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Displaying of GnRH Peptides on Bacteriophage T7 and Its Immunogenicity in Mice Model

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***Abstract*—**T7 phage could be used as a perfect vector for peptides expression and haptens presentation. T7-3GnRH recombinant phage was constructed by inserting three copies of Gonadotrophin Releasing Hormone (GnRH) gene into the multiple cloning site of T7 Select 415-1b phage genome. The positive T7-3GnRH phage was selected by using polymerase chain reaction amplification, and the p10B-3GnRH fusion protein was verified by SDS-PAGE and Western-blotting assay. T7-3GnRH vaccine was made and immunized with 10^{10} pfu in 0.2 ml per dose in mice. Blood samples were collected at an interval in weeks, and anti-GnRH antibody and testosterone concentrations were detected by ELISA and radioimmunoassay, respectively. The results show that T7-3GnRH phage particles confer a high immunogenicity to the GnRH-derived epitope. Moreover, the T7-3GnRH vaccine induced higher level of anti-GnRH antibody than ImproVac®. However, the testosterone concentrations in both immunized groups were at a similar level, and the testis developments were significantly inhibited compared to controls. These findings demonstrated that the anti-GnRH antibody could neutralize the endogenous GnRH to down regulate testosterone level and limit testis development, highlighting the potential value of T7-3GnRH in the immunocastration vaccine research.

***Keywords*—**Gonadotrophin releasing hormone, GnRH, immunocastration, T7 phage, phage vaccine.

I. Introduction

THE growing importance of animal welfare and realizing the inhumane of surgical castration of male pigs have encouraged the seek for more friendly treatments. GnRH has been studied for developing immunocontraceptive vaccines in various mammalian species [1]. Moreover, immunization against GnRH not only inhibits reproductive functions in boars, but also keeps the unpleasant boar taint under control [2]. In past decades, a number of synthetic peptides and genetic engineering vaccines against GnRH have been developed for immunocastration. Immunizing boars against a synthetic analogue of GnRH coupled to a large carrier protein (ImproVac® vaccine) and results in production of antibodies against GnRH, with testis regression and reduction in synthesis and accumulation of steroid hormones [3], [4].

The GnRH is an incomplete antigen due to small molecular mass (10 amino acids), with a very weak immunogenicity [5]. Hence, it needs to couple to a larger carrier molecule to generate strong immune response. The lytic T7 bacteriophage has been extensively studied and engineered to display heterologous peptides (up to 50 amino acids) as a C-terminal fusion protein of 10B capsid protein. T7 phage nanoparticles have been exploited previously for display of peptides, B cell epitopes, and an immuno-dominant region antigen for vaccination purposes [6], [7]. However, there is very little information about using the T7 phage as a large carrier and expression vector to improve the immunogenicity of GnRH peptide vaccine.

In this study, we tested the hypothesis that T7 phage can be used as an effective carrier vector for developing GnRH peptide immunocastration vaccine when relative molecular mass of the aimed antigens is small. The immunocastration effect of T7-GnRH vaccine in mice was evaluated in terms of identifying a specific anti-GnRH antibody response and assessing its effect on serum testosterone levels, testicular weight, and tissue damage.

II. Materials and Methods

A. Cloning of 3GnRH Gene into T7 Select 415-1b Genome

The oligonucleotide sequence encoding three copies of GnRH with a glycine- glycine- glycine- serine (GGGS) linker was artificial synthesized, then cloned into T7 select 415-1b *EcoRI / HindIII* double-digested genomic arms. Recombinant T7 phage genomic DNA was packaged by package protein. T7-3GnRH phage was rescued, and then selected by PCR amplification (T7 Select® system manual).

B. Large-Scale Cultivation and Purification of T7-3GnRH Phage

The T7-3GnRH phage was propagated in *Escherichia coli* strain BL21. Bacteria were infected with the phage nanoparticles at a multiplicity of infection (MOI) of 0.001, and incubated by vigorous shaking at 37 ℃ more than 3h until complete lysis of cells was observed. The lysate was centrifuged for 15 min at 6000 rpm to separate the bacterial debris from the T7-3GnRH phage nanoparticles. The supernatant was tenfold concentrated by ultra-filtration and then centrifuged for 15 min at 12000 rpm and 25 ℃. The resuspended T7-3GnRH phages were extracted with 0.1% Triton-114 to remove endotoxin and with an equal volume of chloroform to remove bacterial debris.

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C. Protein Identification and Vaccine Preparation

T7-3GnRH phage proteins were analyzed on SDS-10% polyacrylamide gel electrophoresis (PAGE) and Western-blotting assay. The purified T7-3GnRH (10^{11} pfu/ml) was emulsified with Montanide ISA206 (54:46, V/V) (Seppic, France) to form a water-in-oil-in-water (W/O/W) blend. A commercial vaccine ImproVac® (Pfizer) was bought from veterinary bio-products market to set as control.

