

Expression of follicle stimulating and luteinizing hormone receptors in porcine testes after timing the first vaccination for immunocastration

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Abstract

This study aimed to examine the expression of follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) in the testes of male fattening pigs based on the timing of the first immunocastration dose. A total of 24 male pigs were randomly assigned to 3 groups: C (n=8) consisted of only intact pigs, T1 (n=8) contained those immunocastrated at 15 and 19 weeks of age, and T2 (n=8) was composed of those immunocastrated at 9 and 19 weeks of age. The expression of FSHR and LHR was evaluated using H-score from immunohistochemistry and protein band intensity from Western blotting. Immunohistochemistry revealed that C pigs exhibited the highest FSHR H-score (82.70 ± 12.20), whereas no significant difference was observed between T1 and T2 (31.61 ± 16.51 vs 36.44 ± 7.77 , $P > 0.05$). The highest LHR H-score was found in C (109.68 ± 3.41), whereas that between T1 and T2 was not different (72.06 ± 5.63 vs 75.71 ± 5.75 , $P > 0.05$). Western blot analysis demonstrated that the highest FSHR protein band intensity occurred in C group (0.66 ± 0.19), while T1 and T2 showed no significant difference (0.29 ± 0.06 vs 0.26 ± 0.10 , $P > 0.05$). LHR protein band intensity was highest in C pigs (0.60 ± 0.38), with no significant difference between T1 and T2 (0.23 ± 0.27 vs 0.17 ± 0.08 , $P > 0.05$). In conclusion, the GnRH vaccine suppressed porcine testicular growth and functions. FSHR and LHR expression did not differ significantly between vaccination protocols.

Keywords: follicle stimulating hormone receptor, GnRH vaccine, immunocastration, luteinizing hormone receptor, pigs, testes

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Introduction

In general, the suckling piglets destined for fattening pigs are usually castrated within their first few weeks of life in order to minimize boar taint accompanied by aggressive and sexual behaviors (Fredriksen *et al.*, 2009). Boar taint is majorly caused by the accumulation of androstene and skatole in adipose tissues. Androstene is a testicular steroid synthesized from Leydig cells under LH control; skatole is produced from L-tryptophan by bacteria in the pig's large intestine (Zamaratskaia and Squires, 2009). Even if the surgical castration effectively reduces boar taint, it is frequently performed without appropriate pain management in many countries, contributing to pain and an increased risk of infection to the piglets. Currently, several countries, particularly in the European Union, are working to phase out surgical castration in suckling piglets without proper analgesia and anesthesia in order to enhance animal welfare (Prunier *et al.*, 2006). For this reason, immunocastration is introduced as an alternative to surgical castration in male pigs.

Gonadotropin releasing hormone (GnRH) vaccine is one of the universal products used for immunocastrating male pigs as it activates antibody production to neutralize endogenous GnRH, subsequently affecting the release of both follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Zamaratskaia *et al.*, 2009). FSH and LH are considered pituitary gonadotropin, and are key factors mediating the communication among the hypothalamus, pituitary glands, and gonads via FSH receptor (FSHR) and LH receptor (LHR) to modulate reproductive functions (Plant, 2008). The expression of FSHR (76 kDa) is mainly found on Sertoli cells, whereas that of LHR is chiefly observed on Leydig cells (McLachlan *et al.*, 2002; Simoni *et al.*, 1997). FSH plays a vital role in the spermatogenesis since it is responsible for development and proliferation of Sertoli cells, which are vital for germ cell development and survival (Sani *et al.*, 2012). Sertoli cells do not only provide histoarchitectural support but also supply essential hormones and nutrients for germ cell differentiation (Patel and Bhartiya, 2016). Moreover, FSH activates the synthesis of cytokines and growth factors from Sertoli cells to stimulate functions of Leydig cells in the interstitial areas of the testes (Rebourcet *et al.*, 2014). Leydig cells are also important for spermatogenesis since they synthesize testosterone following LH release, contributing to the further activation of various steroidogenic enzymes (Shiraishi and Ascoli, 2007). Steroidogenesis within the testes, initiated by the activation of LHR (85-95 kDa) on Leydig cells by LH, results in conversion of cholesterol into testosterone, which is secreted locally within the testes and into the systemic circulation (Corradi *et al.*, 2016).

