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Induction of immunocastration in pre-pubertal boars immunized with recombinant gonadotropin-releasing hormone conjugated with *Salmonella* Typhimurium flagellin fljB

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Abstract

Immunocastration is an alternative method used to replace surgical castration commonly performed in swine farms. In boars, the main effects of immunocastration are reduction of gonadotropin-releasing hormone (GnRH) and the resulting inhibition of testicular function. The aim of this study was to evaluate immunocastration efficacy in pre-pubertal boars vaccinated with a recombinant GnRH protein conjugated with *Salmonella* Typhimurium flagellin fljB (STF2). A total of 35 boars were assigned to three groups: the untreated group (n = 5), the surgically castrated group (n = 5), and the immunocastrated group (n = 25). Pigs in the immunocastration group were immunized with the GnRH-STF2 vaccine at pre-pubertal ages 4 and 8 weeks. All experimental pigs were kept for 26 weeks before slaughter. Anti-GnRH antibody levels of immunocastrated pigs were significantly higher than those of untreated pigs ($P < 0.001$). In contrast, testosterone levels of immunocastrated pigs were significantly lower than those of untreated pigs ($P < 0.001$). Statistical significances were not found in the body weights and backfat thicknesses of untreated vs. immunocastrated pigs. Weights of the testes and epididymides of immunocastrated pigs were significantly lower than those of untreated pigs ($P < 0.001$). Testicular tissues of immunocastrated pigs were severely suppressed compared with those of untreated pigs. In conclusion, immunization with the STF2-GnRH vaccine effectively induced immunocastration in pre-pubertal boars.

Key Words: GnRH, STF2, immunocastration, vaccine, boars

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Introduction

Surgical castration of male piglets is commonly performed to improve growth performance while reducing boar taint in meats.^{1,3)} However, most surgical castration conducted at pig farms causes pain and carries a risk of microbial infection. Therefore, it is recommended that surgical castration be replaced by animal welfare-based methods.^{15,20)} The most promising alternative method is use of an immunocastration vaccine composed of gonadotropin-releasing hormone (GnRH) that induces anti-GnRH antibodies.

GnRH composed of 10 amino acids plays a critical role in reproductive-system development in boars. GnRH is released by the hypothalamus and allows production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary.¹⁹⁾ FSH and LH regulate gonadal functions in boars. FSH is critical for spermatogenesis in the seminiferous tubules, and LH stimulates the secretion of testosterone from the testis.¹³⁾ Anti-GnRH antibodies produced by vaccination with GnRH neutralize GnRH in boars. Deficiency of GnRH leads to insufficient production of FSH and LH, which eventually induces castration effects in boars.

However, the immunogenicity of GnRH is too low to produce enough antibodies, because it is a peptide composed of 10 amino acids. Therefore, most immunocastration vaccines contain multiple copies of GnRH conjugated with various adjuvants to overcome the low immunogenicity.¹⁸⁾ Recognition of the pathogen-associated molecular patterns (PAMPs) by members of the toll-like receptor (TLR) family is important for the induction of innate and adaptive immune responses. It has been suggested that administration of antigens with the PAMPs would provoke potent antigen-specific immune responses in mammals. Bacterial flagellin, a ligand of TLR5, functions as an adjuvant and enhances immunogenicity of antigens.^{2,24)} A new immunocastration vaccine composed of multiple

copies of GnRH conjugated with *Salmonella* Typhimurium flagellin fljB (STF2), the STF-GnRH vaccine, was developed and showed very efficient immunocastration effects in male rats.²³⁾

In boars, immunocastration vaccines composed of GnRH induce meat-quality improvement and growth performance comparable to the effect of surgical castration.⁷⁾ Administration of immunocastration vaccines at 8 and 4 weeks before slaughter (around ages 16 and 20 weeks) was recommended as a standard vaccination method in the initial studies.⁷⁾ However, subsequent studies demonstrated that early vaccination of boars, at ages 10 and 14 weeks, produced more promising castration effects than the standard method.^{5,9)}

Currently, the commercialized immunocastration vaccine Improvac[®] is administered to boars at ages 8 and 12 weeks. When rams were immunized with GnRH vaccine at pre-pubertal ages (3–4 weeks), efficient castration effects were observed for 23–24 weeks.⁴⁾ In boars, the anti-GnRH antibodies were sustained for up to 22 weeks after the second vaccination.²⁶⁾ Pubertal development of boars begins at about 4 weeks of age.¹¹⁾ Therefore, we hypothesized that the newly developed STF-GnRH vaccine could be administered to pre-pubertal boars to maintain immunocastration effects until the fattening period.

