups 3 and 4 that the rise was observed after the booster at week 6. Levels of specific IgG2a against the recombinant peptide showed short increases after the second booster at week 6, only in the animals in group 4 and 5 ($p \leq 0.01$). Animals immunized with Chi-HMW and P-Chi-HMW formulations as adjuvant did not develop an immune response, as measured by the increasing of IgG isotype 2a ($p \leq 0.05$) (Fig. 3c).

3.4. Decrease of steroidogenesis and spermatogenesis

The concentration of serum testosterone in the animals immunized with the recombinant His-GnRH-TR peptide in PBS (group 1) showed constant and increased levels during the assay $p \leq 0.01$ (Fig. 4). At week 4 (Fig. 4a) only the animals in group 2 showed a significant lower concentration of serum testosterone compared with the control ($p \leq 0.05$) in that it agrees with the immunoglobu-

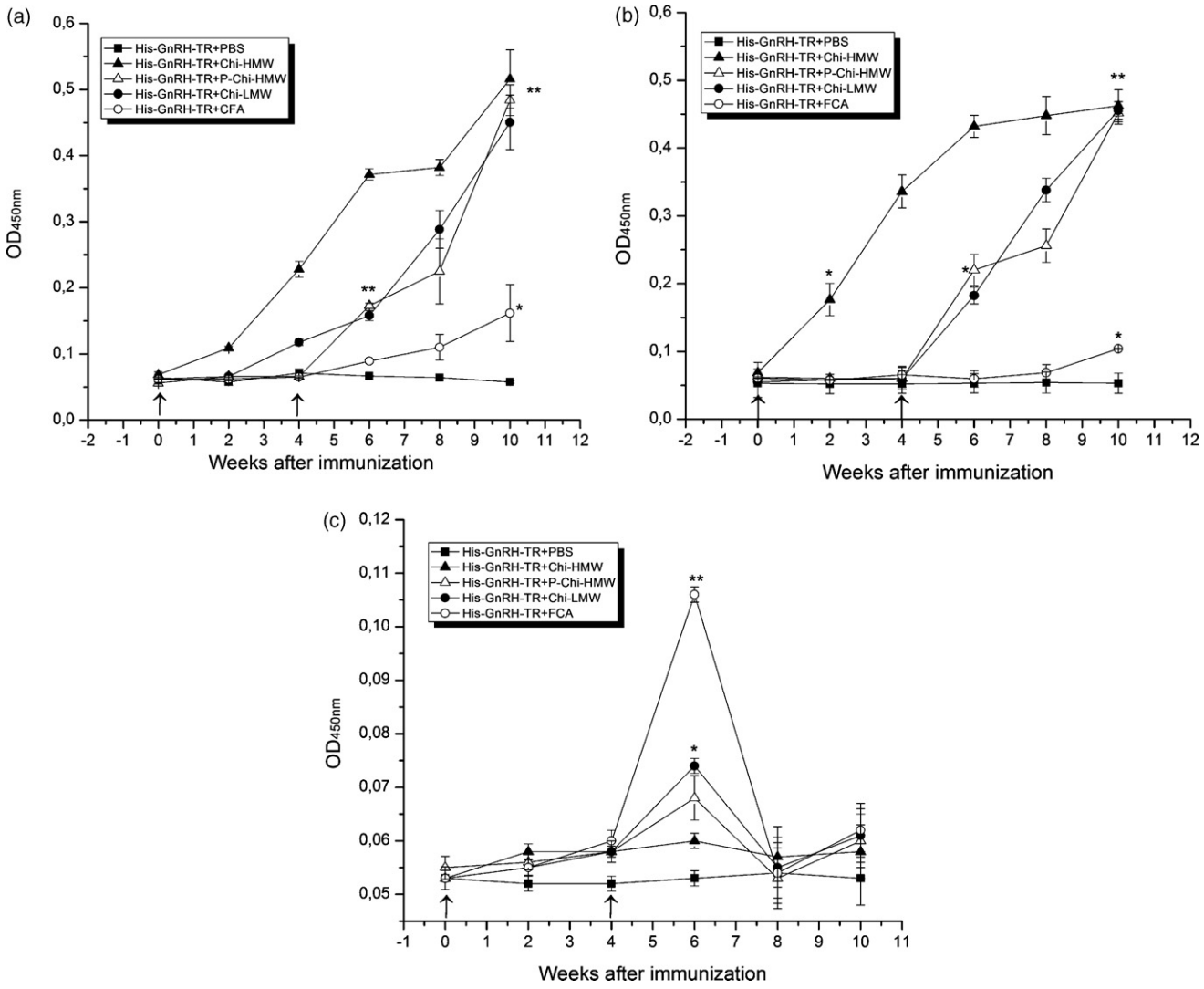

Fig. 3. IgG, IgG1 and IgG2a levels using different solutions of chitosan as adjuvant. Males Sprague–Dawley rats ($n = 3$) were immunized on days 0 and 30 (arrows) with 100 μg of the recombinant His-GnRH-TR peptide in 200 μl of PBS (■) (group 1); in 200 μl of 0.5% (w/v) Chi-HMW (▲) (group 2); in 200 μl of 0.5% (w/v) P-Chi-HMW (△) (group 3), in 200 μl of 0.5% (w/v) Chi-LMW (●) (group 4) or in 200 μl of CFA (○) (group 5). IgG levels (a); IgG1 levels (b) and IgG2a levels (c) were measured on weeks 0, 2, 4, 6, 8 and 10 by ELISA. All data are represented as a mean ± S.E.M. $^{*}p \leq 0.01$, $^{**}p \leq 0.005$

lin high levels at week 4 (Fig. 3). At week 10 all animals immunized showed a lower concentration of testosterone than control group ($p \leq 0.05$) and lower than the group 4 ($p \leq 0.005$).