For effective immunocastration, a two-dose administration of GnRH vaccine is recommended. The first dose should be injected after eight weeks of age, followed by the second dose four weeks later and prior to slaughter (Einarsson *et al.*, 2009). Even though the literature describes the inhibitory effect of the GnRH

vaccine on pituitary FSH and LH release, data relevant to the expression of FSHR and LHR in immunocastrated pigs remain scarce. Moreover, their expression patterns in the testes of male fattening pigs subjected to an adjusted protocol at 9 and 19 weeks have never been demonstrated. Therefore, the present study aimed to examine the expression of FSHR and LHR in the testes of male fattening pigs immunocastrated with the GnRH vaccine at 9 and 19 weeks of age.

Materials and Methods

Animals and general managements: Twenty-four male Landrace x Yorkshire x Duroc crossbred pigs were randomly selected after weaning for the present study. All of them were housed in a fattening facility on a commercial breeding farm in northeast Thailand. Within the facility, pigs were raised in groups at a stocking density of ≥ 2.0 m²/head, fed with the same commercial diet, and provided ad libitum access to water via nipple drinkers throughout the study period. Routine health checks were performed by farm veterinarians according to farm program. All pigs were vaccinated against foot and mouth disease, Aujeszky's disease, classical swine fever, porcine reproductive and respiratory syndrome, porcine circovirus type 2, and mycoplasmosis. All experimental procedures involving animals were approved by the Animal Care and Use Committee of Mahasarakham University (approval number 16/2017).

Timing of immunocastration: All the male fattening pigs were equally allocated into three groups on the basis of the immunocastration protocol: C (n=8) was intact male pigs, T1 (n=8) consisted of those injected with GnRH vaccine at 15 and 19 weeks of age, and T2 (n=8) contained those receiving GnRH vaccine at 9 and 19 weeks of age. For pigs in T1 and T2, 2 ml of GnRH vaccine (Improvac®, Zoetis, Belgium) was intramuscularly administered by an experienced veterinarian throughout the study.

Testicular tissue collection and sample preparation: All of the pigs were slaughtered at 25 weeks of age at the standard slaughterhouse. Immediately after slaughter, both testes from each pig were visually evaluated for gross appearance. Testicular tissue samples (approximately $1 \times 1 \times 1$ cm³) were randomly harvested from each testis. Tissue samples were preserved either in 4% paraformaldehyde for immunohistochemical process or in phosphate-buffered saline (PBS) supplemented with protease inhibitor for Western blot analysis. Tissue samples in the latter medium were kept at -80°C until assay.

Immunohistochemical analysis: Tissue samples were immersed in 4% paraformaldehyde for 48 h and processed using an automatic tissue processor (Tissue-Tek VIP 5 Jr, Tokyo, Japan) for paraffin embedding. Each tissue block was sectioned into 4 μ m thick using a rotary microtome (Anglia Scientific Instrument Ltd., Cambridge, UK) and mounted onto gelatin-coated glass slides.