In this study, for the first time, we demonstrated immunocastration efficacy in pre-pubertal boars immunized with the STF2-GnRH vaccine.

Materials and methods

Expression of Recombinant STF2-GnRH Protein

The recombinant STF2-GnRH protein was produced as previously described.²³⁾ Briefly, the STF2 gene was amplified from *S. Typhimurium* with specific primers. The STF2 gene was ligated with six tandem copies of a swine GnRH gene. The fused STF2-GnRH gene was cloned into a pQE-40 protein expression vector (Qiagen, USA).

The recombinant STF2-GnRH protein was expressed in *Escherichia coli*. The recombinant protein containing six histidine residues was purified by nickel-nitrilotriacetic acid metal-affinity chromatography following the manufacturer's instructions (Qiagen, USA).

Animals, Sampling, and Experimental Design

Animal experiments to evaluate the effect of STF2-GnRH vaccine in boars were approved by the Institutional Animal Care and Use Committee of Konkuk University (IACUC, KU-13164). All animals were kept at a domestic pig farm located in Pocheon, Gyeonggi-do, Korea. A total of 35 commercial crossbred post-weaned male piglets (Large Yorkshire, Danish Landrace, and Duroc) under ad libitum feeding conditions were divided into three groups: an untreated group ($n = 5$), a surgically castrated group ($n = 5$), and the immunocastration (vaccination) group ($n = 25$). The immunocastration group was vaccinated with 3 mg of STF2-GnRH mixed with oil adjuvant IMS 1313 (Seppic, Paris, France) in a 7:3 ratio. Surgical castration and vaccination with the STF2-GnRH were performed at age 3 and 4 weeks, respectively. The second injection of immunocastration vaccine was given at 8 weeks. Jugular-vein blood samples from all groups were collected prior to vaccination at ages 4 and 8 weeks and again at age 26 weeks, before slaughter. Serum samples from blood collected in heparinized Vacutainer tubes (Becton, Dickinson and Co., NJ, USA) were separated by centrifugation and stored at -80°C prior to analysis. When the pigs reached age 26 weeks, they were taken to the slaughterhouse, where the animals' testes and epididymides were removed and weighed as pairs.

Measurement of Anti-GnRH Antibodies and Testosterone Levels

The antibody titers to GnRH in the serum samples were determined using an enzyme-linked immunosorbent assay (ELISA) as previously described.²⁰⁾ Briefly, each well of a microplate was

coated with 100 μL of keyhole limpet hemocyanin (KLH)-conjugated GnRH (10 $\mu\text{g}/\text{mL}$) (Peptron, Daejeon, Korea) at 4°C overnight. After being washed with PBST three times, each well was blocked with 100 μL of 5% skim milk in PBST at 37°C for 2 h. After three washes, 100 μL of 40-fold diluted serum samples in 2.5% skim milk in PBST were added to each well of microplate and incubated for 1 h at room temperature. Then, after five washes, horseradish peroxidase (HRP)-conjugated anti-pig IgG (AbD Serotec, Oxford, United Kingdom) diluted 10,000-fold in 2.5% skim milk in PBST was added to the wells and incubated for 1 h at room temperature. Finally, after five washes, color development was performed using tetramethylbenzidine (TMB) for 10 min at room temperature, and the reaction was stopped by adding 0.4 N H_2SO_4 . Optical density (OD) values were determined at 450 nm by using an ELISA reader (Sunrise, Tecan, Mannedorf, Switzerland). The concentrations of testosterone in the serum samples were measured by enzyme immunoassay (EIA) in Neodin Vetlab, Seoul, Korea.

