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RESEARCH PAPER

Immunocontraceptive potential of a recombinant chimeric protein composed by a single gonadotropin-releasing hormone molecule and B subunit of *Escherichia coli* heat-labile enterotoxin in a mice model

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Vaccine;
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Abstract: The present study aimed to evaluate the immunocontraceptive potential of a chimera containing gonadotropin-releasing hormone (GnRH) and the B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) as a carrier molecule in male BALB/c mice. Mice were randomly divided into six experimental groups, according to the antigen/control inoculation: GnRH/LTB with adjuvant; commercial vaccine; GnRH/LTB; LTB with adjuvant; LTB; and PBS as control group. The humoral immune response was evaluated by indirect ELISA and the contraceptive potential was assessed through histological analysis of the animals' gonads and serum testosterone. Vaccinated groups respond with production of anti-GnRH/LTB antibodies. GnRH/LTB associated with adjuvant induced significant ($P < 0.05$) higher IgG titer after 14 days after the prime vaccination, keeping high titer up to 126 days. The histology results showed that chimeric antigen caused changes in spermatogenesis, and in the gonads tissue. In addition, this group had significantly lower testosterone levels than the control group ($P < 0.05$). The chimera produced through the fusion of a single GnRH molecule with LTB as a carrier are capable of inducing the humoral immune response, blocking endocrine functions related to reproduction, and causing tissue alterations in the gonads of male mice.

Introduction

Non-surgical castration methods have become highly desirable approaches for the control of both animal population and male sexual behavior in animals breed for meat production (Siel et al., 2016). Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide responsible for providing the primary stimulus for the reproductive axis of male and female mammals. Active immunization against GnRH neutralizes endogenous GnRH, creating an immunologic barrier between the hypothalamus and the anterior pituitary gland, preventing GnRH from binding with its pituitary gonadotropin receptors. This process leads to the suppression of gonadotropin secretion,

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thereby inhibiting both steroidogenesis and gametogenesis (Herbert & Trigg, 2005; Han et al., 2016).

The major problem in targeting GnRH for immunocontraception is the molecule's low immunogenicity. Several strategies have been examined to rectify this problem, including, conjugation of multiple hormone copies, association with carrier molecules, and combine with adjuvants (Ferro et al., 2004). So, our approach was to add a carrier molecule and an adjuvant to GnRH and evaluate its immunocontraception potential. The heat-labile enterotoxin B subunit of *Escherichia coli* (LTB) has been used as immune modulator improving vaccine efficacy (Conceição et al., 2006). LTB is characterized as a potent signaling molecule with the capacity to stimulate a strong systemic response against co-administered or coupled antigens (Yamamoto et al., 2001; Conceição et al., 2006). LTB subunit itself is capable of enhancing the immune response to antigens supplemented with it, thus making a perfected candidate as a carrier molecule. This molecule forms a pentameric cylinder-like structure, responsible for binding to a cell surface receptor, the GM1 ganglioside (Nashar et al., 2001). Oil base adjuvant has the capability for generating a rapid, high and long-lasting immune response. Montanide ISA 50 V2 (SEPPIC, France), is an emulsion composed of a mineral oil and a surfactant from the mannide monooleate family, is an adjuvant with enhancing specific antibody titers and cytotoxic T-lymphocyte (CTL) responses (Yamshchikov et al., 2001).The immune enhancing effect of ISA 50 V2 is suggested to be associated with depot formation and slow the release of antigens, leading to an inflammation, which stimulates the recruitment of antigen presenting cells (APCs) (Orr et al., 2013), which stimulates the accumulation of lymphocytes in draining lymph nodes (Aucouturier et al., 2001; Kaeberle, 1986).

The aims of this study were to evaluate the immunogenic potential of a chimera containing the gonadotropin-releasing hormone (GnRH) and the B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) as a carrier molecule adjuvanted with Montanide in male BALB/c mice for developing immunocontraceptive vaccine.

Materials and methods

Compliance with ethical standards

All mice were maintained and handled at the animal care facility from the Universidade Federal de Pelotas (UFPel), Brazil, and were housed in autoclaved cages (Alesco, Brazil) with no food or water restrictions. All procedures were performed in accordance with the Brazilian Committee for animal care and use (COBEA) guidelines and were approved by the UFPel Ethics Committee for animal research (project number 1039). All efforts were made to minimize animal suffering.

Recombinant GnRH/LTB antigen

The gene fragment encoding the carrier protein LTB fused to GnRH was designed based on GenBank sequences, found under accession numbers S60731.1 and AF031653.1, respectively.

