จุฬาลงกรณ์มหาวิทยาลัย
ทุนวิจัย
กองทุนรัชดาภิเษกสมโภช

รายงานวิจัย

ผลกระทบของการติดเชื้อไวรัสพีอาร์อาร์เอสในสุกรสาวทดแทนต่อจำนวนและการเจริญของฟอลลิเคิลในเนื้อเยื่อรังไข่

โดย

เผด็จ ธรรมรักษ์

เมษายน ๒๕๕๘

Chulalongkorn University

Research fund

Rachadapiseksompoch

Research report

Impact of PRRS virus infection in the replacement gilts on number and development of follicles in the ovarian tissues

By

Padet Tummaruk

April 2015

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ชื่อโครงการวิจัย ผลกระทบของการติดเชื้อไวรัสพีอาร์อาร์เอสในสุกรสาวทดแทนต่อจำนวนและการเจริญของฟอลลิเคิลในเนื้อเยื่อรังไข่

ชื่อผู้วิจัย รองศาสตราจารย์ น.สพ. ดร. เผด็จ ธรรมรักษ์

เดือนและปีที่ทำวิจัยเสร็จ เมษายน 2558

บทคัดย่อ

สุกรสาวทดแทนเป็นแหล่งที่มาหลักของการนำโรคพีอาร์อาร์เอสเข้าสู่ฟาร์มสุกร การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อประเมินความชุกของการตรวจพบเชื้อไวรัสพีอาร์อาร์เอสในรังไข่ของสุกรสาวที่ถูกคัดทิ้งเนื่องจากปัญหาทางการสืบพันธุ์ เนื้อเยื่อรังไข่จากสุกรสาวพันธุ์ผสมแลนด์เรซ x ยอร์กเชียร์ในประเทศไทย จำนวน 100 ตัว ถูกใช้ในการทดลอง เชื้อไวรัสพีอาร์อาร์เอสในรังไข่ของสุกรสาวถูกตรวจวิเคราะห์ด้วยวิธีอิมมูโนฮีสโตเคมี โดยเฉลี่ยสุกรสาวถูกคัดทิ้งเมื่ออายุ 277 วัน ที่น้ำหนักเฉลี่ย 148 กิโลกรัม จากสุกรสาวทั้งหมดที่ศึกษา 75 ตัว เข้าสู่วัยเจริญพันธุ์แล้ว และ 25 ตัวยังไม่เข้าสู่วัยเจริญพันธุ์ เชื้อไวรัสพีอาร์อาร์เอสถูกตรวจพบในเซลล์เม็ดขาวชนิดแมคโครฟาจภายในรังไข่ ลักษณะทางจุลกายวิภาคศาสตร์ของเซลล์แมคโครฟาจที่ติดเชื้อไวรัสพีอาร์อาร์เอสในรังไข่มีลักษณะคล้ายกับที่พบในเนื้อเยื่อปอด จากเนื้อเยื่อรังไข่ทั้งหมด (n=100) เชื้อไวรัสพีอาร์อาร์เอสถูกตรวจพบใน 70 (70%) รังไข่ ความถี่ในการตรวจพบเชื้อไวรัสพีอาร์อาร์เอสในรังไข่ไม่มีความแตกต่างกันอย่างมีนัยสำคัญระหว่างสาเหตุการคัดทิ้ง (*P*=0.496) สุกรสาวที่เคยแสดงอาการเป็นสัดแล้วตรวจพบเชื้อไวรัสพีอาร์อาร์เอสน้อยกว่าสุกรสาวที่ยังไม่เคยแสดงอาการเป็นสัด (62.8% และ 92.0% ตามลำดับ *P* = 0.005) สุกรสาวที่มีน้ำหนักตัว 151-160 กิโลกรัม มีความถี่ในการตรวจพบเชื้อไวรัสพีอาร์อาร์เอสมากว่าสุกรสาวที่มีน้ำหนักตัว ≤130 กิโลกรัม (52.9% *P* = 0.025) และ 131–140 กิโลกรัม (50.0% *P* = 0.032) อัตราการตกไข่ในสุกรสาวที่มีเชื้อไวรัสพีอาร์อาร์เอสในรังไข่มีแนวโน้มต่ำกว่ารังไข่ที่ไม่มีเชื้อไวรัสพีอาร์อาร์เอส (14.8 และ 16.7 ใบ ตามลำดับ *P* = 0.196) การค้นพบเหล่านี้บ่งชี้ว่าการสัมผัสเชื้อไวรัสพีอาร์อาร์เอสโดยวิธีธรรมชาติในสุกรสาวทดแทนเป็นสาเหตุให้เกิดการคงค้างอยู่ของเชื้อไวรัสในเนื้อเยื่อรังไข่และอาจจะมีผลกระทบต่อการทำงานของรังไข่ได้

คำสำคัญ อิมมูโนฮิสโตเคมี รังไข่ สุกร พีอาร์อาร์เอส ระบบสืบพันธุ์

Project title: Impact of PRRS virus infection in the replacement gilts on number and development of follicles in the ovarian tissues

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Year: 2015

Abstract

Replacement gilts are a major source of introducing the porcine reproductive and respiratory syndrome (PRRS) virus into the swine breeding herd. The objective of the present study was to determine the prevalence of PRRS virus antigen by detection in the ovary of gilts culled due to reproductive disturbances. Ovarian tissues were collected from 100 Landrace x Yorkshire crossbred slaughtered gilts in Thailand. PRRS virus antigen in the ovarian tissues were detected by using immunohistochemistry. On average, the gilts were culled at 277 days of age at a body weight of 148 kg. Of these gilts, 75 had attained puberty and 25 had not attained puberty. PRRS virus antigens were detected in the macrophages within the ovary. The histological morphology of macrophages containing the PRRS virus in ovarian tissue was similar to that found in lung tissue. Of all the ovarian tissues (n = 100), the PRRS virus was detected in 70 (70.0%) ovaries. The detection frequency of the PRRS virus in the ovarian tissue of gilts did not differ significantly among the culling reasons (P = 0.496). The gilts that had exhibited standing oestrus had a lower PRRS virus detection than those that had never exhibited standing oestrus (62.8% versus 92.0%, respectively, P = 0.005). Gilts with a body weight of 151 – 160 kg had a higher frequency of PRRS virus detection (89.5%) than gilts with a body weight of ≤130 kg (52.9%, P = 0.025) and 131–140 kg (50.0%, P = 0.032). The ovulation rate in the ovary containing the PRRS virus tended to be lower than the ovary without the PRRS virus (14.8 and 16.7 CL, respectively, P = 0.196). These findings indicated that the natural exposure to PRRS virus in the replacement gilts caused an existence of the virus in the ovarian tissues and may subsequently influence the ovarian function.

Keywords: Immunohistochemistry, Ovary, Pig, PRRSV, Reproduction

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CHAPTER I

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a common disease in pigs worldwide (Zimmerman et al. 2012). The disease is caused by the PRRS virus, which was discovered in the United States of America (USA) in 1987 (Keffaber 1989). The virus was identified for the first time in The Netherlands in 1990 (Wensvoort et al. 1991). In Thailand, the PRRS virus has been detected in pigs since 1989 (Damrongwatanapokin et al. 1996). The common clinical symptoms of the disease caused by the PRRS virus in gilts and sows include late-term abortion, mummified foetuses, stillborn piglets, and low viability of piglets at birth (Olanratmanee et al. 2013). In practice, the PRRS virus is controlled by intensive gilt acclimatisation, a regular serological monitoring programme, and vaccination with modified live PRRS virus (MLV) and/or killed vaccines (Díaz et al. 2013). Nevertheless, replacement gilts remain to be a major source of introducing new strains of the PRRS virus into the herd (Tummaruk and Tantilertcharoen 2012; Nilubol et al. 2014). Under field conditions, an intensive acclimatisation of replacement gilts with culled sows or nursery pigs is commonly practised in most swine breeding herds in Thailand. Although the titre of antibody against the PRRS virus is often carefully monitored, detection of the PRRS virus in the reproductive organs of these gilts has never been done (Tummaruk and Tantilertcharoen 2012). Moreover, the titre of antibody against the PRRS virus of the gilts is highly variable both within and between herds (Tummaruk and Tantilertcharoen 2012). This causes difficulties for the farmer when deciding to mate the gilts and occasionally causes infertility problems in some batches of replacement gilts. The previous study found that 73% (122/166) of replacement gilts culled due to reproductive failures (e.g., anoestrus, abnormal vaginal discharge, repeat service, and abortion) had been infected with the PRRS virus (Tummaruk and Tantilertcharoen 2012). Furthermore, PRRS virus antigen could be detected in the uterine tissue in 33.0% of the gilts culled due to reproductive failures (Olanratmanee et al. 2011). This may compromise the fertility of replacement gilts.

In general, the PRRS virus primarily infects pulmonary alveolar macrophages of the pig during the acute stage of infection (Sur et al. 1997). Likewise, macrophages from other organs are also the primary cell type that sustains the *in vivo* replication of the virus (Thanawongnuwech et al. 2000). Our previous study found that the PRRS virus was detected in the cytoplasm of the macrophages in the sub-epithelial connective tissue layers of the endometrium of the gilts culled due to reproductive failures (Olanratmanee et al. 2011). Interestingly, the detection frequency of PRRS virus antigen varied between the herds, from 14.3% to 80.0% (Olanratmanee et al. 2011). Moreover, the PRRS virus exists in the uterine tissue of the infected gilts for several months even though vaccination has been done

(Olanratmanee et al. 2011). The most common reproductive disturbances among replacement gilts is anoestrus and/or delayed puberty (Tummaruk et al. 2009). This problem may be associated with ovarian dysfunction in gilts infected with the PRRS virus during an intensive acclimatisation period. If so, gilt acclimatisation practices need to be revised. Nevertheless, to enhance our understanding of this issue, additional research needs to be carried out on the pathogenesis as well as the factors associated with PRRS virus infection in replacement gilts. To our knowledge, PRRS virus detection in the ovarian tissue of gilts naturally exposed to the PRRS virus associated with the gilts' age, body weight, average daily gain (ADG), ovarian status, and delayed puberty has not been comprehensively determined.

In the boar, the PRRS virus is able to induce apoptosis of the testicular germ cells (Sur et al. 1997), while in the female's gonad, only limited information is known. To our knowledge, only one study has demonstrated that the PRRS virus can penetrate the resident macrophages of the ovary but its involvement in follicle development, ovulation, and corpus luteum formation is not clearly understood (Sur et al. 2001). Under experimental conditions, the PRRS virus was detected in the ovarian tissue of gilts for 21 days post infection (Sur et al. 2001). Nevertheless, the role of natural PRRS virus infection in the existence of the virus in gilts' ovaries has not been elucidated. Also, little is known about the mechanism of pathogenesis underlying ovarian infection by the PRRS virus. The objective of the present study was to determine the prevalence of PRRS virus antigen detection in the ovarian tissue of gilts in relation to the gilts' historical data, e.g., culling reason, and reproductive data, e.g., age, body weight, ADG, ovarian status, and delayed puberty.

CHAPTER II

LITERATURE REVIEW

The selection of high quality replacement gilts is an important key to improving pig production, since production from individual females contributes to the overall productivity of swine herds (Roongsitthichai et al. 2013). Gilts and sows with a history of poor reproductive performance (e.g., prolonged weaning-to-service interval, repeated service and abortion) have suboptimal productivity and short longevity (Takai and Koketsu 2007; Tummaruk et al. 2010). In the last decade, the replacement rate of gilts in substitution for culled sows has been 40% to 60% per year (Engblom et al. 2007). This high replacement rate is because of the high level of unplanned removal and culling of sows at a younger age than in the past (i.e., parities above 6) (Engblom et al. 2007). This has also affected production costs and herd efficiency. In general, the proportion of gilts in commercial swine herds accounts for 20% to 25% of the sows in production. This reflects the importance of selecting quality gilts to be the production units in a herd. In general, age, body weight, and estrous behavior of gilts are the major criteria to be considered before first mating. Based on economic analysis, gilts should conceive before 220 to 230 d of age (Schukken et al. 1994; Koketsu et al. 1999). Koketsu et al. (1999) revealed that if the age of first insemination of gilts was increased, their longevity decreased. Gilts that have a low average daily gain (ADG) tend to have a low conception rate and have to be removed from herds due to reproductive disturbances (Sur et al. 1997; Roongsitthichai et al. 2013). It has been demonstrated that 15% to 20% of sows are removed from a herd after having produced only one litter, and more than 50% of them are removed before attaining the fifth parity (Engblom et al. 2007).

Porcine reproductive and respiratory syndrome (PRRS) virus has been isolated from the ovaries of gilts (Sur et al. 2001). PRRS virus is transmitted following an exposure of naïve animals to infected animals or an expose with semen from infected boars. The duration of viral shedding in the boar semen of the infected boars varies from 2 to 92 days (Karniychuk and Nauwynck 2013). PRRS virus reaches the tissue of the boar reproductive tract by migration of infected macrophages (Prieto and Castro 2005). In the female pigs, endometrial tissue can be infected by PRRS virus contaminated semen and thereafter distribute to regional lymph node and other organs (Prieto and Castro 2005). In boars, it has been clearly demonstrated that the virus is able to induce apoptosis of testicular germ cells (Sur et al. 1997) however, in female gonads, only limited information is available about its impact. To our knowledge, only one study has demonstrated that PRRS virus can penetrate the resident macrophages of the ovary, but its involvement in follicle development, ovulation and corpus luteum formation is not clearly understood (Sur et al. 2001). In the porcine ovary, macrophage are an essential cells for cleaning up apoptotic debris during follicular atresia (Miyake et al. 2006). The presence

of macrophages containing phagocytosed cells in cytoplasm has been reported in follicles of pig (Sur et al. 2001). Furthermore, macrophages also play many important roles in the porcine ovaries, e.g., phargocytosis and degeneration of foreign antigen, matrix dissolution and tissue remodeling, production and secretion of cytokines, chemokines, and growth factors (Miyake et al. 2006). Sine majority of the porcine follicles undergo atresia, macrophages is therefore an important cell for eliminating those follicles that will not be ovulated and thus maintain normal function of the porcine ovary. Since macrophage is an important target cell of PRRS virus infection, the association between some ovarian activities, e.g., follicular growth, and the presence of PRRS virus in the ovarian tissue of replacement gilts is therefore of interest.

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase delta that is necessary for DNA synthesis (Kurki et al. 1986; Bravo and McDonald-Bravo 1987). The expression of PCNA increases during the G1 phase, reaches its highest level in the S-phase and decreases during the G2/M phases of the cell cycle (Kurki et al. 1988). An earlier study has demonstrated that expression of PCNA in the ovaries is involved with follicular development in many species, e.g., rats (Oktay et al. 1995), cows (Wandji et al. 1996), baboons (Wandji et al. 1997), pigs (Phoophitphong et al. 2012) and humans (Kelsey et al. 2010). The aims of the present study were to quantify the number of follicles and determine granulosa cell proliferation in the ovaries of gilts in relation to ovarian status (i.e., prepubertal and cycling ovaries) and PRRS virus detection.

CHAPTER III

MATERIALS AND METHODS

Experimental designs

The present study included two experiments: ***Experimental I*** (Detection of porcine reproductive and respiratory syndrome virus in the ovary of gilts culled due to reproductive disturbances) and ***Experimental II*** (Granulosa cell proliferation in the gilt ovary associated with ovarian status and porcine reproductive and respiratory syndrome virus detection). Experimental I included 100 ovarian tissues and experimental II included 37 ovarian tissues from crossbred Landrace x Yorkshire replacement gilts (see below). Immunohistochemical detection of the PRRS virus was applied in both experiments and immunohistochemistry of PCNA was applied in experimental II. Both PRRS virus detection and proliferation of granulosa cells in the porcine ovaries were carefully determined by using Image-Pro® Plus software under a light microscope.