Measurement of Body Weights and Backfat Thickness

The pigs' body weights were measured at ages 4 and 26 weeks. Backfat thickness was measured at age 26 weeks, following slaughter.

Histological Study

Fixation of the testes samples was performed as previously described.²⁰⁾ Briefly, testes were fixed with 10% neutral buffered formalin, decolorized with 70% ethanol, and embedded in paraffin wax. The testicular sections were then stained with hematoxylin and eosin (H&E) and observed using an Olympus BX41 microscope (Olympus, Tokyo, Japan)

Statistical Analysis

The data were expressed as the mean \pm standard deviation (SD). Statistical analyses of antibody titers to GnRH, testosterone levels,

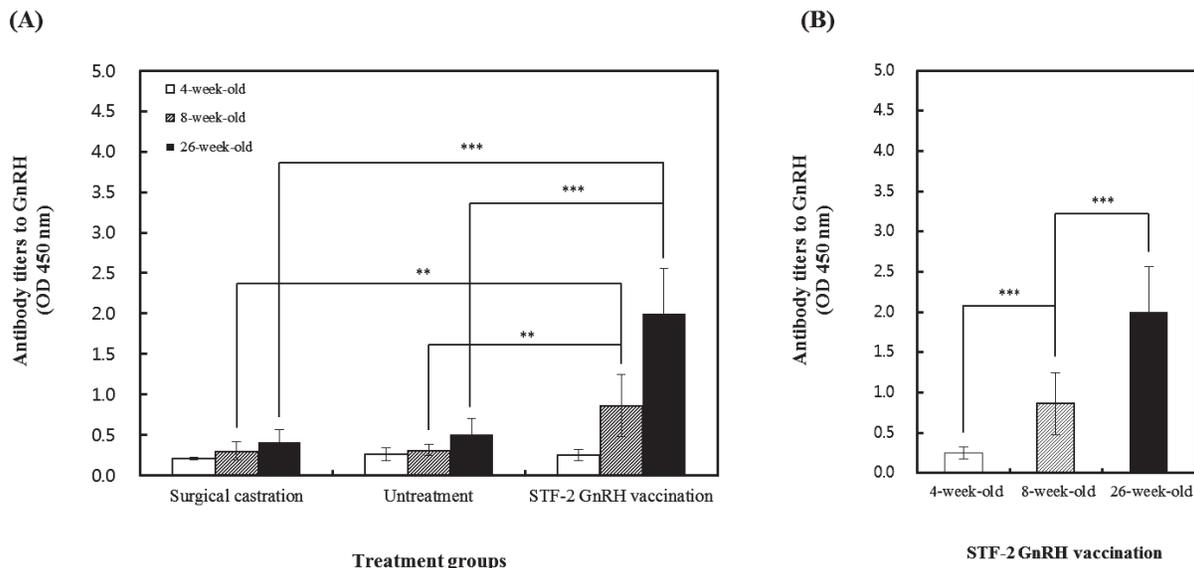


Fig. 1. Anti-GnRH antibody titers in surgically castrated, untreated, and STF2-GnRH vaccinated boars. Anti-GnRH antibody levels in serum samples of boars were determined by ELISA. (A) Statistical differences of anti-GnRH antibody titers were analyzed among the three groups at ages 4, 8, and 26 weeks. (B) Statistical differences of anti-GnRH antibody titers were compared in the vaccinated group between ages. Surgically castrated and untreated groups did not show statistical differences of anti-GnRH antibody titers between ages. ** $P < 0.01$ and *** $P < 0.001$.

body weights, and backfat thicknesses among the three groups of boars (surgically castrated, untreated, and vaccinated with STF2-GnRH) were performed using one-way ANOVA. Anti-GnRH antibody titers and testosterone levels were compared between groups at same age and between different ages within each group by one-way ANOVA. Body weights at ages 4 and 26 weeks and backfat thicknesses at age 26 weeks were also compared between groups by one-way ANOVA. Tukey's multiple-comparisons test was carried out as post hoc test. Statistical analysis for weights of testes was performed using *t* test. The level of statistical significance was set at $P < 0.05$.