The GnRH/LTB sequence was cloned into the pAE vector (Ramos et al., 2004). The recombinant plasmid pAE/GnRH/LTB was transformed into *E. coli* BL21 Star™ (DE3) cells. The GnRH/LTB protein expression and recovery was performed as previously described (Gil et al., 2013). The recombinant protein was purified by affinity chromatography using both HisTrap™ HP 1 ml columns pre-packed with pre-charged Ni Sepharose™ and the AKTAprime™ Automated Liquid Chromatography System (GE Healthcare, Little Chalfont, UK). Purified GnRH/LTB protein was dialyzed in phosphate-buffered saline (PBS) with decreasing urea concentrations for 72 h and was then stored at -20 ºC for further analysis.

SDS-PAGE and Western blotting

Purified protein was boiled in SDS-PAGE loading buffer and separated on a 12% separating gel in a Mini-PROTEAN electrophoresis system (Bio-Rad, Hercules, California, USA). The gel was stained with Coomassie Brilliant Blue R250. For Western blotting, proteins were transferred onto a nitrocellulose membrane using a Bio-Rad Mini Trans-Blot Cell. The membrane was blocked with 5% non-fat dry milk. Antigenic proteins were detected by incubating the membrane first anti-GnRH sera from Bopriva® commercial vaccine (Zoetis, New Jersey, USA) immunized rabbits (1:400) and anti-CT MAb (1:5000) (Sigma-Aldrich, St. Louis, Missouri, USA), and second with anti-rabbit (1:4000) or anti-mouse (1:4000) HRP conjugated immunoglobulins. The blots were revealed using 3'-Diaminobenzidine (DAB, Sigma-Aldrich), per the manufacturer's protocol.

Vaccinations

Sixth male BALB/c, mice five-to-seven weeks of age, were randomly located in groups of 10 as follow: Group 1: 25 µg of GnRH/LTB adjuvanted with Montanide ISA 50 V2 (Seppic Adjuvants, France) (50%, v/v); Group 2: Bopriva® commercial vaccine (Zoetis, New Jersey, USA) using 1/20th of a recommended dose (1 ml cattle dose with 400 µg, mice dose ~80 µg); Group 3: a control group with 25 µg of GnRH/LTB suspended in PBS; Group 4: a control group with 25 µg of LTB adjuvanted with Montanide ISA 50 V2 (50%, v/v); Group 5: a control group with 25 µg of LTB suspended in PBS, and Group 6: a control group inoculated with only PBS. Mice were inoculated intramuscularly (i.m.) with 200 µl, two times at an interval of 14 days with except the commercial vaccine group, inoculated subcutaneously (s.c.) due to adverse reactions showed in animals that received the vaccine intramuscularly (data not shown). Blood samples were collected every 7 days through submandibular puncture until day 42 and a final blood collection was performed on day 126. Sera were separated by centrifugation at 3,000 x *g* for 7 min, then pooled and stored at -20 ºC for further analysis.

IgG dynamic against GnRH/LTB

Indirect ELISAs were performed with individually group serum from each collection. Plates were coated overnight at 4 ºC with 0.2 µg of purified GnRH/LTB per well. Serum samples were diluted 1:200, and HRP-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) was used for detection.

Reactions were visualized with ortho-phenylenodiamine (OPD, Sigma-Aldrich) stopped with 2 N H_2SO_4, and analyzed at OD 492 nm using an ELISA MR 700 Microplate Reader spectrophotometer (Dynatech Laboratories, Germany). To determine the IgG isotype pool serum from the groups 1 and 2 were used. Briefly, plates were coated overnight at 4 ºC with 0.2 µg of purified GnRH/LTB, and pooled sera were diluted 1:200. An ELISA was performed in triplicate according to the instructions of the isotyping kit from Sigma-Aldrich for IgG1, IgG2a and IgG2b.

Effects of GnRH/LTB chimera on the testes

Three animal collections were performed during the experimental period for histological analysis of the testes. For each collection, three animals were removed from each experimental group, and euthanasia was performed. The euthanasia was performed on days 28, 42 and 126 of the study. The testes were surgically removed and processed for histological analysis. The testes were fixed for 12 h in Bouin's solution and were sent to the microscopy laboratory at the Universidade Federal de Rio Grande (FURG), Brazil, where histological analysis was performed. The tissues were dehydrated in ascending concentrations of ethanol, diaphanized in xylene, impregnated and embedded in paraffin Paraplast X-tra® (Sigma-Aldrich, St. Louis, Missouri, USA). Embedded testes were sectioned (5 µm) in a rotary microtome (RM 2255, Leica, Germany). Slides containing tissue sections were deparaffinized in xylene, rehydrated in descending concentrations of ethanol and stained with Mayer's Hematoxylin and Eosin. Photomicrographs for documentation of the testes tissue were taken using a DP-72 digital camera under a BX-51 light microscope at 400× magnification (Olympus, Hamburg, Germany).