Animals, data, and sample collection

Experimental I: Ovarian tissues were collected from 100 Landrace x Yorkshire crossbred gilts from two commercial swine herds in Thailand. Historical data of the gilts including the gilts' identity, the date of birth, date of entry into the herd, date of first observed oestrus, date of culling, body weight at culling, and culling reason were collected. The age at culling was calculated. ADG from birth to culling was calculated: ADG (g/day) = (body weight at culling – 1.5/ age at culling) x 1,000. The ovaries and reproductive tract of the gilts were collected immediately after slaughter at an abattoir and were placed on ice and transported to the laboratory within 24 h. The appearance of the ovaries was used to define the stage of the oestrous cycle. The ovarian appearance was defined according to a previous study (Tummaruk et al. 2009). Briefly, corpora lutea (CL) were the structures on the ovaries with a pink, tan, or yellow colour and with a diameter of between 5 to 15 mm (Figure 1). Corpora albicantia (CA) were regressing and shrunken CL. Follicles were transparent fluid-containing structures in the ovaries (Figure 1). The number of CL was counted and defined as the number of ovulations. The ovary was defined as 'pre-pubertal' when containing only small follicles and having no CL or CA, and was defined as 'cycling' when containing CL or CA and follicles (Figure 1). Of the cycling ovaries, 'luteal phase' was characterized by ovaries that had CL, and 'follicular phase' by ovaries that had follicles of 7 to 12 mm in diameter either with or without CA (Tummaruk et al. 2010). The reasons for culling were classified into five groups, i.e., anoestrus, abnormal vaginal discharge, abortion, repeat breeding, and miscellaneous (e.g., lameness, inverted nipple, and illness) (Tummaruk et al. 2009).

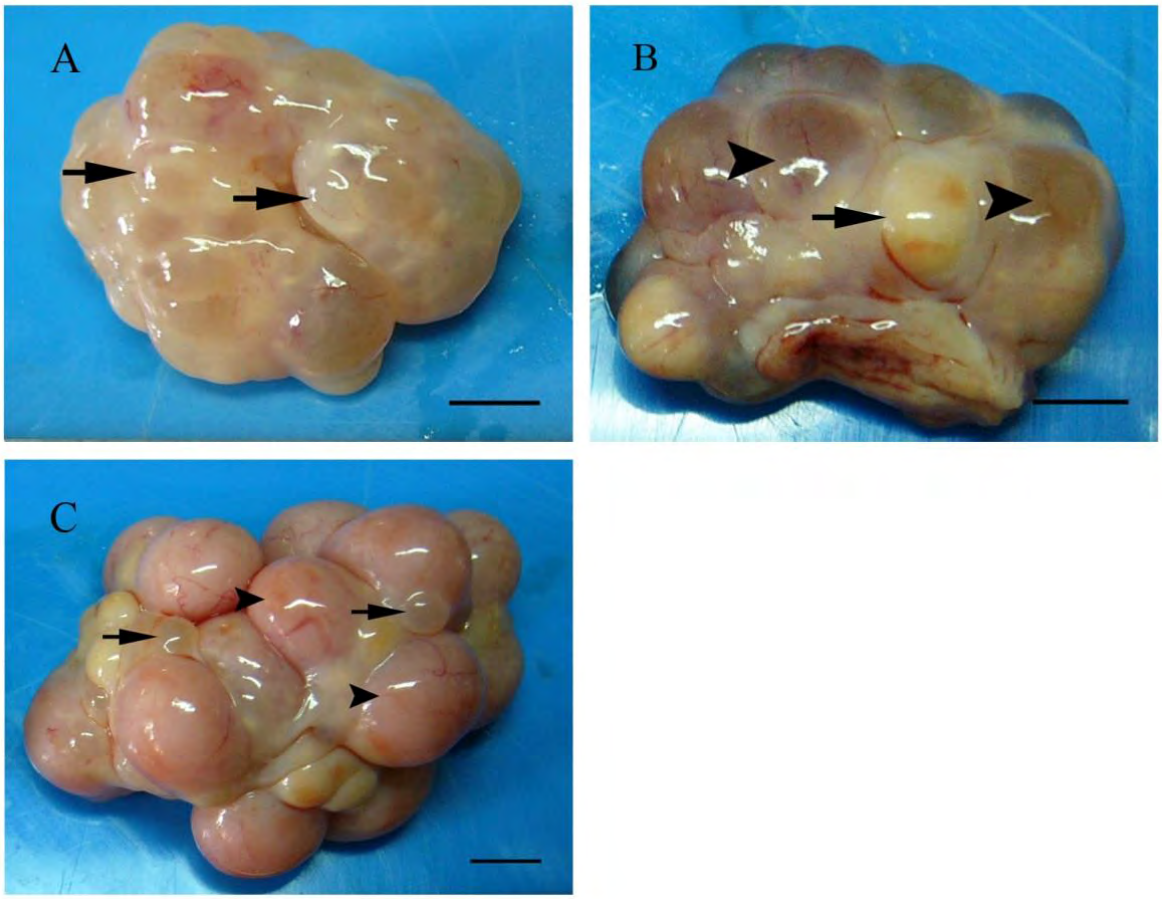

Figure 1 Gross morphology of the gilt's ovary: (A) ovary of a pre-pubertal gilt containing numerous small follicles (arrows); (B) ovary of a pubertal gilt during the follicular phase, containing numerous large follicles (arrowheads) and corpora albicantia (arrow); (C) ovary of a pubertal gilt during the luteal phase, containing numerous large corpora lutea (arrowheads) and small follicles (arrows). Bar = 1.0 cm.

Experimental II: Ovarian tissues of 37 Landrace x Yorkshire crossbred gilts were obtained from slaughter houses. The genital organs and historical data of the gilts were collected from two commercial swine herds in Thailand. The historical data of the gilts consisted of the herd and gilt identity, breed, date of birth, date of entry into the herd, date of first observed estrus, date of culling, and body weight at culling. The age at culling was calculated. The weight of the ovaries was measured using an electronic balance (BJ 210C, max = 210 g, d = 0.01 g, Precisa Instruments Ltd., Switzerland). The appearance of the ovaries was used to define the stage of the oestrous cycle. The ovarian structures were defined according to a previous study (Tummaruk et al. 2009a). Briefly, corpora lutea (CL) were the structures on the ovaries with a pink, tan or yellow colour and a diameter of between 5 and 15 mm. Corpora albicantia (CA) were regressing and shrunken CL. Follicles were transparent fluid-containing structures in the ovaries (Figure 2). The number of CL was counted and defined as the number of ovulations. The ovary was defined as 'prepubertal' when it contained only small follicles (diameter ≤3

mm) (Evans and O'Doherty 2001; Tummaruk et al. 2009a) and had no CL or CA (Figure 2A), and as 'cycling' when it contained CL or CA and follicles (Figure 2B).

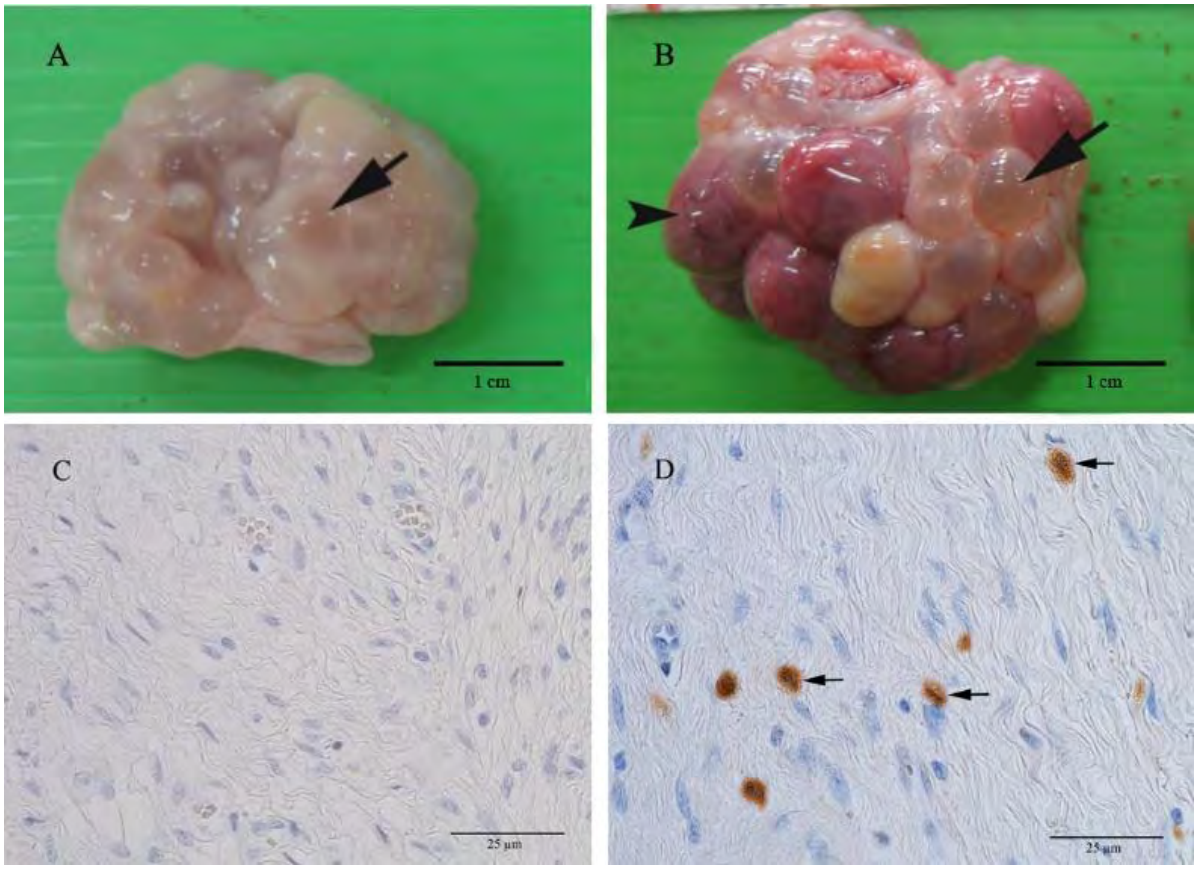

Figure 2 (A-B) Small follicles (arrow) in prepubertal ovaries and corpus luteum (arrow-head), corpus albicantea and follicles in cycling ovaries (bar = 1 cm) (C-D) Immunohistochemical expression of negative (A) and positive (B) PRRSV in macrophages in gilt ovarian tissue

General management and vaccination

The herds in the present study were breeding herds in the north-eastern (Herd A) and middle (Herd B) parts of Thailand. The numbers of sows-on-production were 2,000 (Herd A) and 3,500 (Herd B) sows/ herd. Herd A produced replacement gilts within the herd using their own grandparent stock, while Herd B bought replacement gilts from another breeder. The gilts in both herds were housed in a conventional open-housing system facilitated with a water sprinkler and fan for reducing heat stress. The health status of the herds was monitored routinely by the herd veterinarian. In general, the recommended gilt vaccination programme consisted of foot-and-mouth disease, classical swine fever, Aujeszky's disease, and porcine parvovirus at between 22 – 30 weeks of age. In both herds, the replacement gilts were also vaccinated with a PRRS MLV vaccine (Ingelvac® PRRS™ MLV, Boehringer-Ingelheim Vetmedica Inc., St. Joseph, Missouri, USA) before being sent to the breeding house.

Management of gilts and oestrus detection

In general, the replacement gilts were kept in pens in groups of 6 to 15 gilts/pen with a space allowance of 1.5 to 2.0 m^2/gilt. Oestrus detection was performed daily using observations for vulva symptoms (i.e., reddening, swelling, and mucous discharge) and a back pressure test in the presence of a mature boar (Tummaruk et al. 2007). The gilts were provided water ad libitum using water nipples and they were fed twice a day (about 3 kg of feed/gilt/day) with a corn-soybean-fish ration containing 16 to 18% crude protein, 3,000 to 3,250 kcal/kg metabolisable energy and 0.85 to 1.10% lysine. In general, the herds recommended breeding the replacement gilts from 32 weeks of age onwards at second or later estrus and at a body weight of at least 130 kg. All herds used conventional artificial insemination.

Tissue processing

The ovaries were fixed in 10% neutral-buffered formalin for 24 – 48 h, processed by an automatic tissue processor (Tissue-Tek VIP 5 Jr., Sakura, Tokyo, Japan), and embedded in a paraffin block (Tissue-Tek TEC, Sakura, Tokyo, Japan). The paraffin embeddings were cut into 4 μm thickness using a microtome (Shandon, Anglia scientific instrument ltd., Cambridge, UK). The tissue sections were used to detect PRRS virus infection using immunohistochemistry.

Immunohistochemical detection of the PRRS virus

PRRS virus detection in the gilt's ovary was carried out by immunohistochemistry according to our previous protocol (Olanratmanee et al. 2011). Briefly, the ovarian tissue sections were placed on 3-aminopropyltriethoxysilane-coated slides. The sections were deparaffinised in xylene and rehydrated in graded alcohol. A polymer-based non-avidin-biotin technique was applied. An antigen-retrieval technique was used in order to enhance the reaction between antigens and antibodies by enzymatic treatment using 0.1% trypsin at 37°C for 30 min. After washing in phosphate-buffered saline (PBS), endogenous peroxidase activity was inhibited by immersing the sections in 0.3% hydrogen peroxide (H_2O_2) in absolute methanol for 30 min at room temperature. Thereafter, the sections were blocked with 1.0% bovine serum albumin at 37°C for 30 min and incubated at 4°C overnight (12 – 15 h) with a diluted (1:1000) primary monoclonal antibody SDOW17 (Rural technologies, Inc., USA). After washing in PBS, dextran coupled with peroxidase molecules and goat secondary antibody (Dako REAL™Envision™/HRP, Rabbit/Mouse®, Dako, Denmark) was applied to the tissue sections and incubated at 37°C for 45 min. In the final step, the colour of the bound enzyme (brown colour) was obtained by incubating with 0.05% 3,3'–diaminobenzidine tetrahydrochloride (DAB) for 4 – 15 min. All sections were counterstained with Mayer's haematoxylin, dehydrated, and mounted for investigation under a light microscope. Negative control procedures included the omission of primary antibody. Known PRRS virus–infected

lung tissues served as positive controls. The sections were interpreted as positive if they contained at least one positive cell (brown intracytoplasmic staining) (Figure 3).

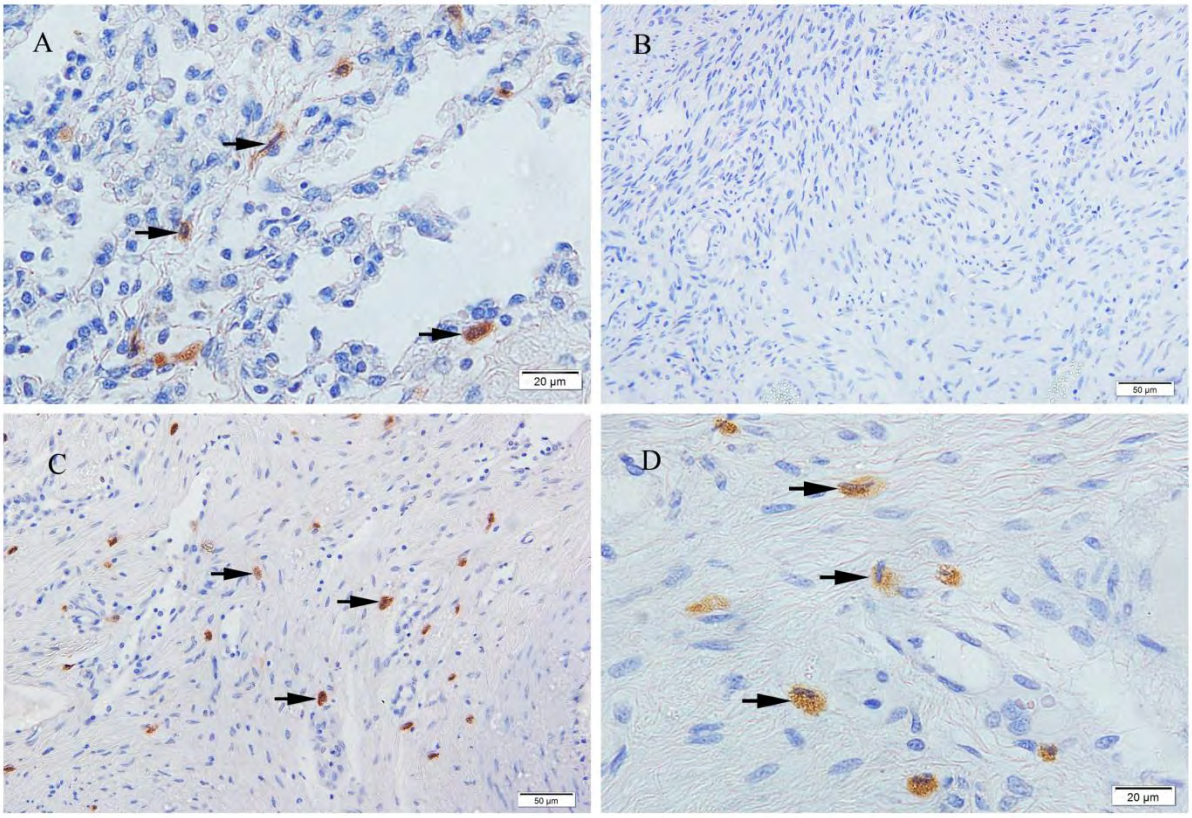

Figure 3 Immunohistochemical staining of porcine reproductive and respiratory syndrome (PRRS) virus antigen (arrows) in (A) positive control (lung tissue), (B) negative control, and (C – D) ovarian tissue.