Results

Anti-GnRH Antibody Titers

Antibody titers against GnRH and testosterone levels were measured with serum samples collected at ages 4, 8, and 26 weeks. Antibody titers to GnRH were negligible in boars in all three

experimental groups at age 4 weeks (Fig. 1A). Anti-GnRH antibody titers of vaccinated boars were significantly higher than those of surgically castrated and untreated boars at ages 8 ($P < 0.01$) and 26 weeks ($P < 0.001$). However, no statistical differences in anti-GnRH antibodies were found between surgically castrated boars and untreated ones at ages 8 and 26 weeks. In the vaccine group, the first vaccination induced significantly higher anti-GnRH antibodies at age 8 weeks ($P < 0.001$) (Fig. 1B). At age 26 weeks, antibodies against GnRH in the boosted boars were significantly higher than those determined at age 8 weeks ($P < 0.001$). However, there were no significant increases of anti-GnRH antibodies in boars that were surgically castrated and untreated throughout the study period.

Testosterone Levels

Very low concentrations of testosterone were detected in all the study boars at age 4 weeks. The testosterone levels of vaccinated and surgically castrated boars were significantly lower than those of untreated boars at ages 8 and 26 weeks

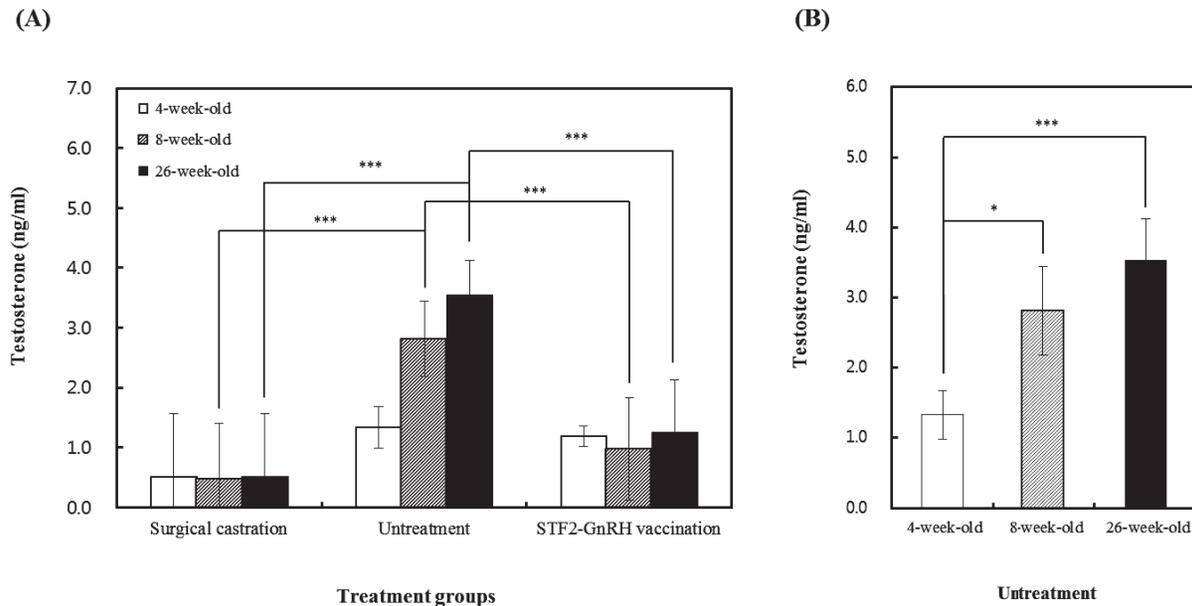


Fig. 2. The concentration of testosterone in serum samples of surgically castrated, untreated, and STF2-GnRH-vaccinated boars. Testosterone levels in serum samples of boars were determined by EIA. (A) Statistical differences of testosterone levels were analyzed among the three groups at ages 4, 8, and 26 weeks. (B) Statistical differences of testosterone levels were analyzed in the untreated group between ages. Surgically castrated and vaccinated groups did not show statistical differences of testosterone levels between ages. * $P < 0.05$ and *** $P < 0.001$.