The decrease on steroidogenesis caused reduction of the spermatogenesis in all groups in which the recombinant peptide and adjuvants were used, visualized by the absence of spermatozoa within the seminiferous tubules. However, in the animals in groups 2, 3 and 4 in which chitosan was used as adjuvant, the histological analysis showed a marked atrophy of the seminiferous tubules evidenced by a notorious reduction in size of tubule and numbers of cellular layers accompanied by the absence of spermatozoa. In the control group the size of tubules was 212.5 ± 25 μm, the somatic and germinal cells (6.33 ± 1.8 cellular layers) and spermatozoids were observed in the seminiferous tubule (Fig. 5a) and spermatozoids in epididymis (Fig. 5b). The animals in group 5 showed a decrease of the height of cellular layers (4.85 ± 1.18 layers) and spermatozoid content in the seminiferous tubule (Fig. 5c) and epididymis (Fig. 5d). The animals of group 2 showed a marked testicular atrophy with a reduction in the size of the seminiferous tubule (68 ± 25 μm) and decrease of the cellular layers (2.6 ± 0.68 layers) (Fig. 5e) and azoospermia (Fig. 5f).

4. Discussion

The gonadotrophin releasing hormone is one of the few peptide hormones that have been effectively immunoneutralized with important results, principally related to the reproductive control in veterinary medicine or as a target in certain types of hormone-dependent on cancers in human medicine (Hsu et al., 2000; Finstad et al., 2004; Conforti et al., 2008; Gonzales et al., 2007; Khan et al., 2007).

In the present article, we demonstrate that it is possible to generate an effective decrease of steroidogenesis and spermatogenesis in animals immunized with three chitosan formulations used as adjuvant and a peptide without immunogenic properties, that have inserted a tandem-repeat of GnRH-I sequence without carrier molecules or epitopes of T lymphocytes associated with pathogens.