D. Immunization of Mice and Evaluation of Immunological Effect

24 male Balb/c mice (six weeks old) were randomly divided into three groups and were used in immunization experiments in accordance with the approval of Animal Use and Care Committee. Mice in group T7-3GnRH were immunized with 10^{10} pfu in 0.2 ml vaccine administered subcutaneously. Positive control mice were immunized with 0.2 ml ImproVac®, while there was no injection for negative control mice. The immunized mice were given a booster injection four weeks later with the same dose. Blood samples were collected via tail vein puncture prior to immunization and every two-week interval after the boost immunization for antibody and testosterone detection. All mice were sacrificed at week 12^{th} after immunization, and the testis was picked for weighing and histological assay.

III. Results

A. Characterization of T7-3GnRH Phage

The recombinant DNA was packaged with T7 extracts and yielded 3.5×10^3 pfu/ml of recombinant phage particles. The positive recombinant phage clone coding the 3GnRH fragment produced a PCR product of approximately 250 bp (Fig. 1 (A)), and was verified by DNA sequencing. A positive recombinant clone was propagated, purified, and analyzed on SDS-10% polyacrylamide gel (Fig. 1 (B)). The recombinant protein of about 43 kDa was detected by the T7·Tag® Antibody HRP Conjugate (Navagen) in Western blot analysis (Fig. 1 (C)). This is consistent with the calculated molecular mass of expected 10B-3GnRH (43 kDa).

B. Evaluation of Anti-GnRH Antibody

Artificial synthesized GnRH peptides were chemical conjugated with BSA protein, and then coated ELISA plate wells for antibody detection. Mice serum was diluted 1:20. T7-3GnRH vaccine induce anti-GnRH antibody faster and higher than ImproVac® (Fig. 2). Antibody reaches the highest level two weeks after booster, and gradually decreases until 12-week post immunization. Negative control mice have bottom level of antibody.

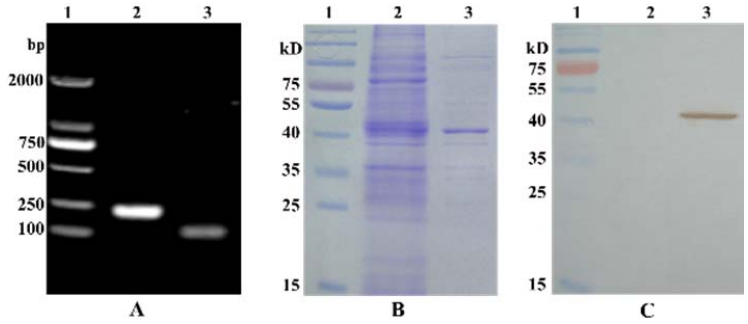


Fig. 1 Identification of recombinant phage: (A). PCR identification of recombinant phage. Line 1, DL2000 marker; Line 2, T7-3GnRH phage; Line 3, T7 wide type phage. (B) SDS-PAGE on 12% polyacrylamide gel. Line1, Protein marker; Line 2, BL21 host cell lysate; Line 3, Purified T7-3GnRH phage. (C) Western blotting assay. Line1, protein marker; Lane 2, BL21 host cell lysate; Lane 3, Purified T7-3GnRH phage

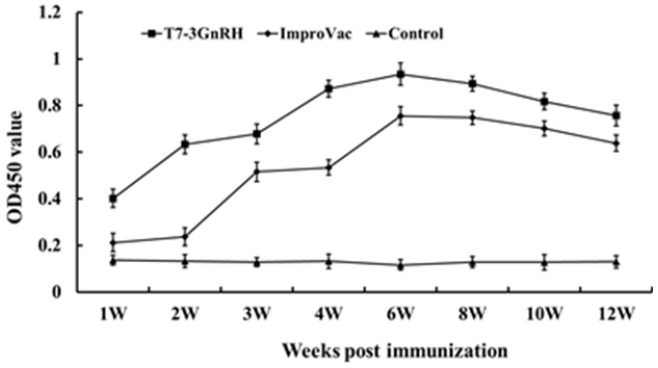


Fig. 2 ELISA antibody titer against GnRH. Artificial synthesized GnRH peptides were conjugated with BSA, and then coated ELISA plate wells to detect the anti-GnRH antibody. Serum was diluted 1:20. Means ± SE are shown

