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Research article

Estrogen receptor alpha expression in fattening pig's testes by the timing of the first injection for immunocastration

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Abstract

The purpose of this study was to examine the expression of estrogen receptor alpha (ERα) in the testicular tissues of male fattening pigs injected with the first gonadotropin-releasing hormone (GnRH) vaccine (Improvac™, Zoetis, Thailand) for immunocastration 6 weeks earlier than the standard protocol. All pigs (n=24) were divided into three groups on the criterion of immunocastration protocol: A (n=8) was injected with GnRH vaccine at 15 and 19 weeks old, B (n=8) received GnRH vaccine at 9 and 19 weeks old, and C (n=8) remained untreated. Expression of ERα was investigated using an immunohistochemistry, appraised by an image analysis application (3DHISTECH, Budapest, Hungary), and reported as a H-score. The results revealed that testicular histoarchitecture of the immunocastrated pigs was less developed than that of the intact pigs. ERα was localized both in the seminiferous tubules and interstitial areas of all groups. ERα H-score of the C pigs was lowest (5.49±4.17) among groups and significantly lower than that of both A and B groups ($P<0.05$). However, the H-score of ERα between A and B groups was not different from each other (21.41±12.61 vs 23.91±11.47, $P>0.05$). In summary, the first injection of GnRH vaccine either at 9 or 15 weeks of age contributed to a similar result of testicular ERα expression.

Keywords: Estrogen receptor alpha, Gonadotropin-releasing hormone vaccine, Immunocastration, Pigs

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INTRODUCTION

In the swine breeding industry, castration of male suckling pigs is usually performed within the first week after birth to annihilate boar taint and minimize aggressive behaviors (Fredriksen et al., 2009). Boar taint, an unfavorable odor from heated pork products, is mainly caused by the gradual collection of skatole and androstenone in swine adipose tissues (Dunshea et al., 2001). Castration in many countries is performed by surgery within the farrowing houses. In addition, that surgical castration is usually performed without appropriate anesthesia and analgesia, leading to high infection risk and pain in the piglets (Prunier et al., 2006). In order to enhance animal welfare, several countries especially in the European Union have made an effort to halt the practice of surgical castration without suitable anesthesia and analgesia (Einarsson et al., 2009). As a result, castration based on immunological processes is an alternative for suppressing boar taint and dangerous behaviors since an immunocastration is proven as a welfare-promoting technique to control animal reproductive functions and sexual behaviors (Zamaratskaia et al., 2008). To date, immunocastration has focused on stimulating antibodies against gonadotropin-releasing hormone (GnRH) to neutralize endogenous GnRH via active immunization. This is because GnRH regulates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) which, in turn, control testicular steroidogenesis (Cook et al., 2000) and development of reproductive organs (Dubois et al., 2002).

GnRH, 1.18 kD, is a short neuropeptide containing 10 amino acid residues. Since it is non-immunogenic, it requires to be modified by combining with a macromolecule carrier protein to improve its immunogenicity. Moreover, it was reported that the substitution of the sixth and tenth amino acid residues elicits anti-GnRH antibodies effectively (Xu et al., 2018). In male pigs, the active immunization with GnRH vaccine is proved to activate immune response against endogenous GnRH released from the hypothalamus; thereupon, it suppresses the biological processes of both FSH and LH (Zamaratskaia et al., 2009), affecting testicular development and sex steroidogenesis (Einarsson et al., 2009).

Testes are one of the primary sources of steroidogenesis to control male reproductive functions (Pearl et al., 2007). In pigs, estrogen is a major sex steroid hormone regulating the male reproductive system, especially spermatogenesis and sperm maturation, via estrogen receptor (ER). ERα and ERβ are two major subtypes with approximately 95% homology in their DNA binding sites; however, their ligand-binding domain is about 55% homologous (Cowley and Parker, 1999). As a result, they show similar affinity to only estradiol but act as selective receptor modulators (Riggs and Hartmann, 2003). In pigs, both ER isoforms are extensively distributed along the male genital tract, especially in testes and epididymides. Moreover, porcine testes are an important source for producing high quantity estrogen, particularly in the form of estrone sulphate (Mutembei et al., 2005).