The ability of chitosan as adjuvant has been previously described (Zaharoff et al., 2007a, b). The authors report that the adjuvant ability of chitosan is mediated by its high viscosity, which favors a gradual release from the antigen to immune system cells and induces lymphatic activation and proliferation. However, we have obtained an equivalent immune response using a formula-

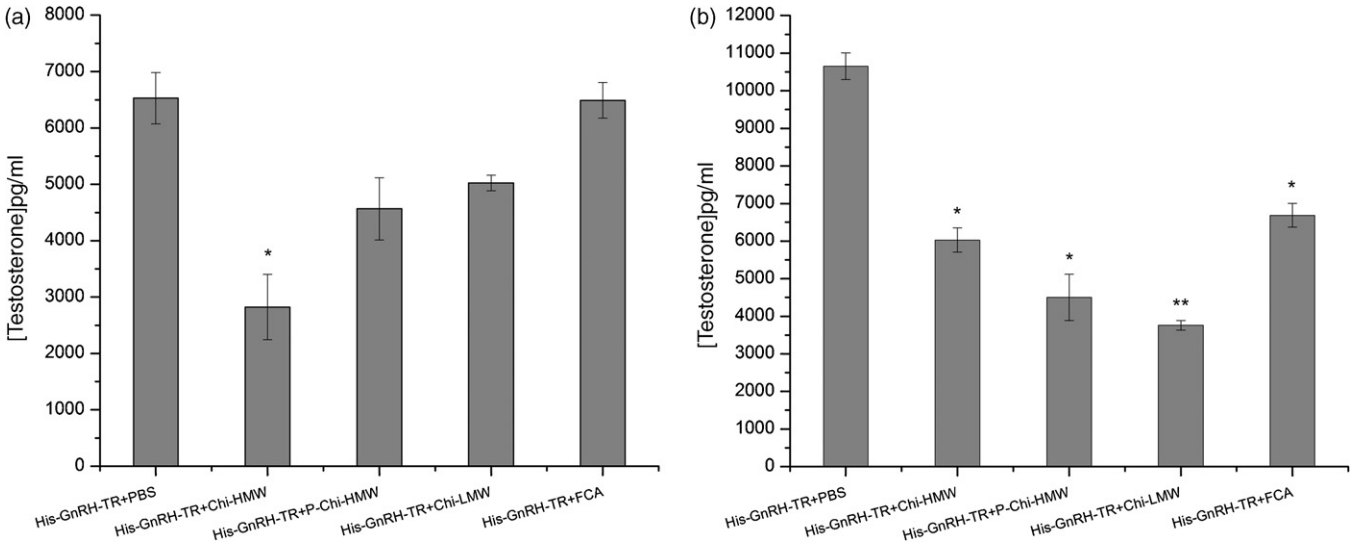

Fig. 4. Serum testosterone levels using different solutions of chitosan as adjuvant. Males Sprague–Dawley rats ($n = 3$) were immunized by 0 and 30 with 100 μg of the recombinant His-GnRH-TR peptide in 200 μl of PBS (group 1); in 200 μl of 0.5% (w/v) Chi-HMW (group 2); in 200 μl of 0.5% (w/v) P-Chi-HMW (group 3), in 200 μl of 0.5% (w/v) Chi-LMW (group 4) or in 200 μl of CFA (group 5). Serum testosterone concentration at week 4 (a) and 10 (b) were measured by a Competitive Enzyme Immunoassay. All data are represented as a mean ± S.E.M. $^{*}p \leq 0.05$, $^{**}p \leq 0.005$.

tion of low molecular weight of chitosan and with low viscosity (20,000 cps) which is 40 times lower than high molecular weight chitosan (800,000 cps). Moreover, the solution of Chi-LMW was able to induce a temporary increase in the levels of IgG isotype 2a which is associated with type Th1 immune response. It is safe to notice, that a reduced immune response was obtained when phophorylated Chi-HMW of the same viscosity of its non-phosphorylated form is used. This could be explained by the fact that the phosphates are chemically bonded to carbons 3 and 6 of N-acetyl-D-glucosamine units (Jayakumar et al., 2008), thus modifying the chemical group that is responsible for the improvement of the immune response (see below). Therefore, our results suggest that the viscosity of chitosan is not the only factor involved in the improvement of immune response associated with peptide vaccination, but the chemical nature of the N-acetyl-D-glucosamine unit must also be taken into account.