The deparaffinization was performed with xylene; the rehydration was conducted through a descending ethanol series. The sample sections, thereafter, were immersed in 0.01 mol/L citrate buffer and heated at 800 Watt for 15 min for antigen retrieval. In order to block an endogenous peroxidase activity, all samples were kept in freshly prepared 3.0% hydrogen peroxide in methyl alcohol at room temperature. For blocking non-specific bindings, sample sections were incubated with normal serum (Vector Laboratories Inc., CA, USA). Rabbit polyclonal antibody against FSHR (clone N-20, sc-7798, Santa Cruz Biotechnology Inc., TX, USA; manufacturer-reported molecular weight $\approx$ 75 kDa) and rabbit polyclonal antibody against LHR (clone H-50, sc-25828, Santa Cruz Biotechnology Inc., TX, USA; manufacturer-reported molecular weight $\approx$ 85-95 kDa) were used as primary antibodies for examining the expression of FSHR (1: 100) and LHR (1: 100), respectively. For negative controls, non-immune serum was used instead of the primary antibodies. Afterwards, sample sections were washed with PBS, incubated with biotinylated secondary antibody (Vector Laboratories Inc., CA, USA) for 30 min, then incubated with horseradish peroxidase avidin-biotin complex (Vectastain®, ABC kit, Vector Laboratories Inc., CA, USA) for 30 min, followed by additional PBS washes. Immunoreactivity was visualized using 3',3'-diaminobenzidine chromogen (ImmPACT™ DAB Peroxidase substrate kit, Vector Laboratories Inc., CA, USA) for 1-2 min before the sections were counterstained with Mayer's hematoxylin for 1-2 min, mounted with glycerin, gelatin, and prepared for digital image analysis.

Mounted sections were scanned for their entirety using a panoramic digital slide scanner (3DHISTECH, Budapest, Hungary) to generate digital slides. The expression of FSHR and LHR was evaluated based on staining intensity using CellQuant software (3DHISTECH, Budapest, Hungary). The default of each intensity class (low, moderate, and high) was calibrated digitally prior to image evaluation to ensure consistent scoring criteria across all sections. Expression of each receptor was quantified using an H-score, calculated as: $H\text{-score} = (\% \text{ high} \times 3) + (\% \text{ moderate} \times 2) + (\% \text{ low} \times 1)$ (Prapaiwan *et al.*, 2017). In order to evaluate nuclear expression of FSHR and LHR, NuclearQuant software (3DHISTECH, Budapest, Hungary) was used following the same scoring algorithm.

Western blot analysis: The expression of both FSHR and LHR evaluated by immunohistochemistry was validated using Western blot analysis. PageRuler™ Plus Prestained Protein Ladder (10-250 kDa; Thermo Fisher Scientific Inc., MA, USA) was used as a molecular weight standard. Thawed testicular tissue samples were homogenized, centrifuged at $10,000 \times g$ at 4°C for 15 min, and protein concentrations in the supernatant were tested using the Bradford protein assay. Equal amounts of protein (3.0 $\mu\text{g}/\text{ml}$) from T1, T2, and C groups were resolved in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.45 μm

polyvinylidene fluoride (PVDF) membranes. 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1 Tween-20 (TBST) was used to block non-specific protein bindings. Membranes were washed five times with TBST and incubated with primary antibodies against FSHR or LHR at 4°C for 16 h. Thereafter, membranes were incubated with horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (1: 2,000; Abcam, Cambridge, UK) for 1 h at room temperature. Target protein bands were visualized using ECL Western Blotting Detection Reagents (GE Healthcare). Beta-actin was utilized as the reference protein. Western blot imaging and densitometry were performed by ChemiDoc Touch Imaging System (Bio-Rad Laboratories). The expression of target proteins was carefully examined and reported as a ratio of protein band intensity to beta-actin band intensity. All Western blot analyses were performed in triplicate.

Statistical analyses: Data were analyzed statistically using STATA version 14.1 (StataCorp LLC, Texas, USA). Data normality was tested using the Shapiro-Wilk W test. Descriptive statistics were expressed as mean $\pm$ SEM. Mean H-scores of FSHR and LHR, as well as ratio of protein band intensity to beta-actin band intensity, were compared using the Kruskal-Wallis test. Multiple comparisons were conducted by Dunn's test with Bonferroni correction. Values with $P < 0.05$ were statistically different.