PCNA immunohistochemistry

A PCNA immunostaining technique was carried out according to our previous study (Phoophitphong et al. 2012). Briefly, the pig ovarian tissues were deparaffinized and placed in an antigen retrieval solution (0.01 M citrate buffer, pH 6.0) in a microwave oven at 600 watts for 10 min. Endogenous peroxidase was blocked using freshly prepared 3.0% hydrogen peroxide at room temperature for 10 min. Nonspecific staining was blocked with 1.0% bovine serum albumin at 37 °C for 30 min. The slides were incubated with mouse monoclonal anti-PCNA (clone PC10, DAKO, Carpinteria, CA, USA) as a primary antibody at a dilution of 1:200 at 4 °C overnight. After incubation with the primary antibody, the sections were incubated with DAKO EnVision™ reagent for 45 min at room temperature. Staining was developed with 3, 3'-diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma, Germany) for 3 min at room temperature. The sections were counterstained with Mayer's hematoxylin for 1 min, dehydrated through a graded series of ethanols, placed into xylene and mounted with mounting media. During the steps in the staining procedure, the slides were washed 3 times

in PBS for 5 min each time. For the negative control, PBS was used instead of the primary antibody (Figure 4A).

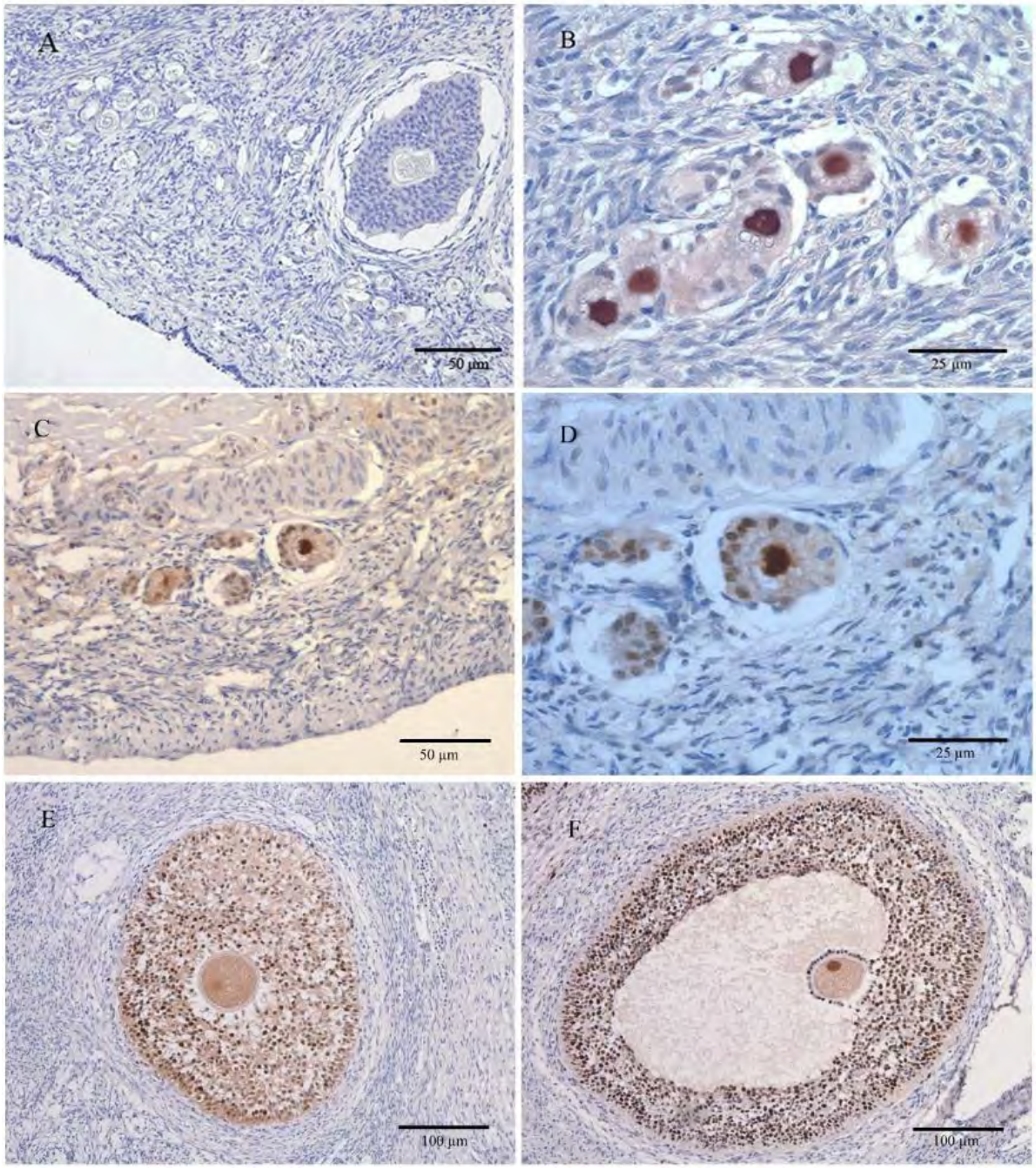

Figure 4 Hematoxylin staining (A, negative control) and PCNA immunostaining of primordial follicles (B) primary follicles (C-D), pre-antral (E) and antral (F) follicles at magnification: X 20 (A, C, E and F) and X 40 (B and D)

Follicle categorization

The follicles were classified into three categories as described earlier (Phoophitphong et al. 2012), i.e., (I) "primordial follicles" were follicles having an oocyte surrounded by one or

more flattened pre-granulosa cells at the periphery of the follicle and no cuboidal cells (Figure 4B); (II) “primary follicles” were follicles having a central oocyte surrounded by either a mixture of flattened pre-granulosa cells and plumb cuboidal granulosa cells or a single layer of cuboidal granulosa cells (Figure 4 C, D); and (III) “growing follicles” including both pre-antral (secondary follicle) and antral follicles (tertiary follicle) and consisting of a visible zona pellucida and/or multiple layers of granulosa cells (Figure 4 E, F). Pre-antral follicles were follicles having a central oocyte and visible zona pellucida surrounded by multiple layers of granulosa cells with no antral formation (Figure 4 E). Antral follicles were follicles having an oocyte and zona pellucida surrounded by multiple layers of granulosa cells with antral formation (Figure 4 F). For each category of follicle, both the number and proportion of follicles were calculated. The number of follicles counted per 100 μm^2 of the real tissue section was calculated by: the number of follicles per 100 μm^2 = (number of counted follicles/ tissue area) x 100. The proportion of follicles was calculated by: the proportion of each category of follicles = (number of each category of follicles/ total number of all follicles) x 100. The total number of follicles counted per 100 μm^2 was the sum of primordial follicles, primary follicles and growing follicles per 100 μm^2.

Determination of granulosa cell proliferation

Granulosa cell proliferation was determined in pre-antral (n = 197) and antral (n = 76) follicles from 37 ovaries. All of the visible pre-antral and antral follicles in the ovarian tissue sections of the gilts were evaluated. In total, 273 follicles were evaluated. On average, 7.3 follicles (range 1 to 18) per ovary were identified. Granulosa cells with positive PCNA staining were evaluated using Image-Pro® Plus software under a light microscope (Figure 5). The percentage of PCNA positive granulosa cells was calculated as: PCNA positive cells (%) = [(number of PCNA positive cells/ total number of granulosa cells counted) x 100]. The follicles were classified according to their ovarian status i.e., prepubertal (n = 59) and cycling (n = 214) ovaries. Also, the gilts were classified into four groups according to age: 229 – 258 d (n = 55), 259 – 272 d (n = 125), 274 – 284 d (n = 33), and 287 – 345 d (n = 60).

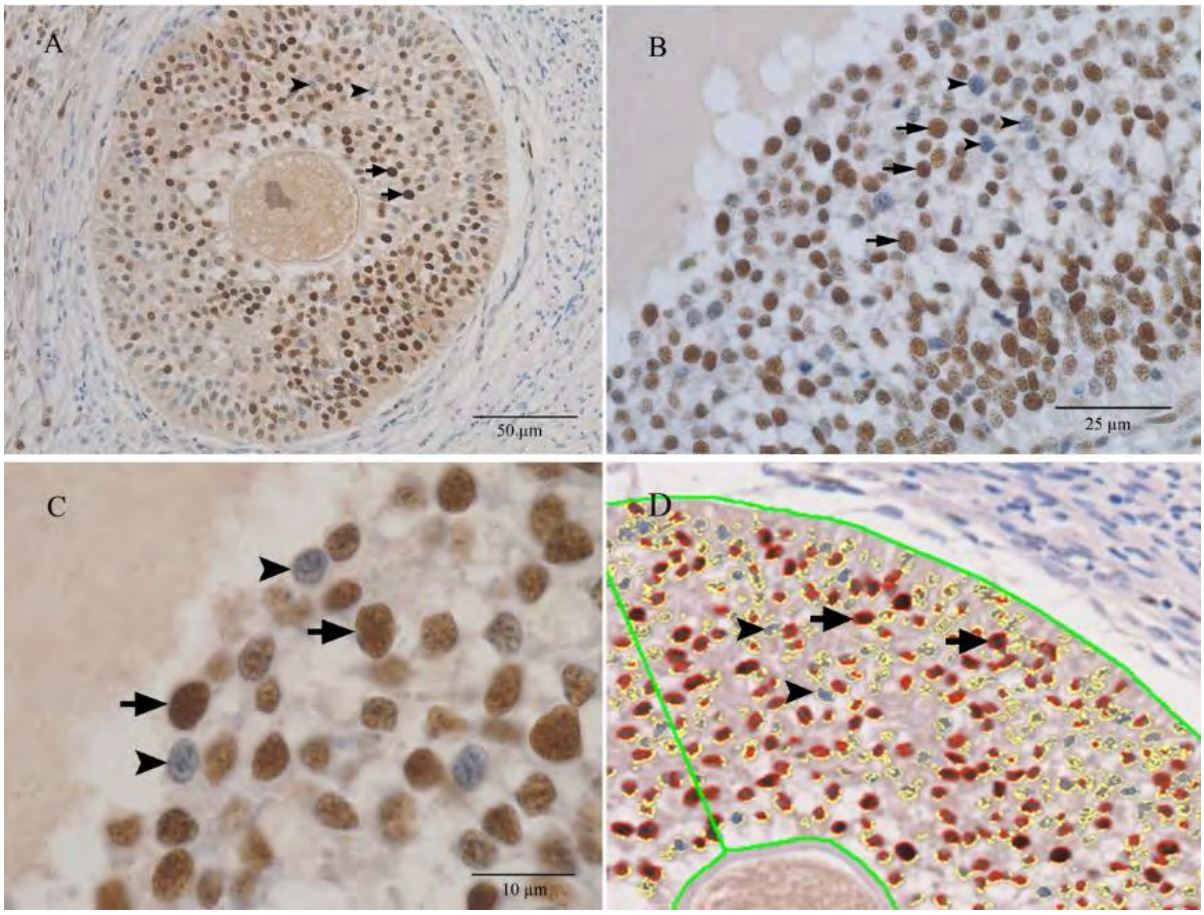

Figure 5 PCNA immunostaining of granulosa cell proliferation in pre-antral follicle and antral follicles at magnification: X 20 (A), X 40 (B) and X 100 (C) and demonstration of image analysis (D). Positive immunostaining for PCNA in granulosa cells stained dark brown (arrow) and negative immunostaining in granulosa cells stained blue (arrow-head)

Statistical analyses

Statistical analyses were carried out by using SAS (SAS, 2002). Data in experimental I are presented as percentages. The frequency of PRRS virus detection in the ovarian tissue of gilts was compared by culling reasons, ovarian status (pre-pubertal, follicular and luteal phases), age classes, body weight classes, ADG classes, season, oestrus behaviour, and ovarian pathology, and the number of ovulation classes was compared by using Chi-square and Fisher's exact test.

The numbers of ovarian follicles are presented as mean ± SD. Multiple analysis of variance (ANOVA) was used to analyse continuous data using the general linear model procedure of SAS. Dependent variables included the numbers of each type of follicle counted per 100 μm^2 [i.e., primordial follicles, primary follicles and growing follicles (both pre-antral and antral follicles)], the proportion of each category of follicle, the total number of follicles counted per 100 μm^2, the weight of the ovary and ADG. The statistical model included the effect of ovarian status (prepubertal and cycling ovary), age class (229 – 258, 259 – 272, 274 –

284, and 287 – 345) and PRRS virus detection (positive or negative). The number of CL (i.e., ovulation rate) was analysed by multiple ANOVA. The statistical model included the effect of age class and PRRS virus detection. Least square means were calculated from the statistical models and compared using the least significant difference test with Tukey-Kramer adjustment for multiple comparisons. Pearson's correlation was performed to determine the associations among the percentage with granulosa cell proliferation (i.e., percentage of positive PCNA immunostaining per follicle) and age of the gilts, body weight, ADG, number of ovulations and weight of the ovaries. The percentage of positive PCNA immunostaining per follicle was analysed by multiple ANOVA using the general linear mixed model procedure of SAS. The statistical model included the fixed effect of follicle type (pre-antral and antral), PRRS virus detection, ovarian status, age class, the interaction between follicle type and PRRS virus detection and the interaction between ovarian status and PRRS virus detection. Other interactions were also tested but excluded from the final statistical models due to insignificance ($P > 0.1$). The gilt's identity was included in the statistical model as a random effect. Least square means were obtained from the statistical models and were compared among each category of factors using a least significant difference test with Tukey-Kramer adjustment for multiple comparisons. $P < 0.05$ was regarded as statistically significant.

CHAPTER IV

RESULTS

Experimental I Detection of porcine reproductive and respiratory syndrome virus in the ovary of gilts culled due to reproductive disturbances

Descriptive statistics

Descriptive statistics on the reproductive data of the replacement gilts included in the present study are presented in Table 1. On average, the gilts were culled at 277 days of age at a body weight of 148 kg. Of these gilts, 75 had attained puberty and 25 had not attained puberty. The average age at first observed oestrus was 214 ± 18.8 days. For the gilts that had not attained puberty (n = 25), the average age at culling was 256 ± 16.1 days. Based on the age and the ovarian status (i.e., pre-pubertal phase), 25 % of the gilts were defined as delayed puberty.