Testosterone analysis

Testosterone concentrations were determined using a direct competitive radioimmunoassay. Samples were analyzed by Pasin Clinical Analysis Laboratory (Santa Maria, Brazil). The analyses were validated with pooled serum samples at a dilution of 1:20 and had a detection limit of 12 ng/dl.

Statistical analysis

Data were analyzed using GraphPad Prism 6 (GraphPad Software, La Jolla, California, USA). ELISA and testosterone concentrations were analyzed by two-way ANOVA followed by Tukey: Compare all pairs columns ($P < 0,05$).

Results

Cloning, expression and characterization of GnRH/LTB

The GnRH/LTB sequence was ligated to the pAE expression vector, which is controlled by a bacteriophage T7 RNA polymerase promoter. The pAE expression vector was selected because of the capacity to insert a 6xHis tag in the N-terminal position of the protein. The resulting expression construct, pAE/GnRH/LTB, was used to transform *Escherichia coli* BL21 Star™ (DE3) cells.

After expression, cells recovery and protein purification, SDS-PAGE was performed and showed a band of approximately 25 kDa (Figure 1A), which suggest the GnRH/LTB expression. Recombinant GnRH/LTB proteins were detected by Western blotting with rabbit polyclonal anti-GnRH and mouse monoclonal anti-CT antibodies (Figure 1B). Bands with approximately 25 kDa of molecular weight and others of different weight were observed. Bands with approximately 25 kDa of molecular weight and others of different weight were observed. Couriously, could be observed bands with different molecular weights on the SDS-PAGE, and some of these bands was identified in the Western blot as well, using the rabbit polyclonal anti-GnRH sera and mouse monoclonal anti-CT antibodies, suggesting the formation oligomers by the recombinant chimera.

IgG dynamic against LTB/GnRH

Antibodies level was evaluated by ELISA and the reactivity against GnRH/LTB are expressed as the absorbance means at 492 nm ± standard deviation. Mice vaccinated with GnRH/LTB-Montanide showed antibodies after the prime vaccination (day 14), the level has increased after the boost and reaching the highest response (1.44 ± 0.08) at day 42, keeping high titre up to 126 days (Figure 2A). Commercial vaccine group had also show humoral response with significant antibodies at day 42th and 126th. The group vaccinated with GnRH/LTB without adjuvant have not presented significant antibody

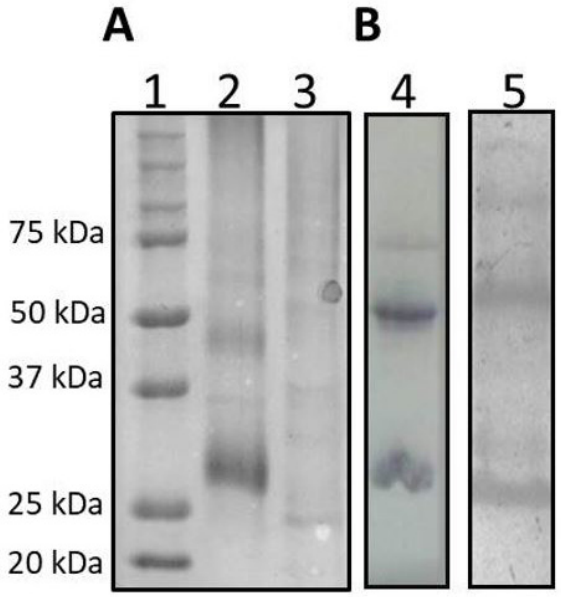

Figure 1. Expression and characterization of GnRH/LTB. (A) Analyzed by 10% SDS-PAGE and Coomassie staining. Lane 1: molecular mass marker (kDa); Lane 2: Expression of GnRH/LTB in *E. coli*; 3. Expression of LTB in an *E. coli*; (B) Immunoblot analysis of GnRH/LTB. Lane 4: anti-GnRH 1:400 sera; Lane 5: anti-CT MAb 1:5000 sera.