Results

At slaughter, testes from C pigs were the largest among all groups. Histological examination revealed that seminiferous tubules of C pigs exhibited the largest diameter compared with those of T1 and T2 groups. Moreover, the seminiferous tubules of C pigs consisted of normal Sertoli cells and complete spermatogenesis. However, tubules of T1 and T2 groups exhibited residual germ cells and sustentacular cells, while mature spermatozoa were scarcely found. In the interstitial areas, Leydig cell development was most prominent in the testes of C pigs, whereas Leydig cells in T1 and T2 groups were less-developed (Fig. 1).

Digital image analysis revealed the mean H-scores of FSHR and LHR expressions as presented in Table 1. The expression levels of both FSHR and LHR were significantly higher in C than in the immunocastrated groups; however, no significant differences were detected between groups T1 and T2 ($P > 0.05$). Immunolabeling demonstrated that FSHR was localized to both the seminiferous tubules and the interstitial areas in all groups. Within the seminiferous tubules, FSHR expression of C pigs was detected in Sertoli cells and most spermatogenic cell types, excluding spermatozoa; whereas that of T1 and T2 pigs was restricted to Sertoli and remaining germ cells. In the interstitial areas, moderate FSHR expression was observed in Leydig cells of C pigs, whereas faint staining was noted in those of both T1 and T2 pigs (Fig. 1). As for LHR, immunostaining in the seminiferous tubules of C pigs was obviously evident in both Sertoli and all spermatogenic cells; meanwhile,

weak immunoreactivity was observed in those of T1 and T2 pigs. In the interstitial areas, LHR was strongly expressed in the Leydig cells of C pigs, but barely detectable in the Leydig cells of T1 and T2 pigs (Fig. 2).

Western blot analysis confirmed the presence of FSHR and LHR in the testicular tissues of all groups

(Figs. 3 and 4). Densitometric quantification demonstrated that the ratio of protein band intensity to beta-actin band intensity of both FSHR and LHR was highest in C pigs; whereas relative protein levels of FSHR and LHR did not differ significantly between T1 and T2 groups (Table 2).