($P < 0.001$) (Fig. 2A). The testosterone levels of vaccinated boars were not significantly different from those of surgically castrated boars at ages 8 and 26 weeks. In untreated boars, the testosterone levels determined at ages 8 ($P < 0.05$) and 26 ($P < 0.001$) weeks were significantly higher than those determined at age 4 weeks (Fig. 2B). However, when their testosterone levels were compared between 8 and 26 weeks, statistical differences could not be found. The testosterone levels of surgically castrated and vaccinated boars did not increase during the study period.

Body Weight and Thickness of Backfat

The body weights of boars vaccinated with STF2-GnRH, those surgically castrated, and those untreated were 78.9 ± 9.0 , 81.2 ± 6.4 , and 88.4 ± 7.4 kg, respectively, at age 26 weeks (Fig. 3A). The body weights of boars vaccinated with STF2-GnRH were lower than those of boars in the two other groups, but there were no statistical differences among them. Backfat thicknesses at age 26 weeks were 16.9 ± 4.7 ,

21.7 ± 3.8 , and 21.4 ± 1.5 cm in the vaccinated boars, surgically castrated boars, and untreated boars, respectively (Fig. 3B). Even though the backfat thickness of the vaccinated boars seemed to be thinner than those of the surgically castrated and untreated boars, no statistical differences were found.

Weights and Histological Features of Testes

The weights of testes of all the pigs were measured after removal of the outer membrane and skin. Immunocastrated boars had significantly lower weights of testes (228.9 ± 43.7 g) than untreated boars (402.4 ± 50.1 g) ($P < 0.001$) (Fig. 4). Histologically, the testicular tissues of the untreated boars had evenly distributed spermatogenic cells that become spermatozoa throughout the developmental process of spermatogonia, spermatocytes, and spermatids (Fig. 5A). They also showed properly developed seminiferous tubules and abundant Leydig cells (Fig. 5C). However, the testicular tissues of the STF2-GnRH- vaccinated boars had almost empty

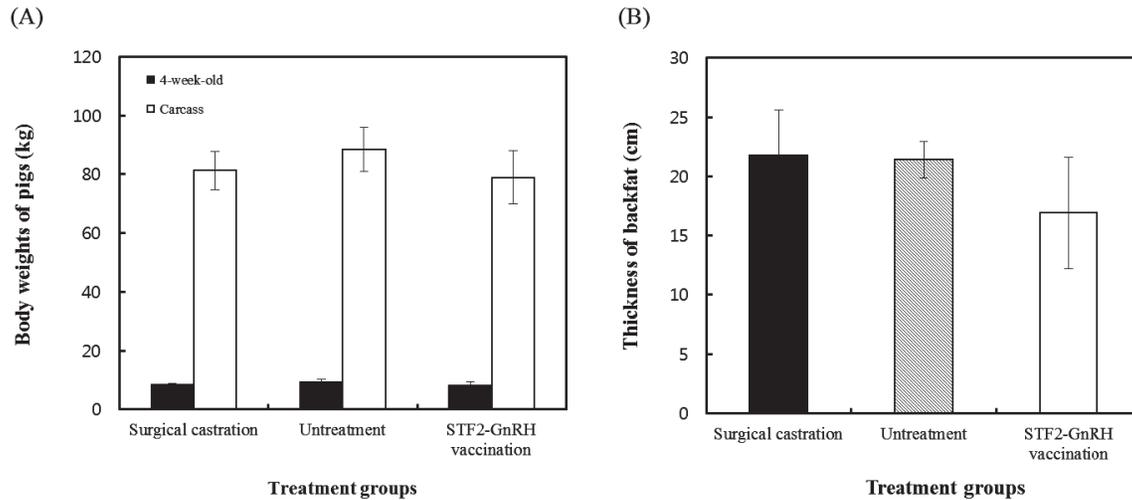


Fig. 3. Measurement of body weights and backfat thicknesses of surgically castrated, untreated, and STF2-GnRH-vaccinated boars. (A) Body weights and (B) backfat thicknesses of boars in the three groups were not significantly different at age 26 weeks.