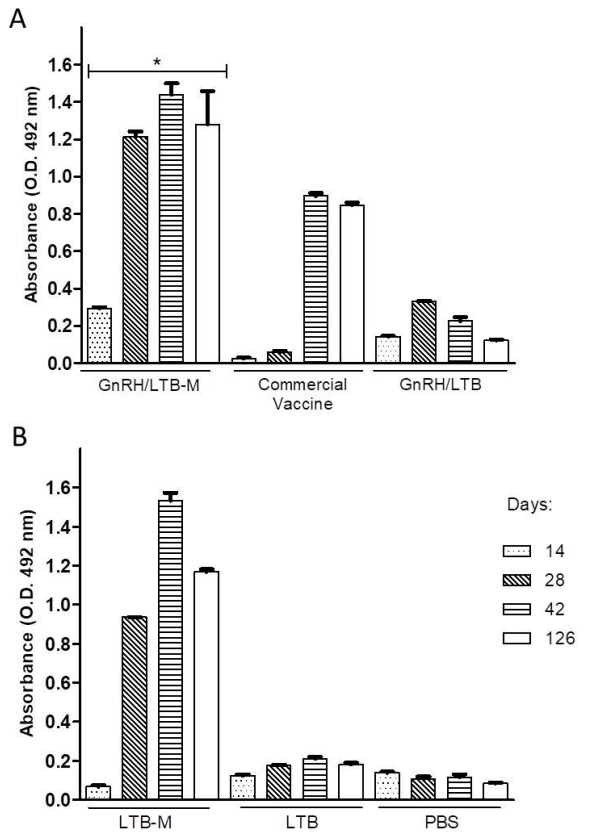

Figure 2. Total IgG dynamics. (A) The data represent the means (+/- standard error of the mean) of the absorbance of total sera IgG antibodies against GnRH/LTB from mice vaccinated with GnRH/LTB-Montanide (GnRH/LTB-M), and commercial vaccine, GnRH/LTB (A), and LTB-Montanide (LTB-M), LTB and PBS (B). Days 14, 28, 42 and 126 individually serum samples were characterized at a single serum dilution (1:200) with anti-mouse IgG secondary antibodies. The mean optical density ($OD_{492\ nm}$) ± standard deviation (bars) from duplicates test is shown. Statistically significance was determined by two-way ANOVA (Tukey multiple comparison) analysis, the presence of asterisk (*) indicates a significant difference ($P < 0.05$) between the groups.

level, but was statistically ($P < 0.05$) superior than the control (PBS) group at day 28th and 42th. Mice group vaccinated with LTB-Montanide showed significant antibodies level against GnRH/LTB, but not the group vaccinated with LTB without adjuvant (Figure 2B).

The IgG isotype profile was characterized with the presence of IgG1, IgG2a, and IgG2b in GnRH/LTB-Montanide and commercial vaccine groups. Both groups showed similar pattern, but with different titers. All isotypes were detected after the first dose and increased after the boost (Figure 3). The GnRH/LTB-Montanide vaccinated group showed similar levels of IgG1, 2a and 2b isotypes, except in the analysis made after a single vaccine dose (day 14), in which the IgG1 level was higher than the other isotypes. Also, the GnRH/LTB-Montanide group showed a statistically significant difference ($P < 0.05$) by the day 14th and 126th in the IgG1 titer comparing with the commercial vaccine group (Figure 3A). The IgG2a titer were significant higher ($P < 0.05$), at every time point evaluated, comparing with the commercial vaccine group (Figure 3B). With the same observation for IgG2b (Figure 3C). The commercial vaccine group presented a similar pattern for the isotypes during the experimental period, with higher IgG1 levels (days 28 and 42) when compared to the others IgG2a and 2b. Noteworthy, that all isotypes titer drop significantly at day 126 of the experiment.

Effects of GnRH/LTB chimera on the testes

Gonadal histologic examinations were conducted on days 28, 42 and 126 after the primary vaccination. The control groups (LTB-Montanide and PBS), tissues showed normal seminiferous tubules with spermatogenic cells in all developmental stages, which included spermatogonia, primary spermatocytes, spermatids and spermatozoa (Figures 4G, 4H, 4I, 4J, 4K, 4L). The GnRH/LTB groups showed visible and well-distributed tissue alterations with the most severe injuries at the end of the experiment, in the adjuvant group. These groups had atrophy of all seminiferous tubules, an absence of spermatogenesis and a large reduction in the number of Leydig cells (Figures 4A, 4B, 4C, 4D, 4E, 4F).