Table 1 Descriptive statistics on reproductive data of 100 Landrace x Yorkshire crossbred gilts in Thailand

Items	Mean ± SD	Range
Age at culling (day)	277 ± 33.4	209 – 406
Body weight at culling (kg)	148 ± 16.8	105 – 203
Average daily gain (g/day)	529 ± 67.4	150 – 693
Age at first observed oestrus (day)	214 ± 18.8	156 – 272
Ovulation rate	15.9 ± 4.3	1 – 26
Weight of an ovary (g)	6.3 ± 3.2	1.8 – 16.6

SD, standard deviation

Localisation of the PRRS virus

Histological examination of ovarian tissues of the gilts revealed that PRRS virus antigens were detected in the macrophages within the ovary (Figure 3C). The virus was found as brown staining in the cytoplasm of the macrophages (Figure 3D). The histological morphology of macrophages containing the PRRS virus in ovarian tissue was similar to that found in lung tissue (Figure 3A). Of all the ovarian tissues (n = 100), the PRRS virus was detected in 70 (70.0%) ovaries.

Factors influencing the detection frequency of the PRRS virus in ovarian tissue

The detection frequency of the PRRS virus in ovarian tissues of the gilts classified by culling reasons is displayed in Table 2. As can be seen from the table, the detection frequency

of the PRRS virus in the ovarian tissue of gilts did not differ significantly among the culling reasons (P = 0.496). Nevertheless, the PRRS virus was detected in the gilts culled due to anoestrus more frequently than the gilts culled due to miscellaneous causes (78.9% versus 53.9%, P = 0.073).

Table 2 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by culling reason

Culling reason	Number of gilts	Number of gilts with PRRS virus detection	Percentage
Anoestrus	51	40	78.4[a]
Vaginal discharge	17	11	64.7[a]
Abortion	10	6	60.0[a]
Repeat breeding	9	6	66.7[a]
Miscellaneous	13	7	53.9[a]
Total	100	70	70.0

[a]Same superscript letter within a column denotes data that do not differ significantly (P>0.05)

The number and percentage of PRRS virus detection in the ovarian tissue of gilts by ovarian status are presented in Table 3. The ovarian status (i.e., pre-pubertal, follicular and luteal phases) did not influence the frequency of PRRS virus detection (P = 0.225). The percentage of PRRS virus detection in ovarian tissue by age groups of the gilts is presented in Table 4. The frequency of PRRS virus detection was not dependent on the age of the gilts (P = 0.683).

Table 3 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by ovarian status

Ovarian status	Number of gilts	Number of gilts with PRRS virus detection	Percentage
Pre-pubertal phase	26	20	76.9[a]
Follicular phase	13	11	84.6[a]
Luteal phase	61	39	63.9[a]

[a]Same superscript letter within a column denotes data that do not differ significantly (P>0.05)

The influences of body weight and ADG of the gilts on the frequency of PRRS virus detection are displayed in Table 5 and 6, respectively. Gilts with a body weight of 151 – 160 kg had a higher frequency of PRRS virus detection (89.5%) than gilts with a body weight of ≤ 130 kg (52.9%, P = 0.025) and 131 – 140 kg (50.0%, P = 0.032). Likewise, gilts with an ADG of

500 – 550 g/day and 550 – 600 g/day had a higher frequency of PRRS virus detection than gilts with an ADG of < 500 g/day (81.8 %, 81.8 %, and 46.2 %, respectively, $P < 0.02$).

Table 4 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by age group of the gilts

Age group (days)	Number of gilts	Number of gilts with PRRS virus detection	Percentage
< 250	17	11	64.7[a]
251 – 300	65	45	69.2[a]
> 300	18	14	77.8[a]

[a]Same superscript letter within a column denotes data that do not differ significantly ($P>0.05$)

Table 5 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by body weight group of the gilts

Body weight group (kg)	Number of gilts	Number of gilts with PRRS virus detection	Percentage
< 130	17	9	52.9[a]
131 – 140	12	6	50.0[a]
141 – 150	28	20	71.4[ab]
151 – 160	19	17	89.5[b]
> 160	24	18	75.0[ab]

[a,b]Different superscript letters within a column denote data that differ significantly ($P<0.05$)

Table 6 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by average daily weight gain (ADG) group of the gilts

ADG group (gram/day)	Number of gilts	Number of gilts with PRRS virus detection	Percentage
≤ 500	30	14	46.7[a]
501 – 550	33	27	81.8[b]
551 – 600	22	18	81.8[b]
> 600	15	11	73.3[ab]

[a,b]Different superscript letters within a column denote data that differ significantly ($P<0.05$)

The percentage of PRRS virus detection did not differ significantly between cool and hot seasons (73.8 % versus 64.1 %, respectively, $P = 0.304$). The gilts that had exhibited standing oestrus had a lower PRRS virus detection than those that had never exhibited standing oestrus (62.8 % versus 92.0 %, respectively, $P = 0.005$) (Table 7). The percentages of gilts with PRRS

virus detection in ovarian tissue by ovarian pathology are presented in Table 8. Although the PRRS virus was detected in 80% of ovaries with multiple cysts, it was also detected in 72% of ovaries with a normal appearance ($P > 0.05$). Nevertheless, PRRS virus detection in ovaries with a single cyst was lower than that in ovaries with multiple cysts ($P = 0.03$). Although the frequency of PRRS virus detection was not related to the number of ovulations, the virus was frequently detected in gilts with a low number of ovulation (i.e., ≤ 13 CL) (Table 9). Furthermore, the ovulation rate in the ovary containing the PRRS virus tended to be lower than the ovary without the PRRS virus (14.8 and 16.7 CL, respectively, $P = 0.196$).

Table 7 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by oestrus behaviour

Oestrus behaviour	Number of gilts	Number of gilts with PRRS virus detection	Percentage
Anoestrus gilts	25	23	92.0[a]
Pubertal gilts	75	47	62.8[b]

[a,b]Different superscript letters within a column denote data that differ significantly ($P = 0.005$)

Table 8 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by ovarian pathology

Ovarian pathology	Number of gilts	Number of gilts with PRRS virus detection	Percentage
Normal	79	57	72.2[a]
Single cyst	7	2	28.6[b]
Multiple cyst	10	8	80.0[a]
Miscellaneous	4	3	75.0[ab]

[a,b]Different superscript letters within a column denote data that differ significantly ($P < 0.05$)

Table 9 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by number of ovulations

Number of ovulations	Number of gilts	Number of gilts with PRRS virus detection	Percentage
≤13	11	9	81.8[a]
14–17	33	18	54.6[a]
≥18	15	10	66.8[a]

[a]Same superscript letter within a column denotes data that do not differ significantly ($P>0.05$)

Experimental II Granulosa cell proliferation in the gilt ovary associated with ovarian status and porcine reproductive and respiratory syndrome virus detection

Descriptive data

Descriptive statistics on reproductive data and the density of follicles in the gilt ovarian tissues are presented in Table 10. The average age and live weight at culling of the gilts were 270.7 ± 24.5 d and 144.5 ± 15.0 kg, respectively. On average, each 100 μm^2 of ovarian tissue consisted of 17.0 primordial follicles, 5.0 primary follicles, and 0.5 growing follicles. The proportion of primordial, primary and growing follicles was 71.9%, 25.4%, and 2.6%, respectively. Of the 37 gilts, 27 gilts (73%) had cycling ovaries and 10 gilts had prepubertal ovaries (27%). All of the gilts with cycling ovaries had dominant CL (Figure 2). The ovulation rate was 16.7 ± 3.4 (ranged 9.0 to 23.0).

Table 10. Descriptive statistics on gilts' historical data, gross morphology and density of follicles in their ovarian tissue (n = 37)

Parameter	Mean±SD	Range
Age at culling (d)	270.7 ± 24.5	229.0 – 345.0
Body weight at culling (kg)	144.5 ± 15.0	117.0 – 179.0
Age at first observed oestrus (d)	214.2 ± 17.6	178.0 – 272.0
Average daily gain (g/d)	530.2 ± 55.1	438.0 – 665.0
Weight of the ovary (g)	5.7 ± 2.3	2.9 – 10.1
Ovulation rate	16.7 ± 3.4	9.0 – 23.0
Total number of follicles per 100 μm^2	22.5 ± 10.9	4.3 – 54.2
Number of primordial follicles (follicles/100 μm^2)	17.0 ± 10.3	2.0 – 46.8
Number of primary follicles (follicles/100 μm^2)	5.0 ± 2.9	0.4 – 14.0
Number of growing follicles (follicles/100 μm^2)	0.5 ± 0.2	0.1 – 1.0
Granulosa cell proliferation (%)	61.4 ± 33.6	0.2 – 99.9

Effect of PRRS virus

Of these ovaries (n = 37), PRRS virus was detected in 20 (54.1%) and was not detected in 17 (45.9%). Figure 2 demonstrates positive immunohistochemical expression of PRRS virus in the ovarian tissue of replacement gilts. Reproductive data, follicle density and granulosa cell proliferation in the ovarian tissue of gilts with and without PRRS virus are presented in Table 11. On average, the total number of follicles per 100 μm^2 of ovarian tissue in gilts without PRRS virus (23.7±2.8) did not differ significantly compared to those with PRRS virus (24.5±2.8, $P > 0.05$). The density of primordial, primary and growing follicles per 100 μm^2 of ovarian tissue in gilts with and without PRRS virus is presented in Table 11. As can be seen

from the table, neither the number nor the proportion of follicles in the ovarian tissue of gilts with PRRS virus differed significantly compared to those without PRRS virus (Table 11).

Table 11. Gilts' historical data, gross morphology and follicle density in their ovarian tissue with and without porcine reproductive and respiratory syndrome (PRRS) virus detection (least square means ± SEM)

Parameter	PRRS virus negative (n=17)	PRRS virus positive (n=20)	*P* value
Gross			
Age at culling (d)	275.8 ± 3.5	268.2 ± 3.6	0.088
Body weight (kg)	141.9 ± 3.8	141.5 ± 3.9	0.929
Average daily gain (g/d)	510.9 ± 13.7	522.1 ± 14.0	0.510
Weight of the ovary (g)	5.1 ± 0.5	5.2 ± 0.5	0.920
Ovulation rate	17.5 ± 1.0	16.5 ± 0.8	0.445
Follicles (per 100 μm^2)			
Total number of follicles	23.7 ± 2.8	24.5 ± 2.8	0.818
Number of primordial follicles	19.3 ± 2.6	19.6 ± 2.6	0.927
Number of primary follicles	4.0 ± 0.8	4.5 ± 0.8	0.620
Number of growing follicles	0.4 ± 0.1	0.4 ± 0.1	0.762
Proportion of primordial follicles	75.1 ± 3.7	75.0 ± 3.8	0.978
Proportion of primary follicles	22.9 ± 3.6	22.5 ± 3.7	0.922
Proportion of growing follicles	1.9 ± 0.5	2.5 ± 0.5	0.383
Granulosa cells			
Granulosa cell proliferation (%)	59.6 ± 4.8	56.4 ± 5.3	0.637

PCNA immunostaining of granulosa cells in pre-antral and antral follicles is presented in Figure 5. Granulosa cell proliferation was influenced by the type of follicle ($P < 0.05$), ovarian status ($P < 0.05$), the age of the gilts ($P < 0.05$), and the interaction between ovarian status and PRRS virus detection ($P = 0.06$). On average, the proliferation of granulosa cells in follicles without PRRS virus did not differ significantly compared to those with PRRS virus (59.6±4.7% versus 56.4±5.2%, $P > 0.05$). The granulosa cell proliferation in antral follicles was significantly higher than in pre-antral follicles in both PRRS virus positive and PRRS virus negative ovarian tissue (Table 12).

In ovarian tissue from gilts without PRRS virus, granulosa cell proliferation in cycling ovaries was higher than that in prepubertal ovaries (84.0 ± 5.1% and 35.2 ± 8.0%, $P < 0.05$). However, in ovarian tissue containing PRRS virus, the proliferation of granulosa cells in cycling ovaries did not differ significantly compared to that in prepubertal ovaries (68.5 ± 4.3% and

44.4 ± 9.2%, $P > 0.05$). Furthermore, the highest percentage of granulosa cell proliferation (i.e., 84.0%) was found in cycling ovaries without PRRS virus (Table 13).

Table 12. Granulosa cell proliferation in pre-antral and antral follicles in the ovarian tissue of gilts with and without porcine reproductive and respiratory syndrome (PRRS) virus detection (least-squares means ± SEM)

Follicle type	n	Granulosa cell proliferation (%)	
		PRRS virus negative	PRRS virus positive
Pre-antral	197	52.5 ± 5.1^{aA}	46.6 ± 5.4^{aA}
Antral	76	66.7 ± 6.0^{aB}	66.3 ± 6.3^{aB}

^{a,A,B} Small letters indicate significant differences within rows and capital letters indicate significant differences within columns ($P<0.05$)

Table 13. Granulosa cell proliferation in the follicles of gilts with prepubertal and cycling ovaries with and without porcine reproductive and respiratory syndrome (PRRS) virus detection (least-squares means ± SEM)

Ovarian status	n	Granulosa cell proliferation (%)	
		PRRS virus negative	PRRS virus positive
Prepubertal	214	$35.2 \pm 8.0^{a,A}$	$44.4 \pm 9.2^{a,A}$
Cycling	59	$84.0 \pm 5.1^{a,B}$	$68.5 \pm 4.3^{a,A}$

^{a,A,B} Small letters indicate significant differences within rows and capital letters indicate significant differences within columns ($P<0.05$)

Effect of gilts' age and ovarian status

The gilts' historical data, gross morphology, follicle density and granulosa cell proliferation by age group are presented in Table 14. It was found that the total number of follicles per 100 μm^2 and the proliferation of granulosa cells in the ovarian tissue of gilts aged 274 – 284 d were higher than in gilts aged 229 – 258 d ($P < 0.05$) (Table 14).

Historical data, gross morphology, follicle density and granulosa cell proliferation in gilts with prepubertal ovaries and cycling ovaries are presented in Table 15. As can be seen from the table, gilts with cycling ovaries had greater ovarian weight, ADG, and body weight than gilts with prepubertal ovaries. Furthermore, the number of primary follicles and growing follicles per 100 μm^2 of ovarian tissue in gilts that had cycling ovaries was higher than in those with prepubertal ovaries (Table 15). Granulosa cell proliferation in gilts with cycling ovaries was also higher than that in gilts with prepubertal ovaries (76.2±3.4% and 39.8±6.5%, $P < 0.05$).