In order to achieve the effective castration outcome from GnRH vaccine administration, it is basically performed using two injections in practice. The first injection should be conducted after eight weeks old (Brunius et al., 2011) and the second injection is recommended four weeks after the first shot and

four weeks prior to slaughter (Einarsson et al., 2009). Information elaborating the expression of ER, especially the alpha subtype, in the pigs injected with GnRH vaccine has been scarcely reported. Consequently, the current study aimed to investigate the localization pattern and expression level of ERα in the testicular tissues of male fattening pigs immunocastrated with the timing of the first GnRH vaccine.

MATERIALS AND METHODS

Animals and general managements

This study was conducted in a commercial swine herd in northeastern Thailand. All the pigs were Landrace x Yorkshire x Duroc crossbred fattening pigs (n=24) which were accommodated in the same open house with the density of ≥2.0 m2/ head at the end of study. The rearing pens were equipped with 8–10 water nipples for ad libitum water access. Throughout the study, all of the pigs were routinely health checked by registered veterinarians based on the herd health program. To provide disease protection, all pigs were vaccinated against porcine respiratory and reproductive disease syndrome, Mycoplasmosis, porcine Circovirus type 2, and Pseudorabies. All of the pigs were slaughtered at 25 weeks old with the same standard procedure at the same slaughterhouse.

Experimental design

The present study was undertaken on the basis of completely randomized design to investigate the expression of ERα in the testicular tissues of fattening pigs after immunocastration with GnRH vaccine, which is a short neuropeptide integrated with carrier protein for enhancement of immunogenicity. All the fattening pigs were randomly selected from the fattening houses and classified, on the basis of immunocastration protocol, into 3 groups: A (n=8) was castrated with GnRH vaccine at 15 and 19 weeks old, B (n=8) was immunocastrated at 9 and 19 weeks old, and C (n=8) was intact. Immunocastration in this study was performed by an injection of 2 ml GnRH vaccine (Improvac™, Zoetis Co., Ltd., Thailand) via subcutaneous route by the same veterinarian throughout the study. All interventions to animals in the current study were approved by the Animal Care and Use Committee, Mahasarakham University (approval number 16/ 2017).

Sample collection and tissue preparation

Immediately after being slaughtered, both testicles of each pig were carefully collected. Tissue samples were randomly excised from the middle part of each testicle and immediately transferred into 4% paraformaldehyde (w/v) prior to being transported to the laboratory for investigation of ERα expression. Each sample of tissues was immersed in 4% paraformaldehyde for 48 h and then processed with a tissue processor (Tissue-Tek VIP 5 Jr., Tokyo, Japan). Samples were individually embedded into paraffin blocks prior to being cut into 4-µm thick by microtome (Shandon, Anglia Scientific Instrument Ltd., Cambridge, UK), and put onto gelatin-coated slides for immunohistochemical staining.

Immunohistochemistry

All sample sections were first deparaffinized in xylene and rehydrated in graded concentration of ethyl alcohol. They were subsequently immersed in 0.01 M Citrate buffer (pH 6.0) and reheated in a microwave oven at 800 Watt for 15 min in order to retrieve antigen. The slide samples were kept in freshly prepared 3.0% hydrogen peroxide in methanol at room temperature for 10 min to block endogenous peroxidase reaction, and then kept in normal serum (Vector Laboratories Inc., CA, USA.) for 30 min to block non-specific binding. Mouse monoclonal antibody against ERα (1: 50, clone 1D-5, Dako, Denmark) was used as a primary antibody to investigate the expression of ERα, meanwhile non-immune serum (mouse IgG) was incubated with tissue sections prepared as a negative control. Thereupon, all sample sections were washed with phosphate-buffered saline (PBS), incubated with biotinylated secondary antibody (Vector Laboratories Inc., CA, USA) for 30 min, following by horseradish peroxidase avidin-biotin complex (Vectastain®, ABC kit, Vector Laboratories Inc., CA, USA) for 30 min, and washed again with PBS. Subsequently, 3′, 3′ diaminobenzidine chromogen (ImmPACT™ DAB Peroxidase substrate kit, Vector Laboratories Inc., CA, USA) was added onto all sections for 1–2 min. Eventually, they were counterstained with Mayer's hematoxylin for 1–2 min and mounted with glycerin gelatin for further evaluation with digital image analyzer. The immunohistochemistry for investigating ERα expression was conducted in triplicate.