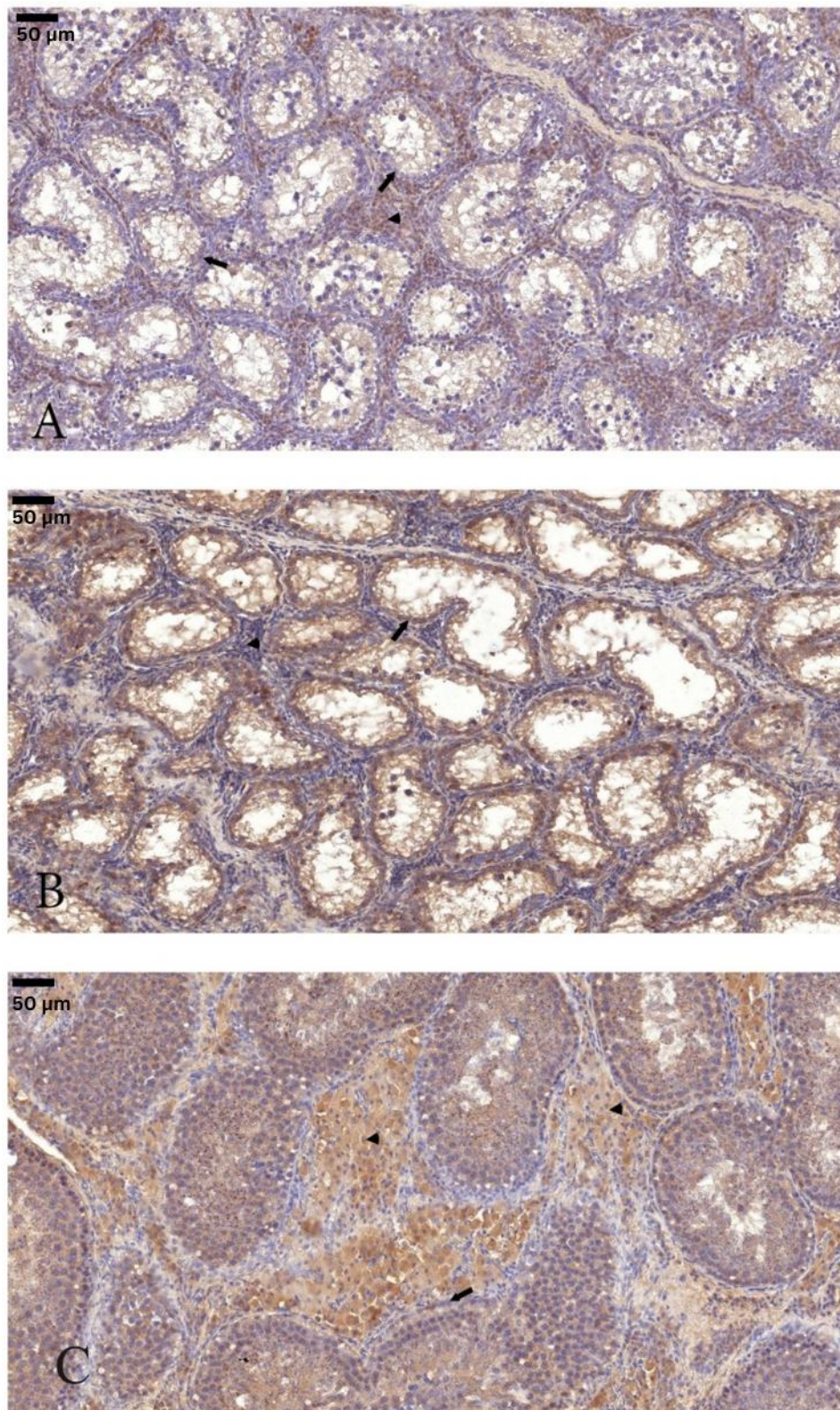


Figure 1 Immunolocalization of follicle stimulating hormone receptor in testicular tissues of the immunocastrated male pigs at 15 and 19 weeks of age (A), at 9 and 19 weeks of age (B), and intact male pigs (C). Arrows demonstrate Sertoli cells, meanwhile arrowheads indicate Leydig cells. A scale bar represents 50 μm.