Table 14. Gilts' historical data, gross morphology, follicle density and granulosa cell proliferation in ovarian tissue from animals culled at different ages (least square means ± SEM)

Parameters	Age at culling (days, range)			
	229 – 258	259 – 272	274 – 284	287 – 345
Gross				
Age at culling (d)	242.7 ± 4.8[a]	266.6 ± 3.4[b]	277.1 ± 6.5[bc]	301.6 ± 5.1[d]
Body weight (kg)	132.2 ± 5.2[a]	143.3 ± 3.6[a]	146.0 ± 7.1[a]	145.2 ± 5.5[a]
Average daily gain (g/d)	540.0±18.0[a]	532.3±13.1[ab]	520.1±25.8[ab]	473.7±20.0[b]
Weight of the ovary (g)	5.5 ± 0.7[a]	4.8 ± 0.5[a]	5.9 ± 0.9[a]	4.5 ± 0.7[a]
Ovulation rate	15.6 ± 1.2[a]	15.1 ± 1.2[a]	19.5 ± 1.9[a]	17.9 ± 1.1[a]
Follicles (per 100 μm^2)				
Total number of follicles	15.5 ± 3.9[a]	23.5 ± 2.6[ab]	34.5 ± 5.2[b]	23.2 ± 4.1[ab]
Number of primordial follicles	11.3 ± 3.5[a]	18.2 ± 2.4[a]	30.3 ± 4.8[a]	18.2 ± 3.7[a]
Number of primary follicles	3.9 ± 1.1[a]	4.8 ± 0.8[a]	3.6 ± 1.5[a]	4.8 ± 1.2[a]
Number of growing follicles	0.3 ± 0.1[a]	0.5 ± 0.1[a]	0.4 ± 0.1[a]	0.3 ± 0.1[a]
Proportion of primordial follicles	63.8 ± 5.1[a]	75.9 ± 3.5[a]	86.8 ± 6.9[a]	73.8 ± 5.4[a]
Proportion of primary follicles	33.1 ± 5.0[a]	21.5 ± 3.5[a]	11.9 ± 6.8[a]	24.4 ± 5.3[a]
Proportion of growing follicles	3.1 ± 0.7[a]	2.6 ± 0.5[a]	1.3 ± 0.9[a]	1.8 ± 0.8[a]
Granulosa cells				
Granulosa cell proliferation (%)	46.4 ± 6.3[a]	60.5 ± 4.4[ab]	74.2 ± 8.2[b]	60.0 ± 6.6[ab]

[a,b,c,d] Different superscripts within rows indicate significant differences (P<0.05)

Table 15. Gilts' historical data, gross morphology, follicle density and granulosa cell proliferation in ovarian tissue with prepubertal ovaries compared with cycling ovaries (least square means ± SEM)

Parameters	Ovarian status		*P* value
	Prepubertal	Cycling	
Gross			
Age at culling (d)	270.7 ± 4.9[a]	273.3 ± 2.7[a]	0.646
Body weight (kg)	134.7 ± 5.3	148.6 ± 2.9	0.028
Average daily gain (g/d)	492.3 ± 19.3	540.8 ± 10.4	0.034
Weight of the ovary (g)	3.5 ± 0.7	6.8 ± 0.4	<0.001
Ovulation rate	0	16.7 ± 3.4[1]	-
Follicles (per 100 μm^2)			
Total number of follicles	24.4 ± 3.9	23.8 ± 2.1	0.895
Number of primordial follicles	21.2 ± 3.6	17.8 ± 1.9	0.407
Number of primary follicles	3.0 ± 1.1	5.5 ± 0.6	0.053
Number of growing follicles	0.3 ± 0.1	0.5 ± 0.1	0.011
Proportion of primordial follicles	71.3 ± 2.8	78.8 ± 5.2	0.211
Proportion of primary follicles	19.4 ± 5.1	26.0 ± 2.8	0.263
Proportion of growing follicles	1.7 ± 0.7	2.7 ± 0.4	0.268
Granulosa cells			
Granulosa cell proliferation (%)	39.8 ± 6.5	76.2 ± 3.4	<0.001

[1]mean±SD

Localization of Estrogen receptor β in the porcine ovary

The immuno-staining of ERβ in the porcine ovaries are demonstrated in Figure 6 and 7. The immuno-staining of ERβ in the porcine corpus luteum are demonstrated in Figure 8. The ERβ immuno-expression was found in both follicles and corpus luteum of the porcine ovary.

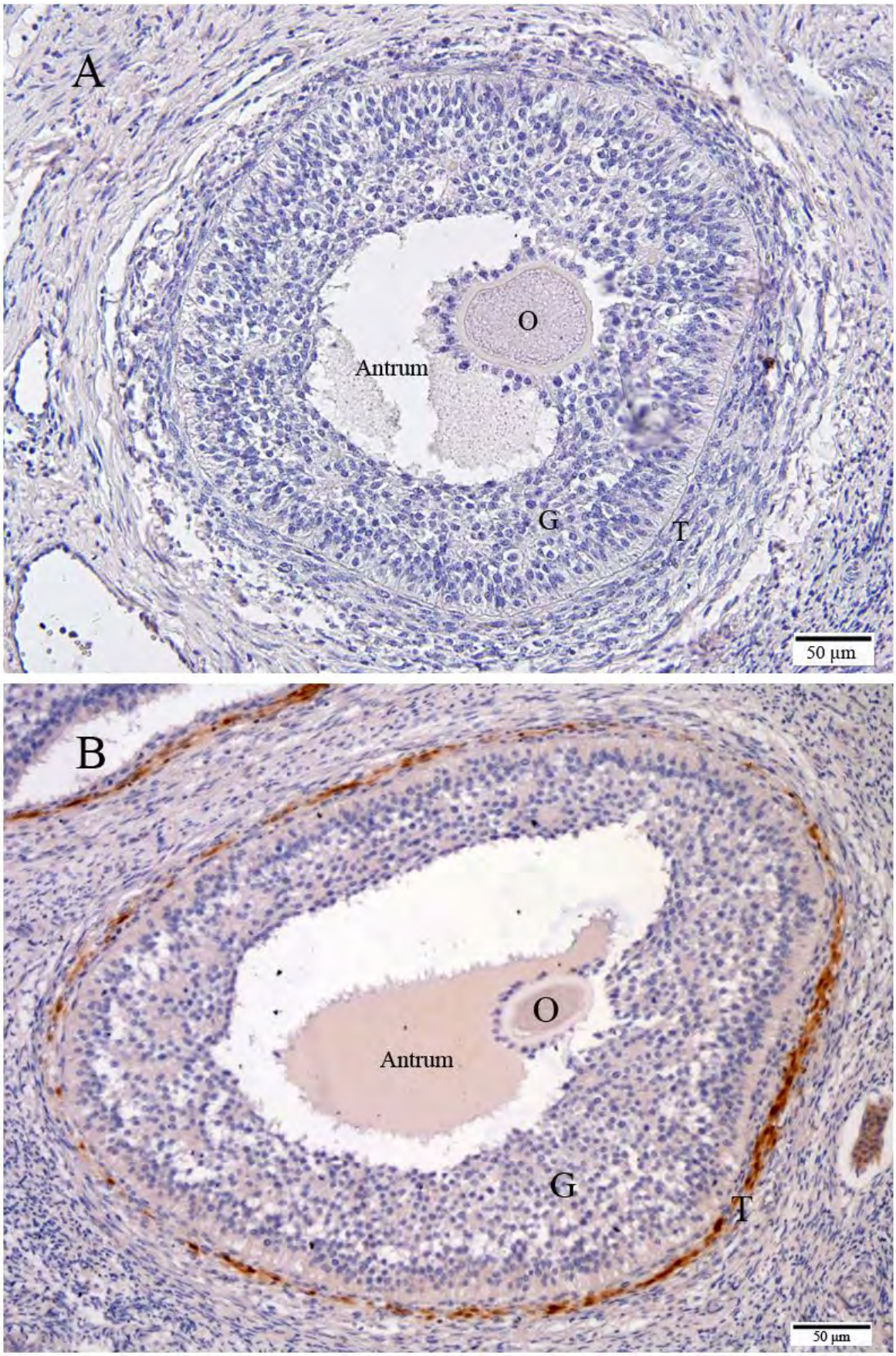

Figure 6 The ERβ immuno-staining in the ovary of pubertal gilts (B) compared with negative control (A). 100x magnification

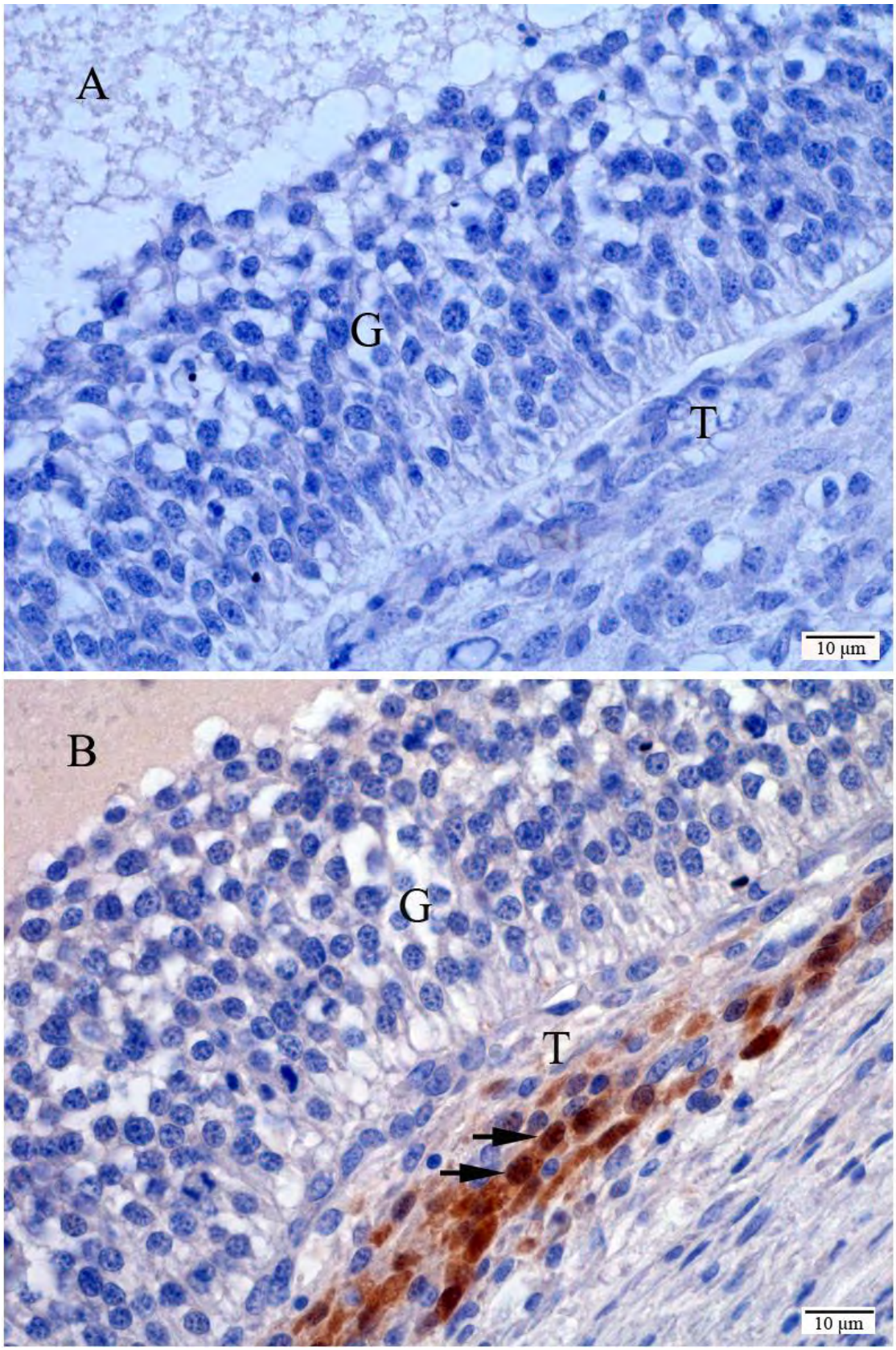

Figure 7 Immuno-staining was found in nuclei and cytoplasm (arrow) of theca cells in follicles (B). Negative control (A). 400x magnification

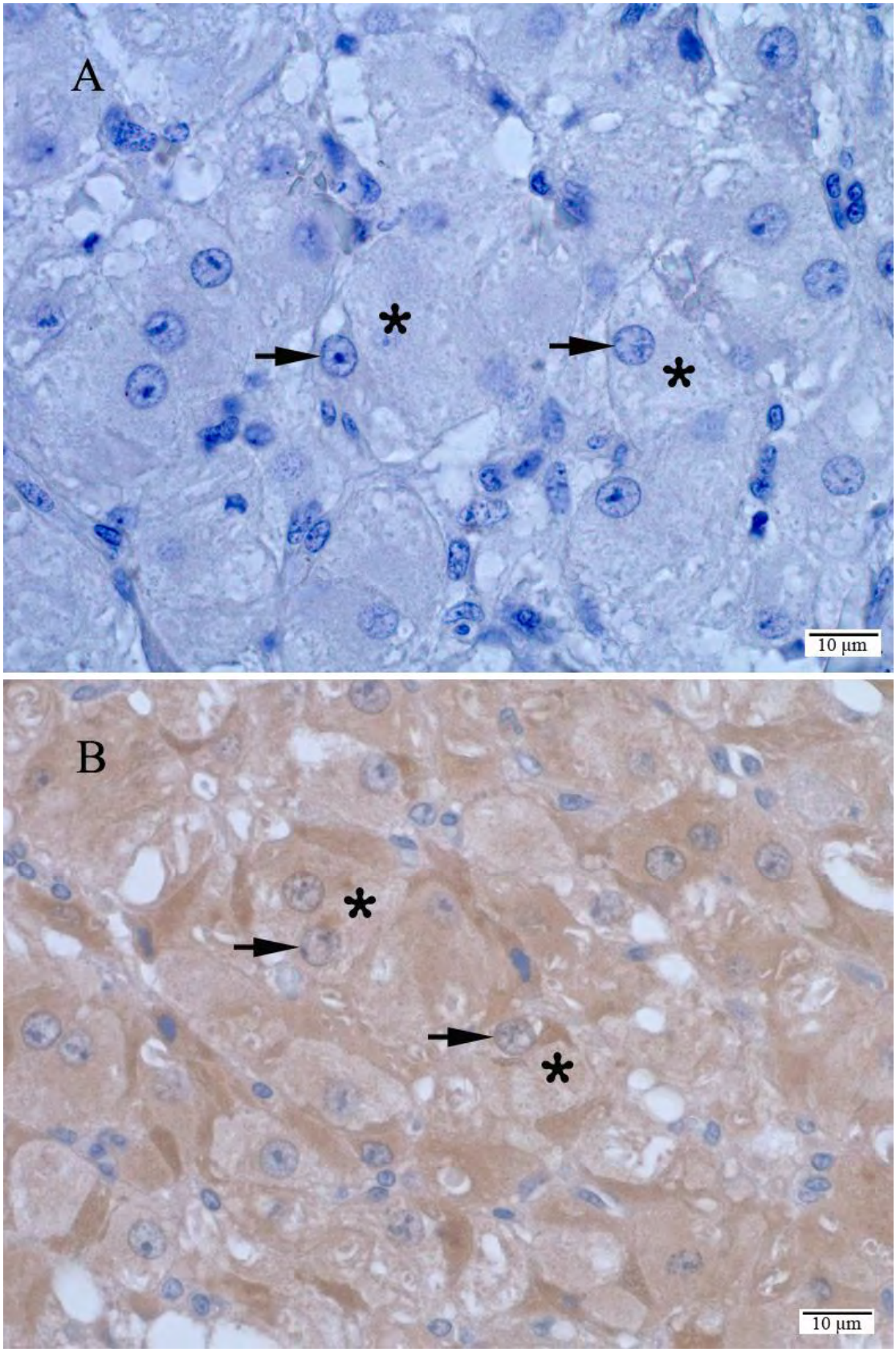

Figure 8 ERβ immunostained was detected in cytoplasm (star) of luteal cells (B) compared with negative control (A) in CL. 400x magnification

Table 16 ERβ immuno-expression in follicles and corpus luteum (CL) in the porcine ovary (mean±SD)

Part of ovary	Number of observation	ERβ expression (%)
Follicles	25	22.6±19.0[a]
CL	25	87.2±9.0[b]

[a,b]different letters within column differ significantly (P<0.05)

The ERβ immuno-staining was mainly detected in the theca cells of the follicles (Figure 7) and in the luteal cells in the CL (Figure 8). In the CL, ERβ immuno-staining located in the cytoplasm of the luteal cells (Figure 8)

In the ovarian follicles, most of the ERβ immunoreaction was found in the cellular nuclei and cytoplasm of the theca cells. This is in accordance with a previous that ERβ protein was exhibited mostly in theca interna cells of the follicle. However, the ERβ immunoreaction was rarely expressed in granulosa cells of the follicle (Figure 6). In the CL, the ERβ immunoreaction was detected in the cytoplasm but not in the nuclei of the luteal cells (Figure 8). This indicated that CL could be a source of estradiol production in the porcine species and also in early luteal phase of bovine species.

The proportion of ERβ expression in the follicles and CL are presented in Table 16. On average, ERβ immunoreaction was detected in 22.6% of the theca cells and in 87.2% of the luteal cells. In conclusion, ERβ immuno-staining was detected in the theca cells and luteal cells in the porcine ovary. This infers that ERβ might play an important role in the porcine ovarian function. Additional study on the variation of ERβ immunoreaction associated with reproductive status will be further investigated.