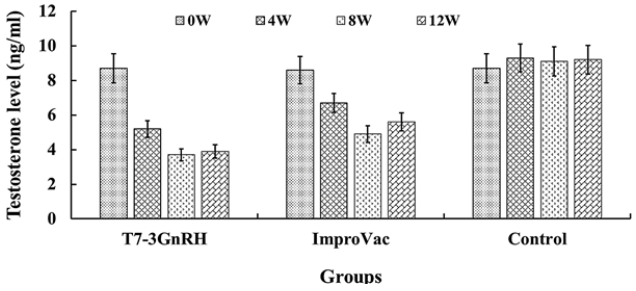


Fig. 3 Testosterone levels in each group after vaccination. Testosterone was detected radio immunosorbent assay. Means ± SE are shown

C. Evaluation of Testosterone Level and Testis Index

The plasma samples were entrusted to professional organization who can operate radioimmunoassay. The difference of testosterone concentration was found between

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the vaccinated mice (Group T7-3GnRH, ImproVac®) and the controls after primary immunization (Fig. 3). Also, the difference was obviously between T7-3GnRH and ImproVac® after week 4 ($p<0.05$). At necropsy, immunized mice were found significant testis poor development.

The testis weight in group T7-3GnRH and ImproVac® were lower than in control group, but on the contrary the, body weight was a little higher than the controls (Table I).

D. Microscopic Evaluation of Testis

The seminiferous tubules in vaccinated mice contained low densities of spermatogonium, spermatocytes, spermatids, and spermatozoa in the seminiferous tubules compared to control group (Fig. 4).

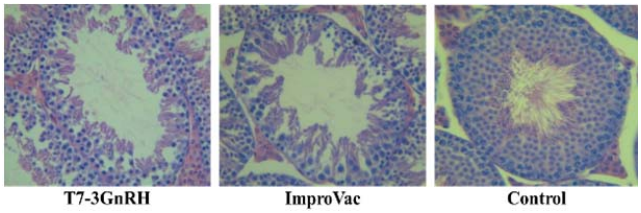


Fig. 4 Microscopic evaluation of mice testes. Mice immunized with T7-3GnRH and ImproVac® showed suppression of spermatogenesis in the lumen of the seminiferous tubules characterized by the reduction of spermatogonium, spermatocytes, spermatids and spermatozoa concentration compared to control

TABLE I
TESTIS INDEX OF EACH GROUP

Groups	Body Weight(g)	Testis Weight(g)	Testis Index
T7-3GRH	43.10±5.19a	0.038±0.032c	0.088±0.073c
ImproVac®	43.07±4.60a	0.065±0.034b	0.150±0.072b
Control	41.16±2.80b	0.138±0.033a	0.335±0.065a

All mice were sacrificed at 12th week after immunization, and the testis was picked out for weighing. Testis Index: (body weight/testis weight) ×100%. a, b, c: differences were considered significant.

IV. DISCUSSION

In China, the surgical castration of male piglets is commonly performed at pig farm to improve growth performance as well as reducing boar taint in meat. However, most surgical castration causes pain and increases a risk of microbial infection. Therefore, it is recommended that surgical castration is replaced by animal welfare-based methods. The most promising approach is use of an immunocastration vaccine composed of GnRH, similar products such as ImproVac®, GonaCon™ have been launched [8]-[11]. Since China has a huge swine population, the seek for alternative castration methods is an urgent issue.

Previous studies have shown that the immunization with multiple repeat copies of GnRH was more effective than using single unit of GnRH [12], [13]. Therefore, in our study three copies of GnRH coding gene was synthesized for surface displaying. The GnRH sequences were designed based on the published data, and modified to increase antigenic responses [14]. In mice, the T7-3GnRH vaccine stimulated specific anti-GnRH antibodies, indicating that displayed of peptides on phage particles was appropriate for recognition by immune system.

In our study, the T7-3GnRH vaccine had no side effects on the injection sites or general health of mice. The anti-GnRH antibody response was detected after the primary immunization. Because the lack of background information of ImproVac®, it is difficult to compare the accurate content of GnRH peptides in two vaccines. Although, the anti-GnRH antibody and testosterone level in T7-

3GnRH group is better than in group ImproVac®, it could not make a hasty judgement about the vaccine's quality. Moreover, the testis weight in immunized mice was significant lower than in control group at week 12. In addition, histological examination of testis in immunized mice showed densities of spermatogonium, spermatocytes, spermatids and spermatozoa in the seminiferous tubules, demonstrating that immunization with GnRH vaccine suppressed spermatogenesis in mice.

In conclusion, the strategy of T7 phage surface displaying GnRH peptide could make an efficient immunocastration vaccine, and stimulated comparable immune response to ImproVac®. T7-3GnRH recombinant phage could be used as a potent antigen to produce immunocastration vaccine. Further studies still are needed to increase the effectiveness and safety in a long perspective.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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