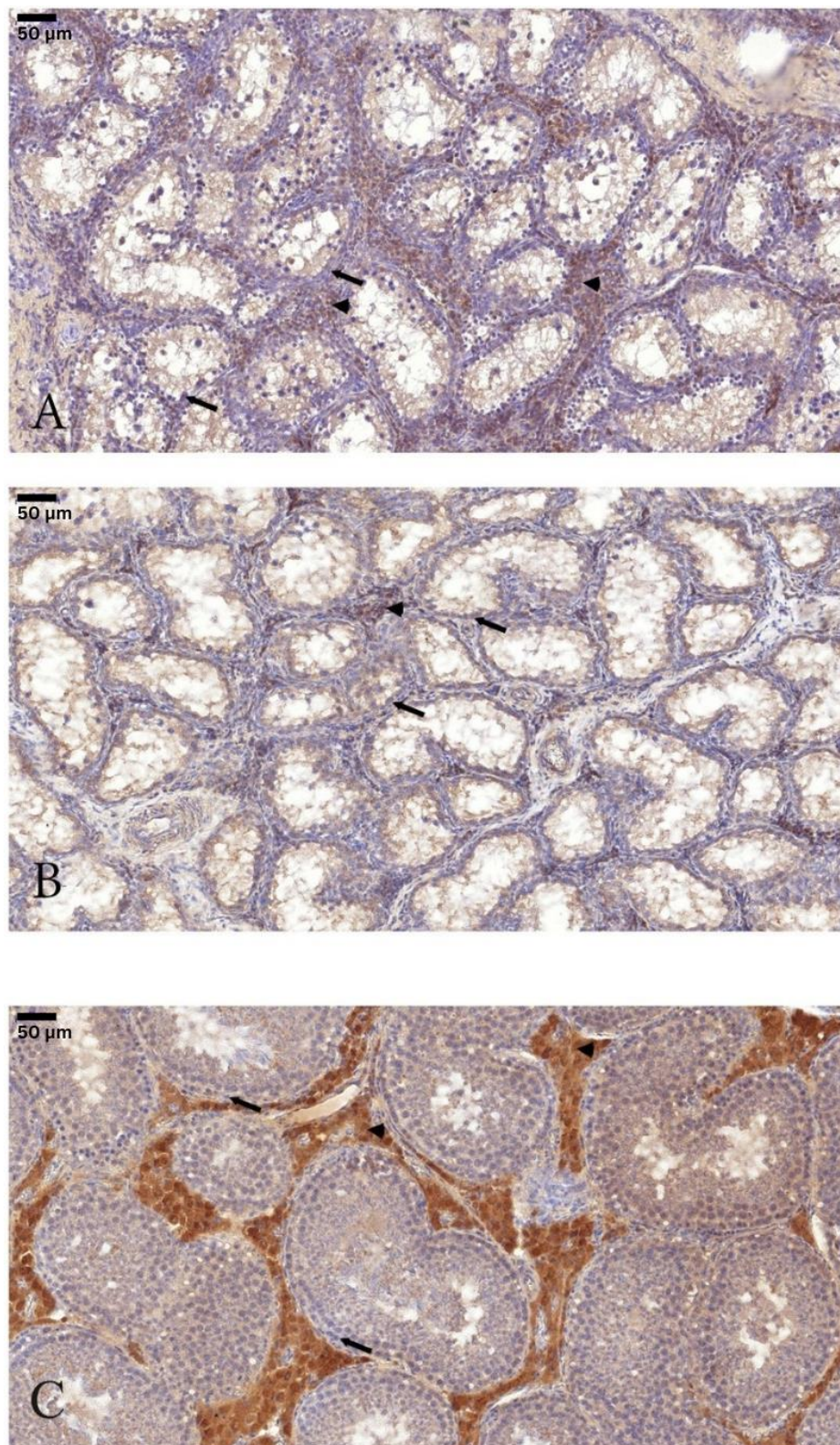


Figure 2 Immunoexpression of luteinizing hormone receptor in testicular tissues of the immunocastrated male pigs at 15 and 19 weeks of age (A), at 9 and 19 weeks of age (B), and intact male pigs (C). Arrows demonstrate Sertoli cells, meanwhile arrowheads indicate Leydig cells. A scale bar represents 50 µm.

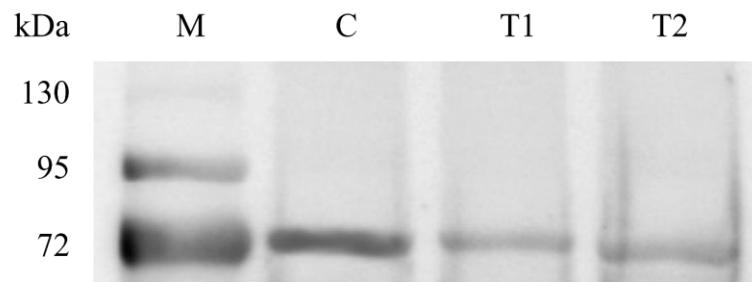


Figure 3 Representative Western blot illustrating protein band intensity of follicle stimulating hormone receptor in testicular tissues of the intact male pigs (C), those immunocastrated at 15 and 19 weeks of age (T1), and those immunocastrated at 9 and 19 weeks of age (T2), and. Protein markers are on the leftmost lane (M).