Impact of PRRS virus infection on the expression of ERβ in the ovary of gilts

Estrogens play the important roles in growth and differentiation of the reproductive system in gilts and sows. The most active estrogen synthesized by antral follicles is estradiol-17β. Theca interna and granulosa cells are major component of the antral follicles that synthesize estrogen. Estrogen regulates reproductive function by binding to specific receptor protein, estrogen receptor (ER). In general, ER is expressed as two isoforms, i.e., ERα and ERβ. ERα is the classical ER that was identified since 1986, while ERβ has been identified later. Although ERα and ERβ are encoded on different chromosome, they have a considerable sequence homology in their domain. Therefore, it can be speculated that the presence of any of the ER subtypes indicate the tissue action of estrogen. The objective of the study was to determine the effect of porcine reproductive and respiratory syndrome (PRRS) virus detection on the expression of ERβ in ovarian follicles of gilt.

Ovarian tissues were collected from 62 Landrace x Yorkshire crossbred gilts. The ovaries were fixed in 10% neutral-buffered formalin and processed histologically. PRRS virus detection in the gilt ovaries was carried out by immunohistochemistry. The ovarian tissues sections were incubated with rabbit polyclonal anti-ERβ as a primary antibody, biotinylated secondary antibody-horse anti-mouse anti-rabbit IgG and avidin-biotin-peroxidase complex. Five follicles per ovarian tissue sections were evaluated using Image-Pro® Plus software. The percentage of ERβ positive cells was calculated. The gilts were classified according to PRRS virus detection as positive (n=38) and negative (n=24). The statistical analyses were carried out using SAS (SAS Cary, NC, USA). Multiple ANOVA was used to analyse the ERβ positive cells using the general linear model procedure of SAS. The statistical model included the effect of reproductive status (prepubertal and cycling gilts), PRRS virus detection (positive and negative) and the interaction between reproductive status and PRRS virus detection. Least-square means were compared using Tukey-Kramer test. $P<0.05$ was regarded as statistically significant.

The result showed that ERβ immunoreaction was found in both pre-antral and antral follicles (Figure 9). On average, ERβ was detected in 23.7% of the theca interna cells surrounding the follicles. The ERβ expression was not detected in the granulosa cells of the porcine ovarian follicles (Figure 9). The percentage of ERβ immuno-staining did not differ significantly between the ovarian tissues detected PRRS virus and those without PRRS virus detection ($P>0.05$). This indicated that although PRRS virus has an effect on reproductive system, the mechanism may not be mediated through the expression of ERβ in the ovary. In conclusions, PRRS virus detection did not significantly influence the ERβ expression in the ovary of gilt.

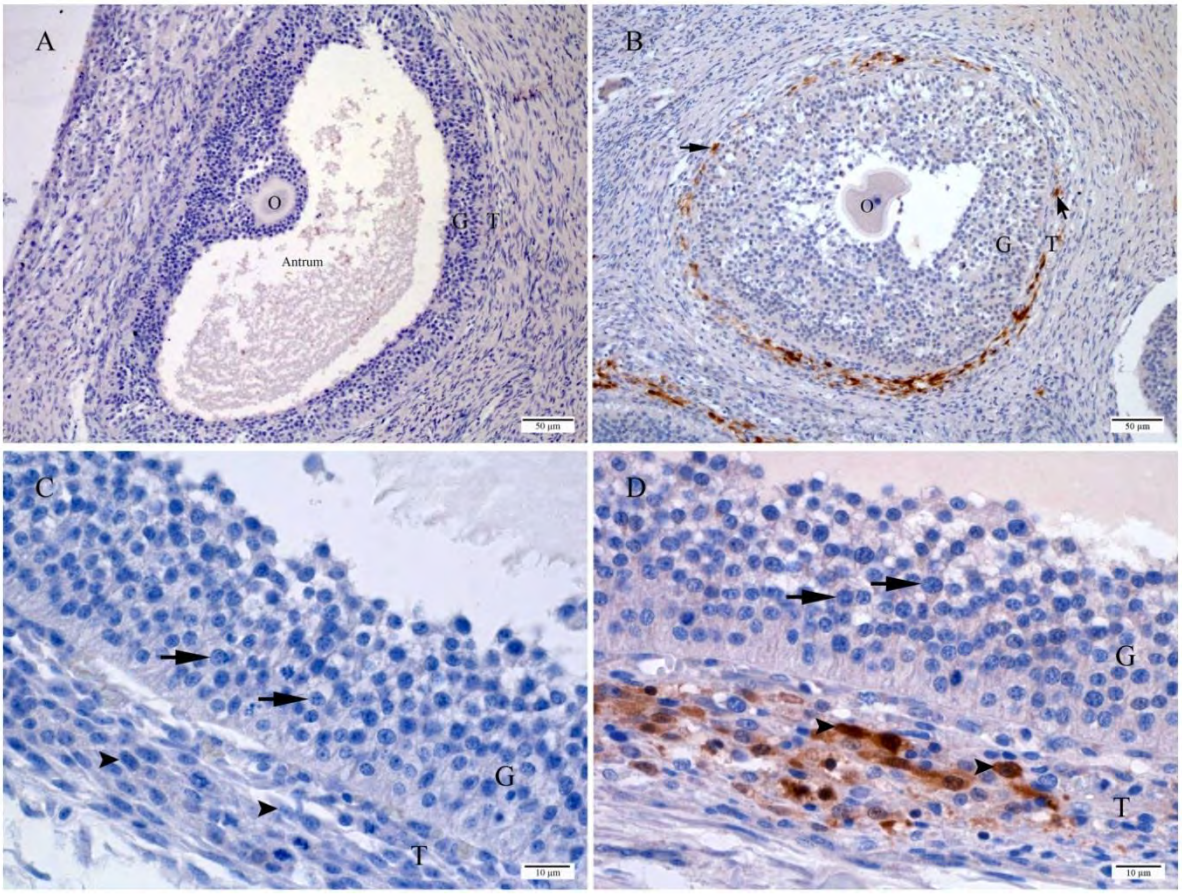

Figure 9 Localization of ERβ protein in the representative paraffin sections of gilt ovarian follicles (A&C) Negative control of ERβ immuno-staining. (B) Black arrows indicate a positive immuno-staining in the ovarian theca interna cells. (D) ERβ positive immuno-staining (arrowhead) was observed only in theca interna cell. Bar = 50 µ.m. O: oocyte; G: granulosa cells; T: theca interna cells. Magnification 40 x (A-B) and 200 x (C-D).

CHAPTER V

DISCUSSION

Experimental I Detection of porcine reproductive and respiratory syndrome virus in the ovary of gilts culled due to reproductive disturbances

The present study reveals the detection of the PRRS virus in the ovarian tissue of replacement gilts under field conditions. In the ovary, the PRRS virus is located in macrophages within the ovarian tissue. This is in accordance with an earlier study (Sur et al. 2001), which found that the PRRS virus could be detected in the ovarian tissue of gilts within 21 days post infection. The study also demonstrated numerous PRRS virus-positive cells in atretic follicles of the ovaries. However, no alternation of the ovarian architecture supporting the possible role of PRRS infection in porcine female infertility could be demonstrated. In contrast, in gestating sows, the experimental challenge of the PRRS virus caused no consistent or significant microscopic lesions in ovarian tissues (Benson et al. 2001). Moreover, no evidence of PRRS virus antigen was detected in the ovarian tissue of sows at 21 days post infection (Benson et al. 2001). This indicates an inconsistency in the appearance of the PRRS virus in porcine ovarian tissue. In the present study, the PRRS virus was detected in 70 % of the ovarian tissues of the replacement gilts culled due to reproductive failure, especially among those culled due to anoestrus. This indicates a possible role of the PRRS virus in ovarian function under field conditions. In general, macrophages in the ovaries are involved in many ovarian functions, e.g., follicular growth, follicular atresia, ovulation, and CL formation (Araki et al. 1996). It was suggested that phagocytic macrophages containing the PRRS virus may deliver the virus to neighbouring cells, such as granulosa cells (Sur et al. 2001). This may subsequently influence granulosa cell function, e.g., steriodogenesis, oocyte maturation, and follicle development. The interruption of these process may influence the oestrus cycle and puberty attainment in gilts. In the present study, the PRRS virus was detected in the gilts with delayed puberty (92.0%) more than those that had already attained puberty (62.8%). The age at culling of these delayed puberty gilts averaged 256 days. Normally, Landrace x Yorkshire crossbred gilts in Thailand attain puberty at 195 days of age (Tummaruk et al. 2007). Thus, the age at puberty is delayed for 61 days. PRRS virus infection in the ovary of replacement gilts may, to some extent, interfere with ovarian functions and contribute to anoestrus problems or delayed puberty in replacement gilts. In boars, PRRS virus infection clearly induces the depletion and death of testicular germ cells and results in hypospermatogenesis (Sur et al. 1997). Moreover, PRRS virus infection in the boar causes a significant reduction in semen quality, e.g., distal cytoplasmic droplet and sperm motility (Prieto et al. 1996; Guérin and Pozzi 2005). The virus might have a role in the ovarian tissue of replacement gilts.

In general, the atretic process of the follicles depends on many factors including the physiological condition of the animal (Orisaka et al. 2009). The atretic process has not yet been well described; however, macrophages play a major role by producing pro-inflammatory mediators, e.g., interleukin 1, interleukin 2, and tumour necrosis factor. A previous study has demonstrated that macrophages are involved in the atretic process in both the pre-pubertal and pubertal stages and macrophages are usually absent in healthy developing swine follicles (Sur et al. 2001). The present study demonstrated the presence of the PRRS virus in both cyclic and non-cyclic gilts. Macrophages infected with the PRRS virus may invade the granulosa cell layer and alter apoptosis and/or proliferation of germinal epithelial cells. However, Sur et al. (2001) did not find any significant differences in ovarian architecture in PRRS virus-infected gilts compared with that in non-infected gilts. Under field conditions, the replacement gilts were exposed to the PRRS virus before the first insemination (Tummaruk and Tantilertcharoen 2012). If the ovaries of the gilts were infected with the PRRS virus, granulosa cell proliferation, steriodogenesis, and oocyte quality may be reduced. This may subsequently cause infertility problems in mated gilts, which is commonly seen under field conditions (Tummaruk et al. 2010). In addition, the PRRS virus is frequently detected in the ovarian tissue of gilts with a relatively good body weight (i.e., 150 – 160 kg), rather than those with a poor body weight. The reason is not known, but this certainly increases the risk of mating the gilts, while the PRRS virus has not been eliminated from their reproductive organs. As a result, fertility of the gilts might be compromised. Based on these findings, it is not recommended to expose gilts to the PRRS virus. Therefore, the acclimatisation of replacement gilts should be omitted or ended for a certain period of time before the first insemination.

In conclusion, the PRRS virus was detected in 70 % of ovarian tissues of the replacement gilts culled due to reproductive failure. The frequency of PRRS virus detection depended on the body weight, ADG, and oestrus cycle. Gilts with a body weight of 151 – 160 kg had a high frequency of PRRS virus detection (89.5 %). This may increases the risk of mating the gilts, while the PRRS virus has not been eliminated from their reproductive organs. The PRRS virus was frequently detected in delayed puberty gilts (92.0 %). The ovulation rate in the ovary containing the PRRS virus tended to be lower than the ovary without the PRRS virus. These findings indicated that the natural exposure to PRRS virus in the replacement gilts cause an existence of the virus in the ovarian tissues and may subsequently influence the ovarian function.

Experimental II Granulosa cell proliferation in the gilt ovary associated with ovarian status and porcine reproductive and respiratory syndrome virus detection

PRRS virus in gilts' ovaries is mainly found in macrophages within the ovarian tissue. Macrophages containing PRRS virus antigen are commonly found in the ovarian medulla, regressing CL and degenerating follicles (Sur et al. 2001). Macrophages in the ovary are involved in many ovarian activities, e.g., follicular growth, follicular atresia, ovulation and CL formation (Araki et al. 1996). In the present study, follicles counted using PCNA immunostaining revealed no differences in follicle density in ovarian tissues with and without PRRS virus. This indicates that PRRS virus does not affect the number of follicles in a gilt's ovaries, in agreement with earlier studies (Benson et al. 2001; Sur et al. 2001). Benson et al. (2001) demonstrated that experimental challenge with PRRS virus in gestating sows caused no consistent or significant microscopic lesions in the ovarian tissues of the sows. Furthermore, no evidence of PRRS virus antigen was detected in the ovarian tissue of sows Day 21 post-infection (Benson et al. 2001). In addition, progesterone levels at 111 d of gestation in PRRS virus-infected sows did not differ significantly compared to those in uninfected sows (Benson et al. 2001). On the other hand, in the testis of boars, PRRS virus infection causes depletion and death of testicular germ cells and results in hypospermatogenesis (Sur et al. 1997). Sur et al. (2001) found that PRRS virus could be isolated from gilts' ovaries at Day 7 post-infection. Numerous PRRS virus-positive cells in the ovaries were found in atretic follicles (Sur et al. 2001). However, no alteration in the ovarian architecture supporting a possible role of PRRS infection in porcine female infertility could be demonstrated. It has been suggested that phagocytic macrophages containing PRRS virus may deliver the virus to neighbouring cells, such as granulosa cells (Sur et al. 2001). In the present study, it was found that granulosa cell proliferation in cycling ovaries was reduced by 18.4% (i.e., from 84.0% to 68.5%, Table 4) in ovarian tissue with PRRS virus compared to ovaries without PRRS virus. The reason might be that PRRS virus infection may interfere with or reduce the synthesis of certain ovarian growth factors, e.g., insulin-like growth factor (IGFs), epidermal growth factor, vascular endothelial growth factor, thrombopoietin, erythropoietin and members of Notch family (Sirotkin 2011). Hence, this may cause poor follicle development and poor steroidogenesis, and may also impair the qualities of the oocytes. For instance, lacking of IGFs decrease the response of granulosa and theca cells and oocytes to gonadotropins (Sirotkin 2011). Although a complete mechanism on the impact of PRRS virus infection on the ovarian function has not been fully elucidated. It is known that macrophage is a target cell of PRRS virus (Olanratmanee et al. 2011) and macrophage plays a critical role on a variety of cellular behavior, e.g., adhesion, migration, invasion and survival (Miyake et al. 2006). This might be in part explain the reduced granulosa cells proliferation in the ovarian tissue infected with PRRS virus.

In general, the atretic process in follicles depends on many factors, including the physiological condition of the animal. The atretic process has not yet been well described; however, macrophages play a major role by producing pro-inflammatory mediators, e.g., interleukin 1, interleukin 2 and tumour necrosis factor. A previous study has demonstrated that macrophages are involved in the atretic process in both the prepubertal and pubertal stages, and are usually absent in healthy developing swine follicles (Sur et al. 2001). We found that PRRS virus was detected in both prepubertal and pubertal gilts. Therefore, PRRS virus infected macrophages may invade the granulosa cell layer and contribute to the apoptosis of germinal epithelial cells (Sur et al. 2001). However, Sur et al. (2001) did not find any significant differences in ovarian architecture in PRRS virus-infected gilts compared to that in non-infected gilts. In the present study, granulosa cell proliferation tended to be reduced in ovarian tissues containing PRRS virus compared to ovaries without PRRS virus. The highest granulosa cell proliferation was found in the follicles of cycling ovaries without PRRS virus detection. A previous study demonstrated that insemination of either seronegative or preimmunized gilts with boar semen containing PRRS virus had no effect on conception or fertilization rates (Prieto et al., 1997a). Furthermore, PRRS virus infection does not appear to affect the embryo before attachment, but may cause embryonic death due to transplacental infection (Prieto et al. 1997b). However, in practice, most replacement gilts were exposed to PRRS virus during the acclimatization period, which takes place sometime before first insemination (Tummaruk and Tantilertcharoen 2012). As indicated by the present results, when the ovaries of the gilts are cycling (puberty), PRRS virus infection may decrease granulosa cell proliferation and possibly cause poor steroidogenesis and compromised oocyte quality. This may subsequently cause infertility problems in mated gilts, which are commonly seen under field conditions (Tummaruk et al. 2010). Based on the information of these findings, pubertal gilts are not recommended to be exposed to PRRS virus. Therefore, the acclimatization process should be done (ended) before the gilts' exhibit first standing heat. On average, Landrace x Yorkshire crossbred gilts in Thailand show first standing heat at 200 d of age (Tummaruk et al. 2009b). Pubertal gilts should be kept in individual stalls for some time (the so called "cool down period") before first insemination takes place.