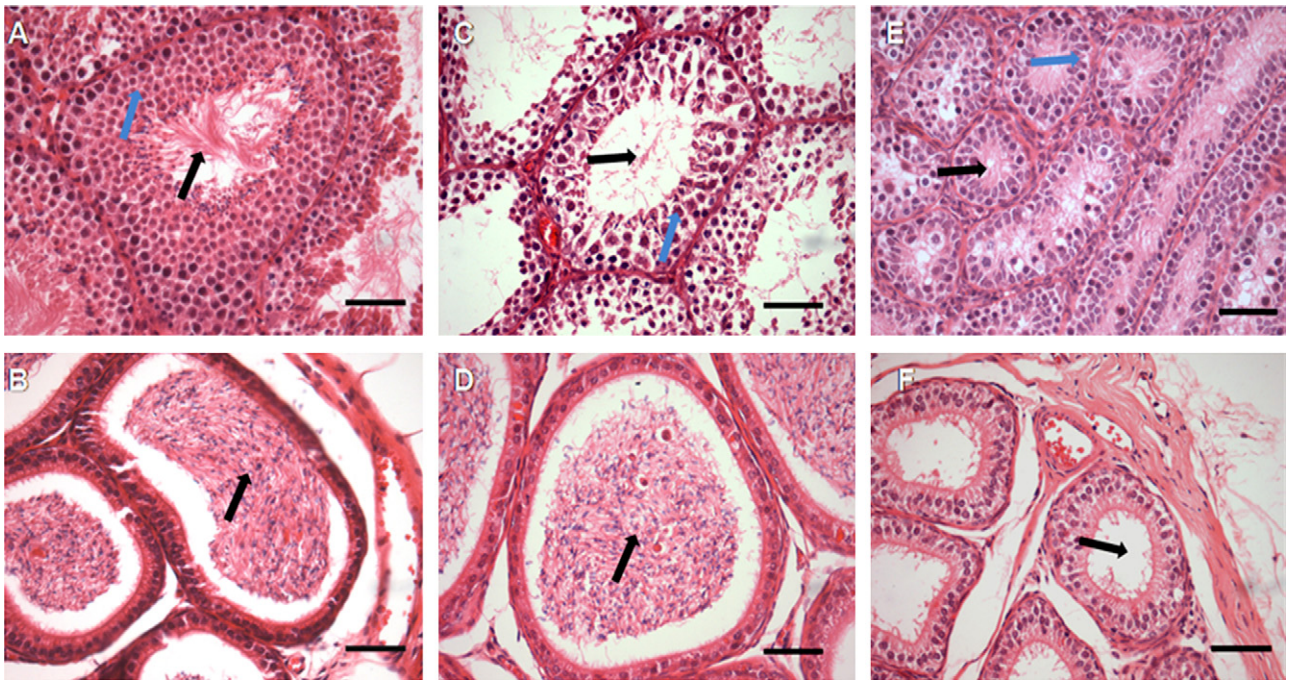

Fig. 5. Histological changes in animals immunized with chitosan as adjuvant. Males Sprague–Dawley rats immunized on days 0 and 30 with 100 μg of the recombinant His-GnRH-TR peptide in 200 μl of PBS (group 1) in 200 μl of 0.5% (w/v) Chi-HMW (group 2) or in 200 μl of CFA (group 5). 5 μm testicular slices were stained with H–E to analyze spermatogenesis in seminiferous tubule and the presence of mature spermatozoa in epididymis. Primordial cells (blue arrow) and spermatozoids (black arrow) were observed in the seminiferous tubule of the control group (a) and spermatozoids in epididymis (b). The animals in group 5 showed a decrease of the primordial cells number (4.85 ± 1.18) and spermatozoid in the seminiferous tubule (c) and epididymis (d). The animals of group 2 showed a marked testicular atrophy with a reduction in the size of the seminiferous tubule (68 ± 25 μm) and decrease of the primordial cells number (2.6 ± 0.8)(e) and azoospermia (f). Bar 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Vaccination with a purified endogenous antigen, without the presence of PAMP, decreases the capacity of eliciting an effective immune response capable of generating immunological memory (O'Hagan et al., 2006). In according with this, we did not detect any effect using our peptide in saline solution for immunization. The last point has been one of the main obstacles in the development of peptide vaccines that has driven the necessity of using different vectors such as viruses or bacteria that deliver these immunostimulating elements (O'Hagan et al., 2006; Reddy et al., 2006).

The Complete Freund's adjuvant contains a series of PAMP carried by the *Mycobacterium* extract that improves the immune response, however, it has demonstrated to be harmful (Lindblad, 2000). Although that chitosan does not contain PAMP; its chemical structure can stimulate an immune response (Mori et al., 2005; Porporatto et al., 2005). This observation could be explained by the presence of N-acetyl-D-glucosamine groups on the chitosan molecule. It is well-known that the N-acetyl-D-glucosamine is an important component of bacterial and fungal cell walls and this molecular unit has been involved in the Toll-like receptor-mediated macrophages and dendritic cells activation (Takeuchi et al., 1999; Girardin et al., 2003; Netea et al., 2006; Zhang et al., 2006). Chi-LMW has lower molecular weight and viscosity in solution, however it contains a greater number of N-acetyl-D-glucosamine units given its lesser degree of deacetylation (75%), which would explain its better immunostimulating ability to generate a type Th1 immune response, as measured by an increase in