ERα expression assessment

The immunoexpression of ERα of all groups was evaluated with the CellQuant application of the computerized image analysis program (3DHISTECH, Budapest, Hungary). In brief, samples from all groups were individually scanned in panoramic view to acquire images of entire testicular tissues in digital formats. The positive expression of ERα was seen as brown on the testicular tissues. Negative staining, together with high-, moderate-, and low-positive staining, was specified as default values prior to initiating the evaluation so that all sections were appraised under the same criteria. Finally, the immunoexpression of ERα was reported as H-score which was calculated from H-score = (% high x 3) + (% moderate x 2) + (% low x 1) (Prapaiwan et al., 2017).

Histomorphometry

All sample slides were investigated for histomorphometry, including the number of seminiferous tubules, the diameter of seminiferous tubules, and the height of spermatogenic epithelia, with CaseViewer version 2.4 (3DHISTECH, Budapest, Hungary) after panoramic digitalization. The number of seminiferous tubules was randomly quantified from 20 microscopic fields of 1 mm2, the diameter of seminiferous tubules and the height of spermatogenic epithelia were arbitrarily investigated from the same tubules from 500 round and roundish tubules (Srisuwatanasagul et al., 2021).

Statistical analyses

Data were managed and analyzed statistically by STATA version 14.1 (StataCorp LLC, Texas, USA). Descriptive data were reported as mean±SD. Expression of ERα, as H-score, among groups was analyzed by Kruskal-Wallis test. Pairwise comparison was performed by Dunn test with Bonferroni adjustment. Histomorphometric parameters (the number of seminiferous tubules, the diameter of seminiferous tubules, and the height of spermatogenic epithelia) among groups were compared with Linear Mixed Model. Values with $P<0.05$ were statistically significant.

RESULTS

Histological results reported in Figure 1 demonstrated that complete spermatogenesis was observed within the seminiferous tubules of C pigs throughout the development from spermatogonia to spermatozoa, meanwhile complete spermatogenesis did not take place within the seminiferous tubules of both A and B pigs. The number of cells within the seminiferous tubules of A and B pigs was obviously fewer than those of C pigs. At the interstitial areas, Leydig cells of C pigs were more developed than those of both A and B pigs. The immunoreaction of ERα was detected both in the seminiferous tubules and interstitial areas as demonstrated in Figure 1. In non-immunocastrated pigs, ERα expression was apparent in the interstitial areas.

Based on panoramic image analysis, it was found that ERα H-score of C pigs was 5.49±4.17, which was lower than that of both A and B pigs ($P<0.05$). Nonetheless, H-score of ERα between A and B groups was not different from each other (21.41±12.61 vs 23.91±11.47, $P>0.05$). Moreover, means of the number of seminiferous tubules, the diameter of seminiferous tubules, and the height of spermatogenic epithelia were as demonstrated in Table 1.