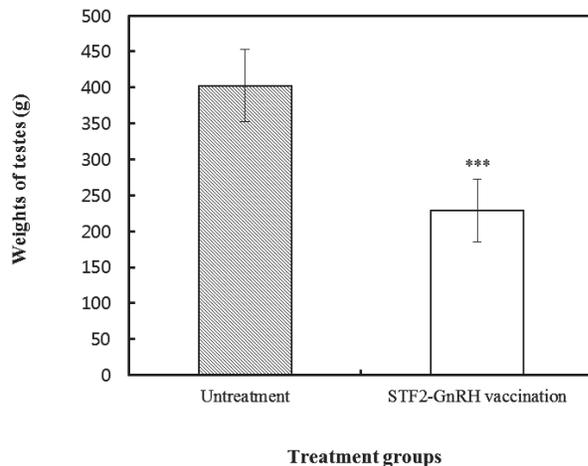


Fig. 4. Weights of testes and epididymides of untreated and immunocastrated boars. The testes weights of immunocastrated boars were significantly lower than those of untreated boars. *** $P < 0.001$.

seminiferous tubules (Fig. 5B) and few spermatogenic cells (Fig. 5D). Moreover, less-developed Leydig cells were observed in the testicular tissues of the STF2-GnRH-vaccinated boars (Fig. 5D).

Discussion

Many researchers have tried to develop immunocastration vaccines using GnRH as an

antigen. However, the GnRH peptide itself is low immunogenic. Therefore, most GnRH vaccines contain multiple copies of the GnRH peptide and carrier proteins to improve efficacy.^{16,21,25} Several immunocastration vaccines in animals have used carrier proteins such as maltose-binding protein, human serum albumin, KLH, bacterial toxoid, ovalbumin, and mycobacterial Hsp70 to increase the peptide's poor immunogenicity. These carrier proteins induced strong anti-GnRH antibody production.^{10,14,25} Interaction of flagellin with TLR5 on the surface of antigen-presenting cells mounts strong innate and adaptive immune responses.²² Because of this advantage, STF2 protein has been widely used as a carrier protein or an adjuvant in influenza and West Nile virus vaccines.^{17,24} We previously developed an immunocastration vaccine composed of STF2 genetically fused with six tandem copies of GnRH. The vaccine produced a high level of anti-GnRH antibodies, eventually inducing a low level of testosterone and atrophy of testes in male rats.²³

In this study, we administered the recombinant STF2-GnRH vaccine to pre-pubertal boars to determine its immunocastration effects. A recent study indicated that pubertal development of boars is characterized by a decrease in anti-Müllerian hormone (AMH) and subsequent