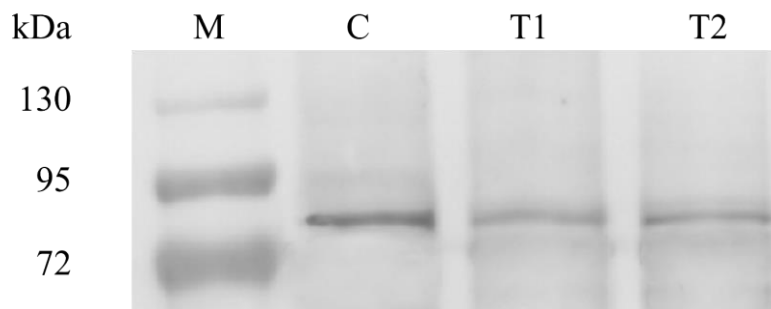


Figure 4 Representative Western blot demonstrating protein band intensity of luteinizing hormone receptor in testicular tissues of the intact male pigs (C), those immunocastrated at 15 and 19 weeks of age (T1), and those immunocastrated at 9 and 19 weeks of age (T2), and. Protein markers are on the leftmost lane (M).

Table 1 H-score (mean±SEM) of follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) in testicular tissues of male fattening pigs based on timing of the first vaccination for immunocastration. C is intact male pigs, T1 is those immunocastrated at 15 and 19 weeks of age, and T2 is those immunocastrated at 9 and 19 weeks of age. a,b different superscript letters within column represent statistical significance ($P < 0.05$).

Experimental group	H-score	
	FSHR	LHR
C (n=8)	82.70±12.20 ^a	109.68±3.41 ^a
T1 (n=8)	31.61±16.51 ^b	72.06±5.63 ^b
T2 (n=8)	36.44±7.77 ^b	75.71±5.75 ^b

Table 2 Protein band intensity (mean±SEM) of follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) in testicular tissues of male fattening pigs with different timing of the first vaccination for immunocastration. C is intact male pigs, T1 is those immunocastrated at 15 and 19 weeks of age, and T2 is those immunocastrated at 9 and 19 weeks of age. a,b different superscript letters within column represent statistical significance ($P < 0.05$).

Experimental group	Protein band intensity	
	FSHR	LHR
C (n=8)	0.66±0.07 ^a	0.60±0.13 ^a
T1 (n=8)	0.29±0.02 ^b	0.23±0.10 ^b
T2 (n=8)	0.26±0.04 ^b	0.17±0.03 ^b

Discussion

The present study demonstrated that immunocastration using a GnRH vaccine significantly affected testicular gross morphology as well as the expression of FSHR and LHR in male fattening pigs. At slaughter, the testes of intact pigs were apparently larger than those of immunocastrated pigs. This aligned with a number of studies reporting that testes of intact pigs were longer (Srisuwatanasagul *et al.*, 2018; Srisuwatanasagul *et al.*, 2021) greater in size (Brunius *et al.*, 2011) and heavier (Einarsson *et al.*, 2011; Zamaratskaia *et al.*, 2008) than those of immunocastrated pigs. This was due to the fact that GnRH vaccine disrupts the hypothalamic-pituitary-

gonadal axis (Einarsson *et al.*, 2009), contributing to an arrest of testicular development.

Histological investigation confirmed that immunocastration interferes with testicular development, as clearly demonstrated by the altered histoarchitecture of both seminiferous tubules and interstitial areas. In non-immunocastrated pigs, in this study, the seminiferous tubules consisted of well-developed Sertoli cells and germ cells at all developmental stages, representing active and complete spermatogenesis. On the other hand, both seminiferous tubules and interstitial areas of immunocastrated pigs were impaired and less developed. This was in accordance with the preceding study showing that immunocastration not only disrupted spermatogenesis, but pyknoses of Leydig

cells were also found in the immunocastrated testes (Einarsson *et al.*, 2011). Histomorphometric analysis from our recent study indicated that the height of spermatogenic epithelia and the diameter of seminiferous tubules were significantly greater in intact pigs than in immunocastrated pigs. Moreover, the number of seminiferous tubules per μm^2 of the immunocastrated pigs is higher than that of the intact pigs (Srisuwatanasagul *et al.*, 2021). Similarly, certain previous studies reported that the diameter of the seminiferous tubules of intact pigs was more than that of the immunocastrated pigs (Einarsson *et al.*, 2011; Hilbe *et al.*, 2006). However, all of the aforementioned histomorphometric variables between T1 and T2 groups were not statistically different (Srisuwatanasagul *et al.*, 2021).