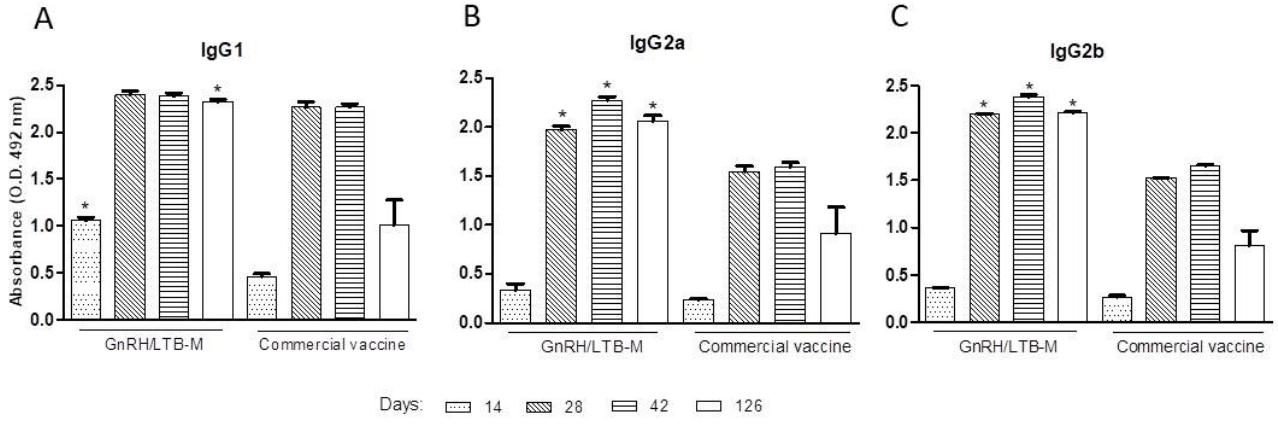

Figure 3. Characterization of the IgG subclass profiles. IgG1 (A), IgG2a (B), and IgG2b (C) levels induced by vaccination with GnRH/LTB-Montanide (GnRH/LTB-M) and Commercial vaccine. Days 14, 28, 42 and 126 pooled serum samples were characterized at a single serum dilution (1:200) with anti-mouse IgG1, IgG2a or IgG2b conjugates. The mean optical density ($OD_{492\ nm}$) ± standard deviation (bars) from duplicates test is shown. Statistically significance was determined by two-way ANOVA (Tukey multiple comparison) analysis, the presence of asterisk (*) indicates a significant difference ($P < 0.05$) between the groups.