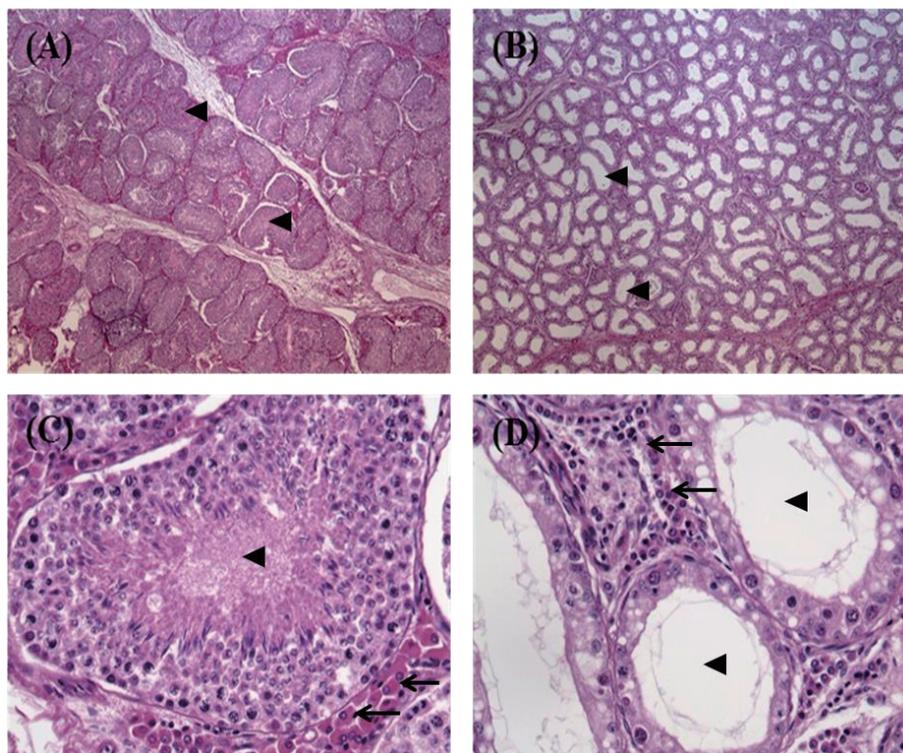


Fig. 5. Testicular tissues of the immunocastrated and untreated pigs. (A) Testicular tissue of an untreated boar stained with H&E was well developed. Abundant spermatogenic cells are shown in a normal seminiferous tubule (arrowhead) (X100). (B) Empty seminiferous tubules (arrowhead) in the testicular tissues of a STF2-GnRH-vaccinated boar (X100). (C) Abundant Leydig cells (arrows) between normal seminiferous tubules (arrowhead) of testicular tissue of an untreated boar (X400). (D) Empty seminiferous tubules (arrowhead) and fewer Leydig cells (arrow) are seen in the testicular tissues of a STF2-GnRH-vaccinated boar (X400).

increase of CDKN1B, a cyclin-dependent kinase inhibitor, in Sertoli cells.¹¹⁾ The decrease of AMH and increase of CDKN1B were observed at ages 28 days and 50–70 days, respectively. In addition, it is known that antibodies against GnRH can persist up to 22 weeks after a second, booster, injection of immunocastration vaccine.²⁶⁾ The advantage of early administration of GnRH vaccine was demonstrated in rams.⁴⁾ Vaccination of pre-pubertal rams with the GnRH vaccine at age 3–4 weeks and a booster 10 weeks later effectively suppressed reproductive function. Immunocastration effects were almost equal in rams that were vaccinated at pre-pubertal or peri-pubertal ages. These results suggest that administration of the GnRH vaccine at pre-pubertal ages would be sufficient for induction of anti-GnRH antibodies and prolonged suppression of testicular function in boars.

Currently, the first vaccination with a commercialized GnRH vaccine, Improvac[®], is done in boars aged 8 weeks. However, the vaccine developed in this study could be administered to post-weaning boars aged 4 weeks. It is expected that administration of the GnRH vaccine to younger boars will greatly reduce workloads on swine farms.

The first administration of the STF2-GnRH vaccine to boars age 4 weeks induced anti-GnRH antibodies and suppression of testosterone synthesis. A booster injection of the vaccine at age of 8 weeks produced very high titers of anti-GnRH antibodies and significant reductions in testosterone level and testis size at age 26 weeks. These data indicate that a considerable amount of antibody to GnRH persisted for at least 18 weeks after the booster vaccination. Our findings are similar to those induced by administration of

Improvac[®], which reduces serum testosterone levels and the size of testes.^{6,8)} These castration effects are supposedly mediated by anti-GnRH antibodies, which block release of GnRH from the hypothalamus and subsequent synthesis of FSH and LH in the pituitary. Deficient FSH and LH may suppress almost all testicular functions, such as spermatogenesis in seminiferous tubules and production of testicular steroids.¹²⁾ These inhibitory effects eventually could lead to testicular atrophy in boars.

Other advantages of the use of immunocastration vaccines may include production of thin backfat, diminished boar taint, and enhanced growth performance through reduction in the boars' aggressive behavior.⁷⁾ In our study, the backfat thickness of boars immunized with the STF2-GnRH vaccine appeared to be less than that of the untreated and surgically castrated pigs, but there were no statistical differences among them. In addition, the body weights of immunocastrated pigs showed no significant differences from those of the untreated and surgically castrated pigs. Since the main objective of this study was to determine the castration efficacy of the STF2-GnRH vaccine, the evaluation of meat quality as determined by the amount of skatole in fat and taste tests was not performed.

In summary, the STF2 protein fused to GnRH acted as an adjuvant, and the STF2-GnRH vaccine induced excellent immunocastration effects when administered to pre-pubertal boars.

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