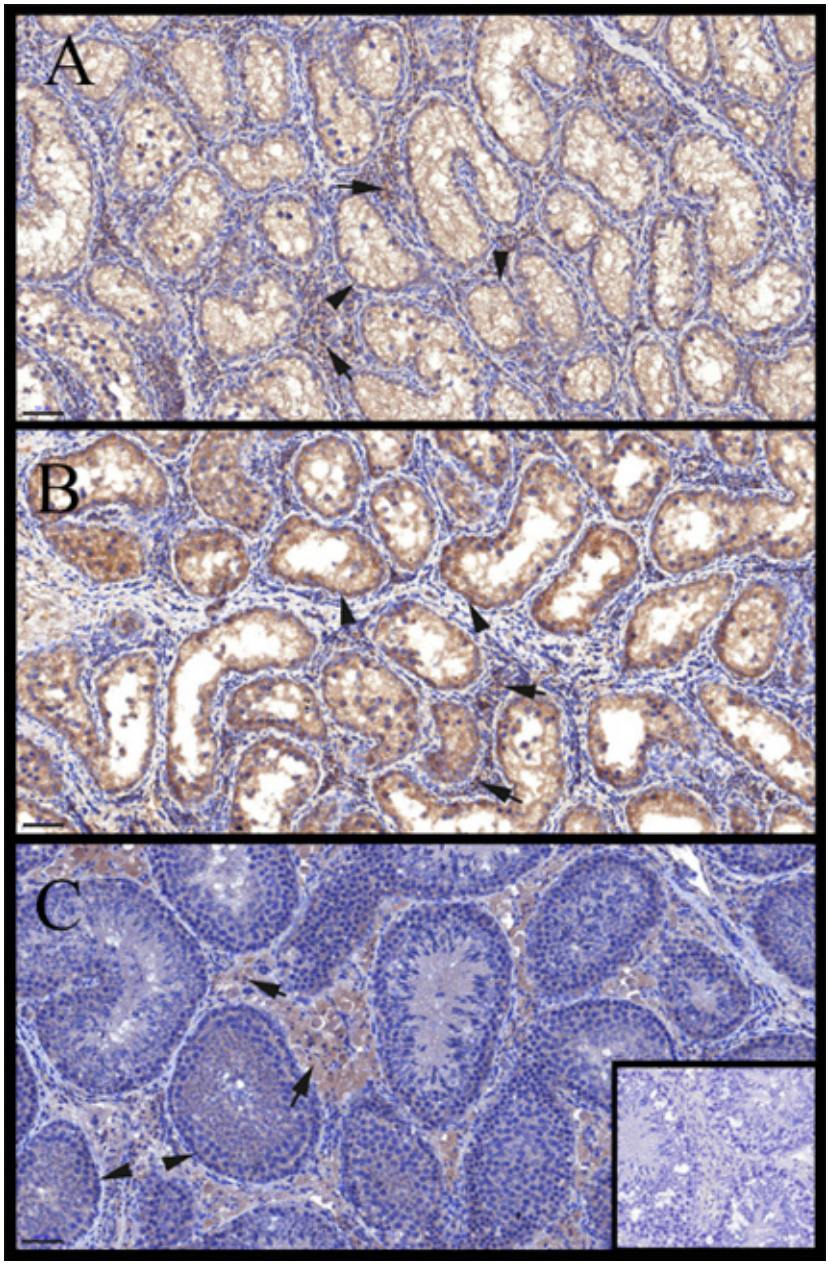

Figure 1 Representative expression of estrogen receptor alpha in the testicular tissues of male fattening pigs immunocastrated at 15 and 19 weeks of age (A), those immunocastrated at 9 and 19 weeks of age (B), and intact pigs (C). Arrows depict Leydig cells and arrowheads represent Sertoli cells. An inset in the lower-right corner illustrates negative control. A scale bar is equivalent to 50 μm.

Table 1 Histomorphometric evaluation (mean±SD) of male fattening pigs with different immunocastration protocols. A is the pigs immunocastrated at 15 and 19 weeks of age, B is those immunocastrated at 9 weeks and 19 weeks of age, and C is intact pigs.

Histomorphometric parameters	Pig groups		
	A (n=8)	B (n=8)	C (n=8)
Number of seminiferous tubules (tubule/ mm^2)	46.50±5.81[a]	42.81±7.53[a]	15.83±2.30[b]
Diameter of seminiferous tubules (μm)	64.49±12.24[a]	70.08±18.99[a]	177.65±25.51[b]
Height of spermatogenic epithelia (μm)	10.67±3.02[a]	11.94±3.71[a]	68.60±8.56[b]

[a, b] different superscript letters within row indicate statistical significance ($P<0.05$).