IgG2a significantly greater than Chi-HMW but less than CFA, which contained fragments of bacterial cell. We have demonstrated that the immunization of animals with Chi-HMW formulation with identical viscosity but with N-acetyl-D-glucosamine chemically modified decreases its adjuvant capacity. This can be visualized in the animals immunized with Chi-HMW which showed a faster response than P-Chi-HMW, expressed by the IgG increase.

Seferian and Martinez (2001), were capable of stimulating the immune system of animals using particles of chitosan together with the antigen, in a similar manner to that observed when aluminum hydroxide was used as adjuvant. Aluminum hydroxide improves the availability of the antigen to macrophages and dendritic cells (Gupta and Rost, 2000). Moreover, it has been described that the chitin and its derivative chitosan can be able to stimulate receptor-mediated phagocytosis by mannose present in certain populations of dendritic cells, which would improve the delivery of the antigen (Apostolopoulos and McKenzie, 2001; Porporatto et al., 2005; Mori et al., 2005).

In our model of vaccine, we can dismiss this stimulation pathway, since our recombinant peptide contains an isoelectric point of 11, much higher than the pK of the chitosan solution (pK = 6.5), making physical interaction mediated by electric charges impossible (Soldier et al., 2001; Pastor de Abram, 2004) and therefore they do not form particles in solution that facilitate phagocytosis. This experimental evidence was corroborated by scanning electron microscopy observation (data not shown). The correct formulation and characterization of microparticles of chitosan charged with antigen could represent an interesting alternative to the use of polymers that have been shown to efficiently activate the immune system but they are of high cost or difficult formulation.

In the present article we have described an important information about the possibility of generating an efficient immune response against GnRH-I, a poor immunogenic endogenous peptide, by using different chitosan formulation by subcutaneous administration. Chitosan of high viscosity and molecular weight was capable of stimulating an effective type TH2 immune response, as measured by IgG isotype 1. Chitosan of low viscosity was equipotent in its capacity to simulate an increase in IgG1 levels, but also capable of stimulating a significant increase in IgG2a levels concordant with a type Th1 immune response. All chitosan formulations used as adjuvant were able to induce a decrease of steroidogenesis and spermatogenesis in the immunized animals, to a greater degree than CFA.

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