The limitation of the present study is that all of the reproductive organs were obtained from only PRRS virus seropositive herds. Of these gilts, PRRS virus was detected in the ovarian tissues of one group, but not in the other. It was found that the presence of PRRS virus in the ovarian tissues of the gilts reduced granulosa cell proliferation to some extent, especially in cycling ovaries. However, it should be remarked that no ovarian tissue from PRRS virus seronegative herd could be obtained. The reason is that PRRS virus seronegative herds are rarely found under field conditions in commercial Thai swine herds (Tummaruk et al. 2013).

In the present study, ovarian status also influenced granulosa cell proliferation. It was clearly demonstrated that granulosa cell proliferation in cycling ovaries was higher than that

in prepubertal ovaries. In fact, the ages of the gilts with cycling and prepubertal ovaries did not differ significantly, while the growth rate and body weight in gilts with cycling ovaries were higher than in those with prepubertal ovaries. This indicated that the physical maturity of gilts with cycling ovaries might be somewhat more advanced than those with prepubertal ovaries. Nevertheless, the total number of ovarian follicles per 100 μm^2 of ovarian tissue as well as the proportion of each category of follicle did not differ significantly between cycling and prepubertal ovaries (Table 6). This indicated that gilts with prepubertal ovaries certainly had a sufficient number of ovarian follicles but failed to accomplish ovulation. The reason might possibly be a lack of steriodogenesis, suboptimal luteinizing hormone (LH) secretion or impaired LH receptors at the ovarian level. The present study demonstrated that granulosa cell proliferation in growing follicles was much lower in prepubertal ovaries than in cycling ovaries (39.8% versus 76.2%, Table 6). In general, it is widely accepted that the quality of granulosa cells is closely associated with oocyte development (Pandy et al. 2010). Therefore, it can be speculated that the poorer granulosa cell proliferation is, the more impaired oocyte development will be. Furthermore, poor granulosa cell development may also subsequently cause poor steroid production and impair ovarian function. Thus, ovulation and puberty attainment will not occur.

It can be concluded that the detection of PRRS virus in the gilt's ovarian tissue was not associated with the number or type of follicles but it was associated with the proliferation of granulosa cells in cycling ovaries. Cycling ovaries had greater granulosa cell proliferation than prepubertal ovaries. These findings imply that PRRS virus detection in gilt ovarian tissue may be one factor that can impair follicle development and oocyte quality, and subsequently lead to abnormal estrous behavior and infertility in replacement gilts.

CONCLUSIONS

- PRRS virus was detected in 70 % of ovarian tissues of the replacement gilts culled due to reproductive failure.
- The frequency of PRRS virus detection depended on the body weight, ADG, and oestrus cycle.
- Gilts with a body weight of 151 – 160 kg had a high frequency of PRRS virus detection (89.5 %).
- The detection of PRRS virus in replacement gilts may increases the risk of mating the gilts, while the PRRS virus has not been eliminated from their reproductive organs.
- The PRRS virus was frequently detected in delayed puberty gilts (92.0 %).
- The ovulation rate in the ovary containing the PRRS virus tended to be lower than the ovary without the PRRS virus.
- These findings indicated that the natural exposure to PRRS virus in the replacement gilts cause an existence of the virus in the ovarian tissues and may subsequently influence the ovarian function.
- The detection of PRRS virus in the gilt's ovarian tissue was not associated with the number or type of follicles but it was associated with the proliferation of granulosa cells in cycling ovaries.
- Cycling ovaries had greater granulosa cell proliferation than prepubertal ovaries.
- These findings imply that PRRS virus detection in gilt ovarian tissue may be one factor that can impair follicle development and oocyte quality, and subsequently lead to abnormal estrous behavior and infertility in replacement gilts.
- ERβ immunoreaction was detected in 22.6% of the theca cells and in 87.2% of the luteal cells.
- PRRS virus detection did not significantly influence the ERβ expression in the ovary of gilt.

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APPENDIX

International publications (2 papers)

1. Tummaruk, P., Phoophitphong, D., Olanratmanee, E.-o., Thanawongnuwech, R., 2015. Detection of porcine reproductive and respiratory syndrome virus in the ovary of gilts culled due to reproductive disturbances. Comparative Clinical Pathology 24: 903-910.
2. Phoophitphong, D., Srisuwatanasagul, S., Tummaruk, P., 2015. Granulosa cell proliferation in the gilt ovary associated with ovarian status and porcine reproductive and respiratory syndrome virus. Comparative Clinical Pathology 24: 1385-1394.

International proceedings (2 papers)

1. Phoophitphong, D., Olanratmanee, E., Srisuwatanasagul, S., Tummaruk, P., 2013. Effect of PRRS virus infection in the ovarian tissue on follicle growth in prepubertal and pubertal gilts. Proc. 38th International Conference on Veterinary Science 2013 FAO Joint Symposium. 16-18 January 2013, Grand Diamond Ballroom, IMPACT Forum, Muang Thong Thani, Thailand. P. 311-313.
2. Phoophitphong, D., Olanratmanee, E., Srisuwatanasagul, S., Wangnaitham, S., Thanawongnuwech, R., Tummaruk, P., 2012. Follicle development and number of ovulation in the ovarian tissue of gilts infected by porcine reproductive and respiratory syndrome virus. Proc. 22nd International Pig Veterinary Society Congress, 10-13 June, 2012, Jeju, South-Korea. P. 277.

National proceedings (6 papers)

1. Phoophitphong, D., Olanratmanee, E., Srisuwatanasagul, S., Tummaruk, P., 2013. Impact of PRRS virus detection in the ovarian tissue of replacement gilts on granulosa cells proliferation in the developing follicles. Proc. 51th Kasetsart University Annual Conference, 5-7 February 2013, Bangkok, Thailand, 7 pages.
2. Boonwong, N., Chumtong, W., Saengthong, W., Jiraphan, S., Phoophitphong, D., Srisuwatanasagul, Tummaruk, P., 2014. Expression of Estrogen Receptor alpha in the endometrium of gilts infected with porcine reproductive and respiratory syndrome virus. Thai J. Vet. Med. 44 (Suppl. 1): 161-162.
3. Choonnasart, A., Prayoonwiwat, N., Wuttiwongtanakorn, P., Butrak, C., Phoophitphong, D., Srisuwatanasagul, S., Tummaruk, P., 2014. Impact of porcine reproductive and respiratory syndrome virus infection on the expression of estrogen receptor beta (ERβ) in the ovary of gilts. Thai J. Vet. Med. 44 (Suppl. 1): 163-164.
4. Limchoochua, W., Tokhomkham, K., Mora, S., Wiriyaprapakul, W., Roongsitthichai, A., Tummaruk, P., 2014. Serum leptin concentration in relation to breed, body weight,

backfat thickness and age at first observed estrus in gilts. Thai J. Vet. Med. 44 (Suppl. 1): 165-166.

5. Phoophitphong, D., Srisuwatanasagul, S., Tummaruk, P., 2014. Localization of estrogen receptor β in the porcine ovary. Thai J. Vet. Med. 44 (Suppl. 1): 147-149. (poster award).
6. Tummaruk, P., Phoophitphong, D., Srisuwatanasagul, S., 2012. The association between number of follicles and body weight of the gilts. Proceedings of the 11th Chulalongkorn University Veterinary Annual Conference, May 18, 2012. P. S38.

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ORIGINAL ARTICLE

Detection of porcine reproductive and respiratory syndrome virus in the ovary of gilts culled due to reproductive disturbances

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Abstract Replacement gilts are a major source of introducing the porcine reproductive and respiratory syndrome (PRRS) virus into the swine breeding herd. The objective of the present study was to determine the prevalence of PRRS virus antigen by detection in the ovary of gilts culled due to reproductive disturbances. Ovarian tissues were collected from 100 Landrace x Yorkshire crossbred slaughtered gilts in Thailand. PRRS virus antigen in the ovarian tissues were detected by using immunohistochemistry. On average, the gilts were culled at 277 days of age at a body weight of 148 kg. Of these gilts, 75 had attained puberty and 25 had not attained puberty. PRRS virus antigens were detected in the macrophages within the ovary. The histological morphology of macrophages containing the PRRS virus in ovarian tissue was similar to that found in the lung tissue. Of all the ovarian tissues (n=100), the PRRS virus was detected in 70 (70.0 %) ovaries. The detection frequency of the PRRS virus in the ovarian tissue of gilts did not differ significantly among the culling reasons (P=0.496). The gilts that had exhibited standing oestrus had a lower PRRS virus detection than those that had never exhibited standing oestrus (62.8 versus 92.0 %, respectively, P=0.005). Gilts with a body weight of 151–160 kg had a higher frequency of PRRS virus detection (89.5 %) than gilts with a body weight of ≤130 kg (52.9 %, P=0.025) and 131–140 kg (50.0 %, P=0.032). The ovulation rate in the ovary containing the PRRS virus tended to be lower than the ovary without the PRRS virus (14.8 and 16.7 corpora lutea (CL), respectively, P=0.196). These findings indicated that the natural exposure to PRRS virus in the replacement gilts caused an existence of the virus in the ovarian tissues and may subsequently influence the ovarian function.

Keywords Immunohistochemistry · Ovary · Pig · PRRSV · Reproduction

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a common disease in pigs worldwide (Zimmerman et al. 2012). The disease is caused by the PRRS virus, which was discovered in the USA in 1987 (Keffaber 1989). The virus was identified for the first time in The Netherlands in 1990 (Wensvoort et al. 1991). In Thailand, the PRRS virus has been detected in pigs since 1989 (Damrongwatanapokin et al. 1996). The common clinical symptoms of the disease caused by the PRRS virus in gilts and sows include late-term abortion, mummified foetuses, stillborn piglets, and low viability of piglets at birth (Olanratmanee et al. 2013). In practice, the PRRS virus is controlled by intensive gilt acclimatisation, a regular serological monitoring programme, and vaccination with modified live PRRS virus (MLV) and/or killed vaccines (Díaz et al. 2013). Nevertheless, replacement gilts remain to be a major source of introducing new strains of the PRRS virus into the herd (Tummaruk and Tantilertcharoen 2012; Nilubol et al. 2014). Under field conditions, an intensive acclimatisation of replacement gilts with culled sows or nursery pigs is commonly practised in most swine breeding herds in Thailand. Although the titre of antibody against the PRRS virus is often carefully monitored, detection of the PRRS virus

in the reproductive organs of these gilts has never been done (Tummaruk and Tantilertcharoen 2012). Moreover, the titre of antibody against the PRRS virus of the gilts is highly variable both within and between herds (Tummaruk and Tantilertcharoen 2012). This causes difficulties for the farmer when deciding to mate the gilts and occasionally causes infertility problems in some batches of replacement gilts. The previous study found that 73.0 % (122/166) of replacement gilts culled due to reproductive failures (e.g. anoestrus, abnormal vaginal discharge, repeat service, and abortion) had been infected with the PRRS virus (Tummaruk and Tantilertcharoen 2012). Furthermore, PRRS virus antigen could be detected in the uterine tissue in 33.0 % of the gilts culled due to reproductive failures (Olanratmanee et al. 2011). This may compromise the fertility of replacement gilts.

In general, the PRRS virus primarily infects pulmonary alveolar macrophages of the pig during the acute stage of infection (Sur et al. 1997). Likewise, macrophages from other organs are also the primary cell type that sustains the in vivo replication of the virus (Thanawongnuwech et al. 2000). Our previous study found that the PRRS virus was detected in the cytoplasm of the macrophages in the sub-epithelial connective tissue layers of the endometrium of the gilts culled due to reproductive failures (Olanratmanee et al. 2011). Interestingly, the detection frequency of PRRS virus antigen varied between the herds, from 14.3 to 80.0 % (Olanratmanee et al. 2011). Moreover, the PRRS virus exists in the uterine tissue of the infected gilts for several months even though vaccination has been done (Olanratmanee et al. 2011). The most common reproductive disturbance among replacement gilts is anoestrus and/or delayed puberty (Tummaruk et al. 2009). This problem may be associated with ovarian dysfunction in gilts infected with the PRRS virus during an intensive acclimatisation period. If so, gilt acclimatisation practices need to be revised. Nevertheless, to enhance our understanding of this issue, additional research needs to be carried out on the pathogenesis as well as the factors associated with PRRS virus infection in replacement gilts. To our knowledge, PRRS virus detection in the ovarian tissue of gilts naturally exposed to the PRRS virus associated with the gilts' age, body weight, average daily gain (ADG), ovarian status, and delayed puberty has not been comprehensively determined.

In the boar, the PRRS virus is able to induce apoptosis of the testicular germ cells (Sur et al. 1997), while in the female's gonad, only limited information is known. To our knowledge, only one study has demonstrated that the PRRS virus can penetrate the resident macrophages of the ovary but its involvement in follicle development, ovulation, and corpus luteum formation is not clearly understood (Sur et al. 2001). Under experimental conditions, the PRRS virus was detected in the ovarian tissue of gilts for 21 days post infection (Sur et al. 2001). Nevertheless, the role of natural PRRS virus infection in the existence of the virus in gilts' ovaries has not been elucidated. Also, little is known about the mechanism of pathogenesis underlying ovarian infection by the PRRS virus. The objective of the present study was to determine the prevalence of PRRS virus antigen detection in the ovarian tissue of gilts in relation to the gilts' historical data, e.g. culling reason, and reproductive data, e.g. age, body weight, ADG, ovarian status, and delayed puberty.

Materials and methods

Animals, data, and sample collection

Ovarian tissues were collected from 100 Landrace x Yorkshire crossbred gilts from two commercial swine herds in Thailand. Historical data of the gilts including the gilts' identity, the date of birth, date of entry into the herd, date of first observed oestrus, date of culling, body weight at culling, and culling reason were collected. The age at culling was calculated. ADG from birth to culling was calculated: ADG (g/day)=(body weight at culling−1.5/age at culling)×1,000. The ovaries and reproductive tract of the gilts were collected immediately after slaughter at an abattoir and were placed on ice and transported to the laboratory within 24 h. The appearance of the ovaries was used to define the stage of the oestrous cycle. The ovarian appearance was defined according to a previous study (Tummaruk et al. 2009). Briefly, corpora lutea (CL) were the structures on the ovaries with a pink, tan, or yellow colour and with a diameter of between 5 and 15 mm (Fig. 1). Corpora albicantia (CA) were regressing and shrunken CL. Follicles were transparent fluid-containing structures in the ovaries (Fig. 1). The number of CL was counted and defined as the number of ovulations. The ovary was defined as 'pre-pubertal' when containing only small follicles and having no CL or CA and was defined as 'cycling' when containing CL or CA and follicles (Fig. 1). Of the cycling ovaries, 'luteal phase' was characterized by ovaries that had CL and 'follicular phase' by ovaries that had follicles of 7 to 12 mm in diameter either with or without CA (Tummaruk et al. 2010a, b). The reasons for culling were classified into five groups, i.e. anoestrus, abnormal vaginal discharge, abortion, repeat breeding, and miscellaneous (e.g. lameness, inverted nipple, and illness) (Tummaruk et al. 2009).