DISCUSSION

The present study illustrated that GnRH vaccine used for immunocastration obviously effected on testicular development and functions, as well as ERα expression of male fattening pigs. It was found that the testes of immunocastrated pigs were less developed than those of the intact pigs. This was lucidly noticed from both testicular histoarchitecture and histomorphometry illustrating that the seminiferous tubules of intact pigs were wider and consisted of complete spermatogenic lineage from spermatogonia to spermatozoa, meanwhile those of immunocastrated pigs were significantly narrower and seldom contained spermatogenic cells. This corresponded with previous studies indicating that the diameter of seminiferous tubules of the intact pigs is wider than those of the immunocastrated pigs (Einarsson et al., 2011; Srisuwatanasagul et al., 2021). In addition, the spermatogenic epithelia of the intact pigs are higher than those of the immunocastrated pigs (Srisuwatanasagul et al., 2021). This was because GnRH vaccine evidently disrupts the release of gonadotropins, in turn, affecting spermatogenesis (Hilbe et al., 2006). Apart from histology, certain studies investigating the testicular gross morphology have reported that the testicles of immunocastrated pigs are smaller and lighter than those of the intact pigs (Zamaratskaia et al., 2008; Brunius et al., 2011; Einarsson et al., 2011; Kubale et al., 2013). Moreover, our previous study demonstrated that testicular length, measured between both apexes, of the intact pigs is more than that of the immunocastrated pigs (Srisuwatanasagul et al., 2018). As for the timing of immunocastration, our earlier study confirmed that the first GnRH vaccine administration either at 9 or 15 weeks of age contributes to comparable testicular length and histomorphometric parameters, including the height of spermatogenic epithelia, the diameter of seminiferous tubules, and the number of seminiferous tubules (Srisuwatanasagul et al., 2021).

According to results for ERα expression, immunohistochemistry is considered a reliable method for investigation since the previous study had verified that the detection of porcine testicular ER with immunohistochemistry provides an identical result to in situ RT-PCR (Mutembei et al., 2005). In this study, immunohistochemical reaction of ERα was detected both in the seminiferous tubules and interstitial areas. Likewise, our earlier study reported that ERβ is also localized both in Leydig cells and seminiferous tubules of the male fattening pigs (Srisuwatanasagul et al., 2018). This was because estrogen

is an indispensable hormone for regulating Sertoli cell development and spermatogenesis within the seminiferous tubules (At-Taras et al., 2006), as well as the development of Leydig cells in the interstitial areas (Abney, 1999).

It has been well-documented that steroid receptors are regarded as ligand-induced transcription factors (Kuiper et al., 1996; Sneddon et al., 2005), implying that the expression of ERα is proportional to the estrogen level. In male pigs, continual reduction of endogenous estrogen level is commensurate with an increasing age of the pigs prior to puberty (Ramesh et al., 2007), suggesting that estrogen concentration of the intact pigs in this study was lower than that of the immunocastrated pigs. As a result, ERα H-score of the intact pigs in this study was significantly lower than that of the immunocastrated pigs. On the other hand, the higher ERα H-score in both treatment groups implied that their testes were less mature than those of the intact pigs; this was supported by our previous study that testes of the immunocastrated pigs expressed more anti-Müllerian hormone (AMH) than those of the intact pigs (Srisuwatanasagul et al., 2021). Between immunocastrated groups, the H-score of ERα was not different from each other, connoting that administering the first GnRH vaccine either at 9 or 15 weeks old contributed to the similar results for the expression of ERα. This reinforced our recent study demonstrating that the injection of GnRH vaccine at 9 and 19 weeks of age could be an alternative to immunocastrate the male fattening pigs as the expression of both AMH and cytochrome P450 aromatase was comparable to that at 15 and 19 weeks of age (Srisuwatanasagul et al., 2021). Moreover, our preceding study reported that the immunocastration with GnRH vaccine at 9 and 19 weeks old contributes to similar H-score of Ki-67 proliferative marker, androgen re

CONCLUSIONS

The first injection of GnRH vaccine either at 9 or 15 weeks of age resulted in similar expression of ERα in the testicular tissues of the male fattening pigs.

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AUTHOR CONTRIBUTIONS

Conceptualization and study design: SS, PY, AR
Sample collection: SS, SJ, RY, AA, PY, AR
Laboratory investigation: SS, KS, RY, AR
Data analyses: SS, SM, AR
Manuscript drafting: SS, SM, AR
Manuscript finalization: AR.

CONFLICT OF INTEREST

None of the authors have conflict of interest to declare.

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