According to the expression of gonadotropin receptors, the FSHR H-score was highest in C group because their seminiferous tubules develop without an interference from GnRH vaccine. As a result, Sertoli cells, the primary target of FSH in regulating spermatogenesis (Meng *et al.*, 2000) and steroidogenesis (Zirkin and Chen, 2000), develop normally and disperse throughout the tubules. In contrast, FSHR H-score of both T1 and T2 groups were significantly lower than that of C group, as Sertoli cell development and maintenance were affected following GnRH vaccine administration. Similarly, C pigs exhibited the highest H-score of LHR expression. This was due to the fact that the testes of non-immunocastrated pigs were composed of well-developed Leydig cells, which serve as the primary target for LH to promote steroidogenesis (Shiraishi and Matsuyama, 2017). In immunocastrated pigs, Leydig cells were markedly less developed than the intact pigs as a result of the injection of GnRH vaccine. These results were corroborated by Western blot analysis confirming that the highest expression of both FSHR and LHR occurred in the testes of intact pigs, with no significant difference observed between T1 and T2 groups. However, circulating hormone concentrations were not evaluated in the present study.

In general, FSHR immunoexpression is majorly detected from spermatogenic and Sertoli cells (Patel and Bhartiya, 2016), while LHR immunolabeling is mainly found in Leydig cells (McLachlan *et al.*, 2002). However, FSHR in the present study was detected from Leydig cells. This might be due to the antibodies used in this study have not been validated for porcine tissues. The possibility of cross-reactivity with related epitopes cannot be entirely excluded, and this finding should be interpreted with appropriate caution.

The current study demonstrated that GnRH vaccine is one of the effective ways of immunocastrating male fattening pigs, as evidenced by regression in gross morphology and histoarchitecture of the testes where the boar taint precursors originated. Furthermore, vaccinating the first dose at 9 weeks of age, with a booster at 19 weeks of age, represented a viable alternative protocol, as it yielded results comparable to that at 15 and 19 weeks of age. Vaccinating at 15 and 19 weeks of age is the manufacturer's standard protocol. However,

vaccinating pigs with this alternative protocol (9 and 19 weeks of age) can provide certain advantages at the farm level. For instance, pigs are lighter at 9 weeks than 15 weeks of age, making the restraint for vaccination easier and more successful. In addition, it provides flexibility for other management practices, such as feeding adjustment, disease treatment, and other vaccinations.

Aside from the expression of FSHR and LHR, the pigs immunocastrated with GnRH vaccine at 9 and 19 weeks of age possessed comparable testicular length, together with the similar testicular expression levels of anti-Müllerian hormone, cytochrome P450 aromatase (Srisuwatanasagul *et al.*, 2021), and estrogen receptor alpha (Srisuwatanasagul *et al.*, 2022) in the testes to those immunocastrated with GnRH vaccine at 15 and 19 weeks of age. Moreover, expressions of Ki-67 proliferative marker, androgen receptor, and estrogen receptor beta in the testes of those vaccinated at 9 and 19 weeks of age were not significantly different from those vaccinated at 14 and 18 weeks of age (Srisuwatanasagul *et al.*, 2018). Understanding the expression of specific reproductive hormone receptors is crucial, as the intricate balance of reproductive hormone receptor expression fundamentally dictates porcine reproductive and testicular functions (Do *et al.*, 2026).

In conclusion, immunocastrating male fattening pigs with the GnRH vaccine impaired testicular growth and function. The first dose of the GnRH vaccine administered at either 9 or 15 weeks of age provided comparable expression of FSHR and LHR in the testicular tissues of male fattening pigs.

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