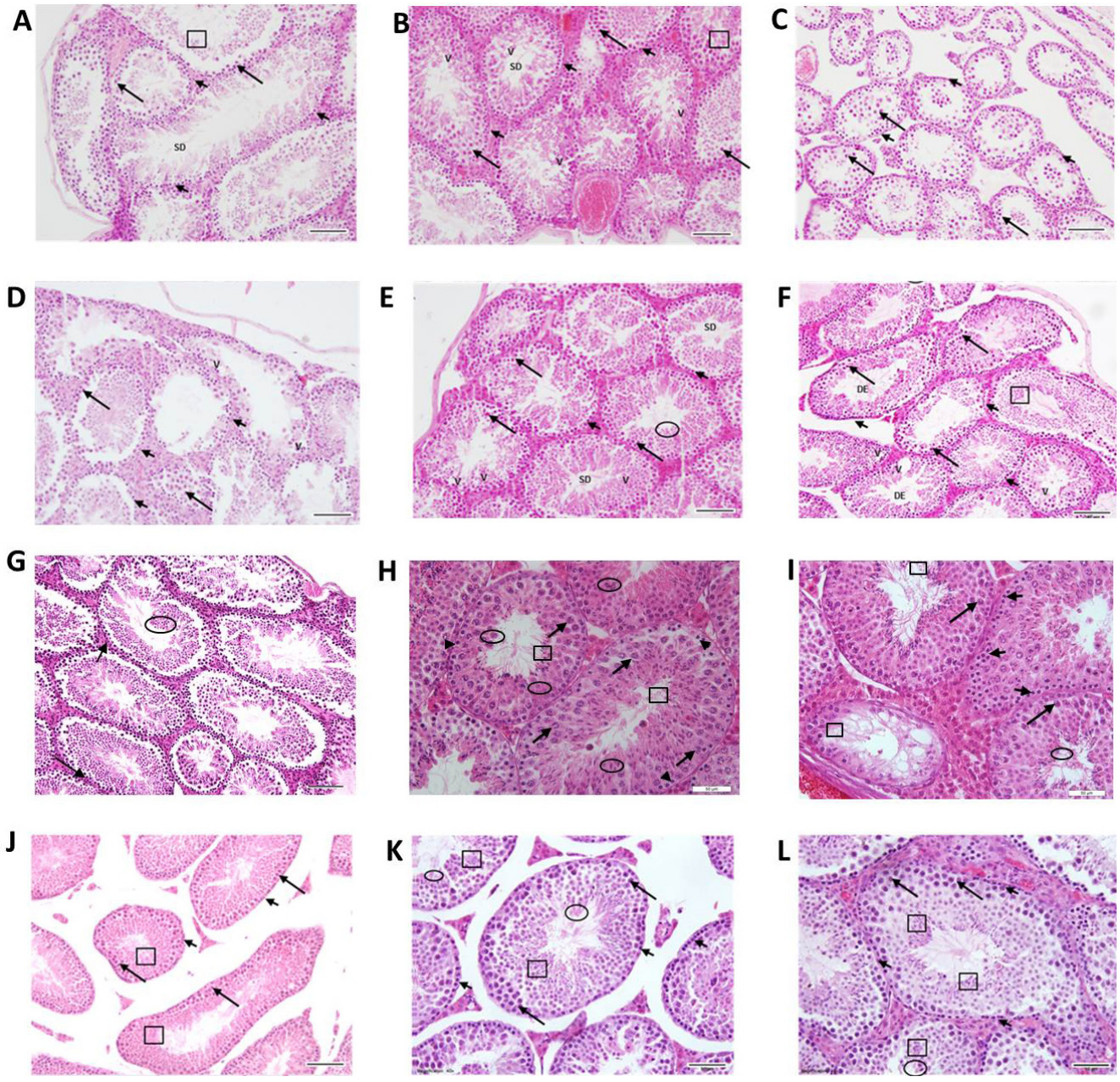

Figure 4. Effects of GnRH/LTB chimera on the testes. Gonadal histology from the GnRH/LTB-M group (A, B and C), the GnRH/LTB group (D, E and F), the LTB-M group (G, H and I) and the PBS group (J, K and L). The first column shows testes sections from day 28, the second column from day 42, and the third column from day 126. Cells in spermatogenesis are shown as spermatogonia (arrows head), primary spermatocyte (arrows), spermatid (ellipse), and spermatozoa (square). V = vesicles. SD = spermatozoa degeneration. Scale bar = 50 µm (H, I, K, L) and 100 µm (A, B, C, D, E, F, G). Testicular tissues were stained by Hematoxylin and Eosin stain.

Testosterone analysis

The mice from the group's GnRH/LTB-Montanide, GnRH/LTB, and commercial vaccine showed lower testosterone level than the control group (Table 1). GnRH/LTB groups had testosterone level on days 14 e 28, significantly lower ($P < 0.05$) than the control group (PBS). We evaluated only the days 14 and 28 since the mice number was bigger (n=9), and we observed a huge variation among the mice in the other time points.

Discussion

The major challenge in the development of an anti-GnRH immunocontraceptive vaccine is the generation of an effective immune response against GnRH. Active immunization against the GnRH molecule results in the production of antibodies able to neutralize the peptide, leading to inhibition of the synthesis and release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Khan et al., 2007). Over the past four decades, several attempts have been made

Table 1. Testosterone concentrations in male BALB/c mice. All data are presented as concentration means (ng/dl) ± S.D. Days 14 and 28 had *n* = 9.

	Day 14 (ng/dl)	**Day 28** (ng/dl)
LTB/GnRH-M	136 (± 175.36) [a]	< 12 (± 0) [a]
Commercial vaccine	153 (±199.40) [a]	168 (± 220.61) [a]
LTB/GnRH	403 (± 86. 26)[a]	131 (± 168.29) [a]
PBS	821.5 (± 122.32)	872 (± 104.65)

[a]Indicate the groups that showed statistically significant differences ($P < 0.05$) when compared with the control group.

to generate anti-GnRH immunocontraceptive vaccines. Most anti-GnRH vaccines are based on the incorporation of a carrier protein to the target molecule; however, reports have shown that the presence of certain carrier molecules leads to the suppression of the antibody response to self-antigens, such as GnRH (Sad et al., 1991). In the present study, the development of a chimeric protein composed by the fusion of a single GnRH molecule with LTB as a carrier molecule was effective to induce antibodies against GnRH, able to modify the gonodal tissue, and modulate the levels of testosterone in male BALB/c mice.

The antibodies level showed that vaccination with GnRH/LTB adjuvanted with Montanide were able to modulate the production of antibodies with just one dose (14th), and after the boost, a significant ($P < 0.05$) increase in anti-GnRH-IgG titer at 28th, 42th, and 126th days of study. Being those anti-GnRH-IgG titers significantly ($P < 0.05$) higher than the commercial vaccine. The commercial vaccine needed the second dose to induced a significant ($P < 0.05$) increase in anti-GnRH-IgG titer (Figure 2). Since we used 1/20th of the recommended commercial vaccine dose for cattle (1 ml, 400 µg) to vaccinate the mice, we assumed that we vaccinated the mice with ~80 µg (200 µl), however we did not quantify the amount of GnRH in this dose. Also, an important point to consider is the adjuvant, the commercial vaccine adjuvant is a synthetic water base, so, some of the difference observed between these groups might reflect the adjuvant/antigen (volume/concentration) used in this study. Likewise, one may suggest that the use of chimeric GnRH/LTB as ELISA antigen to evaluate vaccinal response, might not reflect properly the anti-GnRH-antibodies produced by the commercial vaccine, since the vaccinal antigen may presents different epitopes.

Hsu et al. (2000) reported that using the enterotoxin A from *Pseudomonas* fused with GnRH did not elicited antibodies against GnRH, even using multiple GnRH copies in his chimera. These results were quite different from ours, since we observed significantly higher antibodies against GnRH. One can speculated that the carrier protein used in both studies were different, *Pseudomonas* Exotoxin A vs. *E. coli* LTB, and may play an important role in the immune response, also this point can be stressed by the fact that our chimera has only one GnRH molecule, whereas Hsu et al., (2000) has many.

Remarkable, that the LTB adjuvanted with Montanide group showed a similar total IgG dynamic as the GnRH/LTB-Montanide group (Figure 2), suggesting an important role for the LTB portion of our chimera. However, we did not observe significant IgG titers in the LTB alone group, giving the impression that the LTB concentrations used needs an adjuvant to exercise its immunomodulation.

The nature of an immune response to a defined antigen is established by several factors such as the use of adjuvants, the antigen concentration, the administration route and the host genetics (Ferro & Stimson 1998). One alternative to assess the immune profile induced is to determine the IgG subclasses. In mice, the IgG1 subclass is related to a Th2 immune response, while the IgG2a subclass is more related towards a Th1 immune response (Jegerlehner et al., 2007). Although the LTB mechanisms of action remain unclear, it has been demonstrated that its activity stems from its capacity to bind to GM1 receptors. These ganglioside receptors are ubiquitously distributed through mammalian cells and are recognized as the main receptors for the B subunits of heat-labile proteins from *E. coli* and *V. cholerae* (Williams, 2000). LTB plays a key role by promoting antigen uptake and presentation by dendritic cells, macrophages, and B cells. The use of LTB as adjuvant in subunit vaccines has been an efficient option to overcome the poor immunogenicity of many recombinant subunit vaccines (Wilson-Welder et al., 2009). It was demonstrated that LTB administration was able to modulate the immune response against herpes simplex virus type 1 antigens, leading to a prevalent Th2 response instead of a Th1 response (Richards et al., 2001). As well, it was also demonstrated that, after the parenteral administration of a chimeric antigen containing the R1 region of *Mycoplasma hyopneumoniae* P97 adhesin fused to LTB, there was a significant prevalence of IgG1 antibodies than IgG2a antibodies, suggesting a modulation towards a Th2 immune response (Conceição et al., 2006). These studies suggest that the immune response induced by the parenteral administration of heterologous antigens fused or in association with LTB leads to an induction of an IgG profile towards a Th2 response. Although the LTB mechanisms of action remain unclear, it has been demonstrated that its activity stems from its capacity to bind to GM1 receptors. These ganglioside receptors are ubiquitously distributed through mammalian cells and are recognized as the main receptors for the B subunits of heat-labile proteins from *E. coli* and *V. cholerae* (Williams, 2000). In addition, LTB has shown that it is capable of enhancing both the associated cytokine response of Th1, Th2 and Th17 when fused with different antigens (Zhou et al., 2009).

Additionally, to the LTB role in the vaccine immune response, Montanide also might have a significant role as an adjuvant in the GnRH immune response. The chimeric formulation using Montanide ISA50V2 was able to maintain a significant increase ($P < 0.05$) in the antibody level after the second boost at the 28th day, and maintaining up to 126 days. Our group, studying the immune response of a recombinant glycoprotein gD (rgD5) of Bovine Herpes virus (BoHV-5) adjuvanted with Montanide ISA50V2, in a mice model, showed similar results, where higher antibodies titers were obtained at day 28th and maintaining these high levels up to the end of the experiment (Dummer et al., 2014). Pinheiro et al. (2018), studying the role of different adjuvants in the immune response to a *Neospora caninum* recombinant protein (rNCSRS2), reported very similar results, with a significant vaccinal response after the boost, and an IgG1/IgG2 profile similar to this study, corroborating with a

mixTh1/Th2 modulation of Montanide ISA50V2. In the present study we observed that the isotypes IgG1, IgG2a, IgG2b were detected by day 14th in both groups, but higher levels were found in the GnRH/LTB- Montanide group, remaining higher than the commercial vaccine until day 126th.

Route of vaccine administration plays an important role in the development of immune response because antigen administered via different anatomical sites interacts with diverse subsets of antigen presenting cells. The intramuscular antigen administration is one of the most common routes of vaccine injection and induces specific systemic immunity by increasing the specific antibody titer and activating the cellular immunity (Tanghe et al., 2000; Hunter, 2008; Chauhan et al., 2019). The subcutaneous administration is another route used for vaccination.One characteristic of subcutaneous immune response is the presence of dermal dendritic cells, such as Langerhans cells, which are capable of quick antigen uptake even in low amounts.Moreover, dermal keratinocytes are able to produce inflammatory cytokines and recall APCs to the site. This feature renders the subcutaneous route more efficient when compared to intramuscular route in up-taking low doses of antigen (Chauhan et al., 2019; Smith, 2015; Higgins, 2004). The commercial vaccine group was inoculated subcutaneously, while GnRH/LTB was administrated by intramuscularly route, not allowing a good comparison between them.

The effect of immunization on testosterone levels was not easily accessed in some points in our study. Due to testosterone's pulsatile release pattern, the hormone difference between the immunocastrated groups and the control group was significant during the experiment, also the mice number became reduced by the time point studied. All immunocastrated groups showed statistically significant decreases in testosterone at the days 14th and 28th of the experiment, which were correlated with the histological alterations observed in the gonads. However, the testosterone level analyses showed limitations since the significant standard deviation found in some samples. These observations corroborates with Khan et al., 2007, were they reported that was not possible to determine difference in the levels of testosterone in mice immunized with GnRH-I, GnRH-II and GnRH-III antigens fused with tetanus toxoid (Khan et al., 2007).

Few studies were able to demonstrate an anti-GnRH immune response and gonadal regression in vaccines without Freund's adjuvants (Ferro et al., 2004; Conforti et al., 2008). The authors suggested that this response was due the use of phosphate buffer rather than an emulsion base. Thus far, in our trials, we observed a uniform gonadal regression in the GnRH/LTB-Montanide group and also in the GnRH/LTB group without adjuvant. The histological results for the GnRH/LTB alone was quite intriguing, since the IgG antibodies against GnRH/LTB were relatively low, but able to induce gonodal alterations (Figure 4). This finding suggests that there are no correlations between anti-GnRH/LTB antibodies and gonodal alterations, so this observation needs to be better clarified in a near future.

The GnRH/LTB-Montanide group showed marked gonadal alterations among all groups. At day 126, all animals in the GnRH/LTB-Montanide group had uniform gonadal atrophy, disruption of the interstitial tissue and Leydig cell absence in all the tissues. It was also reported similar results were reported with a modified GnRH peptide fused to tetanus toxin emulsified with different adjuvants (Ferro et al., 2004). These results suggested that the association with adjuvants that permit slow antigen release and prolonged induction of neutralizing antibodies is extremely important for a successful immunocastration. In our study, it was possible to observe disruption in the interstitial tissue and a reduction in the Leydig cells in tissues from GnRH/LTB immunized animals (Figure 4).

We were able to show that a vaccine, containing as an antigen the GnRH molecule fused to the *Escherichia coli* LTB as a carrier adjuvanted with Montanide, are capable of inducing the humoral immune response, blocking endocrine functions related to reproduction, and causing tissue alterations in the gonads of male mice. These results suggest that the chimeric GnRH/LTB present potential to be used for immunocontraception. Further studies need to be done to evaluate it immunocontraception effect on livestock animals.

Author contributions

LBE, REAP, PMMA, CDC performed the experiments; NLC, RCC analyzed the data and wrote the manuscript; FPLL designed the study, analyzed the data and wrote the manuscript.

Conflict of interests

The authors declare that they have no conflict of interest.

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