General management and vaccination

The herds in the present study were breeding herds in the northeastern (Herd A) and middle (Herd B) parts of Thailand. The numbers of sows-on-production were 2000 (Herd A) and 3500 (Herd B) sows/herd. Herd A produced replacement gilts within the herd using their own grandparent stock, while Herd B bought replacement gilts from another breeder. The gilts in both herds were housed in a conventional open-housing

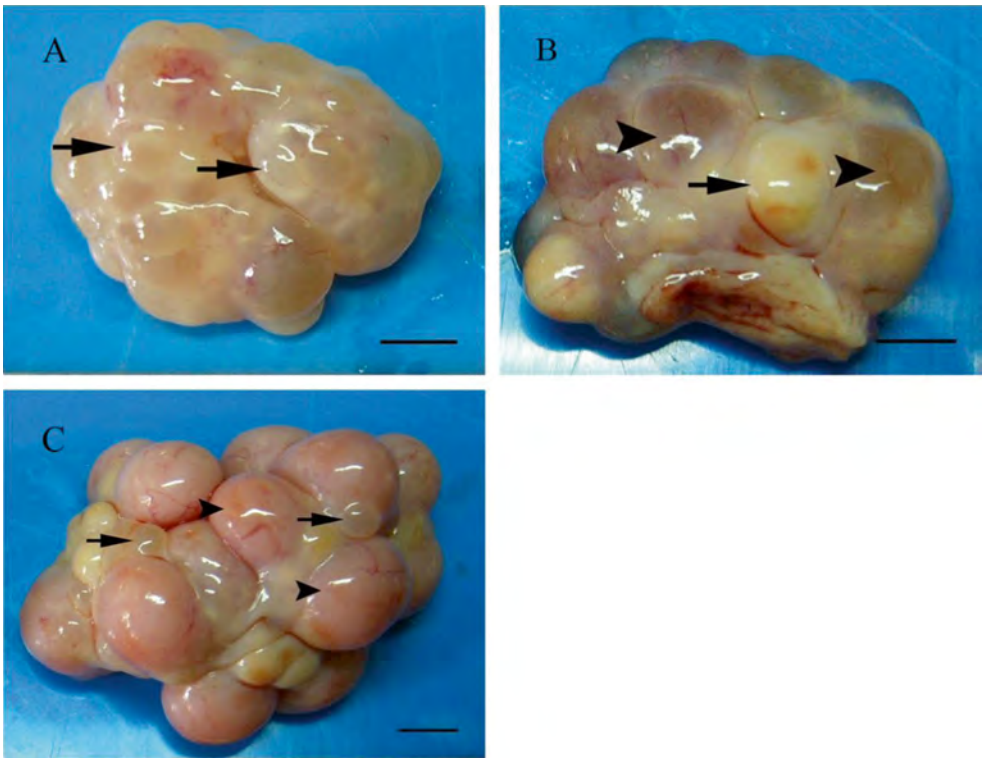

Fig. 1 Gross morphology of the gilt's ovary: **a** ovary of a pre-pubertal gilt containing numerous small follicles (*arrows*); **b** ovary of a pubertal gilt during the follicular phase, containing numerous large follicles (*arrowheads*) and corpora albicantia (*arrow*); **c** ovary of a pubertal gilt during the luteal phase, containing numerous large corpora lutea (*arrowheads*) and small follicles (*arrows*). *Bar*=1.0 cm

system facilitated with a water sprinkler and fan for reducing heat stress. The health status of the herds was monitored routinely by the herd veterinarian. In general, the recommended gilt vaccination programme consisted of foot-and-mouth disease, classical swine fever, Aujeszky's disease, and porcine parvovirus at between 22 and 30 weeks of age. In both herds, the replacement gilts were also vaccinated with a PRRS MLV vaccine (Ingelvac® PRRS™ MLV, Boehringer-Ingelheim Vetmedica Inc., St. Joseph, MO, USA) before being sent to the breeding house.

Management of gilts and oestrus detection

In general, the replacement gilts were kept in pens in groups of 6 to 15 gilts/pen with a space allowance of 1.5 to 2.0 m^2/gilt. Oestrus detection was performed daily using observations for vulva symptoms (i.e. reddening, swelling, and mucous discharge) and a back pressure test in the presence of a mature boar (Tummaruk et al. 2007). The gilts were provided water ad libitum using water nipples, and they were fed twice a day (about 3 kg of feed/gilt/day) with a corn-soybean-fish ration containing 16.0 to 18.0 % crude protein, 3000 to 3250 kcal/kg metabolisable energy, and 0.85 to 1.10 % lysine. In general, the herds recommended breeding the replacement gilts from 32 weeks of age onwards at second or later oestrus and at a body weight of at least 130 kg. All herds used conventional artificial insemination.

Tissue processing

The ovaries were fixed in 10.0 % neutral-buffered formalin for 24–48 h, processed by an automatic tissue processor (Tissue-Tek VIP 5 Jr., Sakura, Tokyo, Japan), and embedded in a paraffin block (Tissue-Tek TEC, Sakura, Tokyo, Japan). The paraffin embeddings were cut into 4 μm thickness using a microtome (Shandon, Anglia Scientific Instrument Ltd., Cambridge, UK). The tissue sections were used to detect PRRS virus infection using immunohistochemistry.

Immunohistochemical detection of the PRRS virus

PRRS virus detection in the gilt's ovary was carried out by immunohistochemistry according to our previous protocol (Olanratmanee et al. 2011). Briefly, the ovarian tissue sections were placed on 3-aminopropyltriethoxysilane-coated slides. The sections were deparaffinised in xylene and rehydrated in graded alcohol. A polymer-based non-avidin-biotin technique was applied. An antigen-retrieval technique was used in order to enhance the reaction between antigens and antibodies by enzymatic treatment using 0.1 % trypsin at 37 °C for 30 min. After washing in phosphate-buffered saline (PBS), endogenous peroxidase activity was inhibited by immersing the sections in 0.3 % hydrogen peroxide (H_2O_2) in absolute methanol for 30 min at room temperature. Thereafter, the sections were blocked with 1.0 % bovine serum albumin at

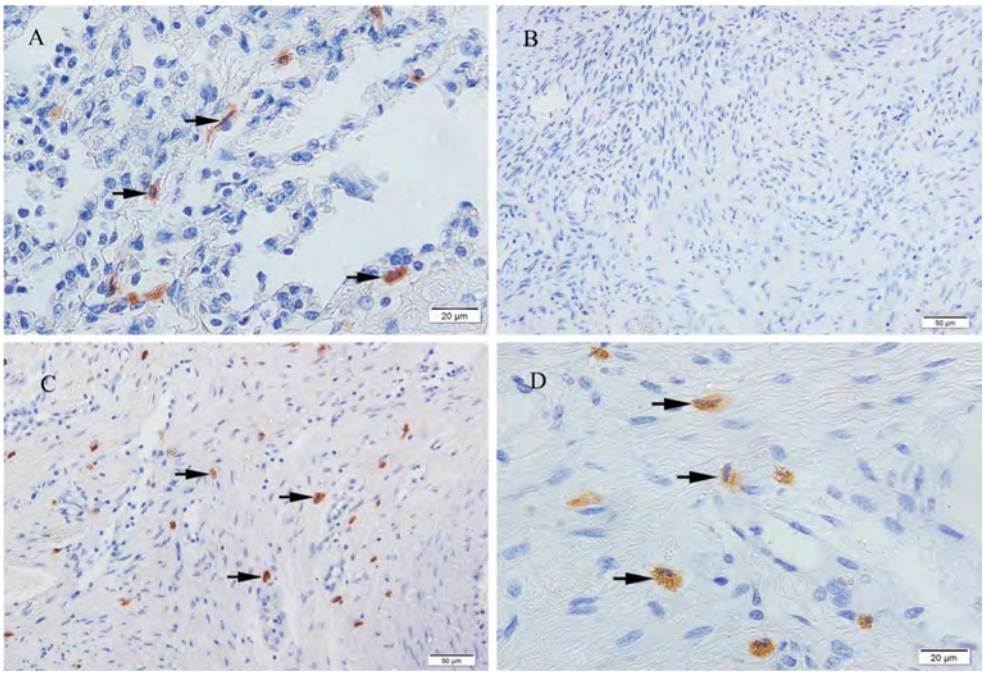

Fig. 2 Immunohistochemical staining of porcine reproductive and respiratory syndrome (PRRS) virus antigen (*arrows*) in **a** positive control (lung tissue), **b** negative control, and **c**–**d** ovarian tissue

37 °C for 30 min and incubated at 4 °C overnight (12–15 h) with a diluted (1:1000) primary monoclonal antibody SDOW17 (Rural technologies, Inc., USA). After washing in PBS, dextran coupled with peroxidase molecules and goat secondary antibody (Dako REAL™Envision™/HRP, Rabbit/Mouse®, Dako, Denmark) was applied to the tissue sections and incubated at 37 °C for 45 min. In the final step, the colour of the bound enzyme (brown colour) was obtained by incubating with 0.05 % 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 4–15 min. All sections were counterstained with Mayer's haematoxylin, dehydrated, and mounted for investigation under a light microscope. Negative control procedures included the omission of primary antibody. Known PRRS virus-infected lung tissues served as positive controls. The sections were interpreted as positive if they contained at least one positive cell (brown intracytoplasmic staining) (Fig. 2).

Statistical analyses

Statistical analyses were carried out by using SAS (SAS, 2002). Data are presented as percentages. The frequency of PRRS virus detection in the ovarian tissue of gilts was compared by culling reasons, ovarian status (pre-pubertal, follicular, and luteal phases), age classes, body weight classes, ADG classes, season, oestrus behaviour, and ovarian pathology, and the number of ovulation classes was compared by using chi-square and Fisher's exact test. $P<0.05$ was considered to be statistically significant.

Results

Descriptive statistics

Descriptive statistics on the reproductive data of the replacement gilts included in the present study are presented in Table 1. On average, the gilts were culled at 277 days of age at a body weight of 148 kg. Of these gilts, 75 had attained puberty and 25 had not attained puberty. The average age at first observed oestrus was 214±18.8 days. For the gilts that had not attained puberty (n=25), the average age at culling was 256±16.1 days. Based on the age and the ovarian status (i.e. pre-pubertal phase), 25.0 % of the gilts were defined as delayed puberty.

Localisation of the PRRS virus

Histological examination of ovarian tissues of the gilts revealed that PRRS virus antigens were detected in the

Table 1 Descriptive statistics on reproductive data of 100 Landrace x Yorkshire crossbred gilts in Thailand

Items	Mean±SD	Range
Age at culling (day)	277±33.4	209–406
Body weight at culling (kg)	148±16.8	105–203
Average daily gain (g/day)	529±67.4	150–693
Age at first observed oestrus (day)	214±18.8	156–272
Ovulation rate	15.9±4.3	1–26
Weight of an ovary (g)	6.3±3.2	1.8–16.6

SD standard deviation

Table 2 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by culling reason

Culling reason	Number of gilts	Number of gilts with PRRS virus detection	Percentage
Anoestrus	51	40	78.4a
Vaginal discharge	17	11	64.7a
Abortion	10	6	60.0a
Repeat breeding	9	6	66.7a
Miscellaneous	13	7	53.9a
Total	100	70	70.0

The same letter within a column denotes data that do not differ significantly ($P>0.05$)

macrophages within the ovary (Fig. 2c). The virus was found as brown staining in the cytoplasm of the macrophages (Fig. 2d). The histological morphology of macrophages containing the PRRS virus in ovarian tissue was similar to that found in lung tissue (Fig. 2a). Of all the ovarian tissues ($n=100$), the PRRS virus was detected in 70 (70.0 %) ovaries.

Factors influencing the detection frequency of the PRRS virus in ovarian tissue

The detection frequency of the PRRS virus in ovarian tissues of the gilts classified by culling reasons is displayed in Table 2. As can be seen from the table, the detection frequency of the PRRS virus in the ovarian tissue of gilts did not differ significantly among the culling reasons ($P=0.496$). Nevertheless, the PRRS virus was detected in the gilts culled due to anoestrus more frequently than the gilts culled due to miscellaneous causes (78.9 versus 53.9 %, $P=0.073$).

The number and percentage of PRRS virus detection in the ovarian tissue of gilts by ovarian status are presented in Table 3. The ovarian status (i.e. pre-pubertal, follicular, and luteal phases) did not influence the frequency of PRRS virus detection ($P=0.225$). The percentage of PRRS virus detection in ovarian tissue by age groups of the gilts is presented in Table 4. The frequency of PRRS virus detection was not dependent on the age of the gilts ($P=0.683$).

Table 3 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by ovarian status

Ovarian status	Number of gilts	Number of gilts with PRRS virus detection	Percentage
Pre-pubertal phase	26	20	76.9a
Follicular phase	13	11	84.6a
Luteal phase	61	39	63.9a

The same letter within a column denotes data that do not differ significantly ($P>0.05$)

Table 4 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by age group of the gilts

Age group (days)	Number of gilts	Number of gilts with PRRS virus detection	Percentage
<250	17	11	64.7a
251–300	65	45	69.2a
>300	18	14	77.8a

The same letter within a column denotes data that do not differ significantly ($P>0.05$)

The influences of body weight and ADG of the gilts on the frequency of PRRS virus detection are displayed in Tables 5 and 6, respectively. Gilts with a body weight of 151–160 kg had a higher frequency of PRRS virus detection (89.5 %) than gilts with a body weight of ≤130 kg (52.9 %, $P=0.025$) and 131–140 kg (50.0 %, $P=0.032$). Likewise, gilts with an ADG of 500–550 g/day and 550–600 g/day had a higher frequency of PRRS virus detection than gilts with an ADG of <500 g/day (81.8, 81.8, and 46.2 %, respectively, $P<0.02$).

The percentage of PRRS virus detection did not differ significantly between cool and hot seasons (73.8 versus 64.1 %, respectively, $P=0.304$). The gilts that had exhibited standing oestrus had a lower PRRS virus detection than those that had never exhibited standing oestrus (62.8 versus 92.0 %, respectively, $P=0.005$) (Table 7). The percentages of gilts with PRRS virus detection in ovarian tissue by ovarian pathology are presented in Table 8. Although the PRRS virus was detected in 80.0 % of ovaries with multiple cysts, it was also detected in 72.0 % of ovaries with a normal appearance ($P>0.05$). Nevertheless, PRRS virus detection in ovaries with a single cyst was lower than that in ovaries with multiple cysts ($P=0.03$). Although the frequency of PRRS virus detection was not related to the number of ovulations, the virus was frequently detected in gilts with a low number of ovulation (i.e. ≤13 CL) (Table 9). Furthermore, the ovulation rate in the ovary containing the PRRS virus tended to be lower than the ovary without the PRRS virus (14.8 and 16.7 CL, respectively, $P=0.196$).

Table 5 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by body weight group of the gilts

Body weight group (kg)	Number of gilts	Number of gilts with PRRS virus detection	Percentage
<130	17	9	52.9a
131–140	12	6	50.0a
141–150	28	20	71.4ab
151–160	19	17	89.5b
>160	24	18	75.0ab

Different letters within a column denote data that differ significantly ($P<0.05$)

Table 6 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by average daily weight gain (ADG) group of the gilts

ADG group (g/day)	Number of gilts	Number of gilts with PRRS virus detection	Percentage
≤500	30	14	46.7a
501–550	33	27	81.8b
551–600	22	18	81.8b
>600	15	11	73.3ab

Different letters within a column denote data that differ significantly ($P<0.05$)

Table 8 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by ovarian pathology

Ovarian pathology	Number of gilts	Number of gilts with PRRS virus detection	Percentage
Normal	79	57	72.2a
Single cyst	7	2	28.6b
Multiple cyst	10	8	80.0a
Miscellaneous	4	3	75.0ab

Different letters within a column denote data that differ significantly ($P<0.05$)

Discussion

The present study reveals the detection of the PRRS virus in the ovarian tissue of replacement gilts under field conditions. In the ovary, the PRRS virus is located in macrophages within the ovarian tissue. This is in accordance with an earlier study (Sur et al. 2001), which found that the PRRS virus could be detected in the ovarian tissue of gilts within 21 days post infection. The study also demonstrated numerous PRRS virus-positive cells in atretic follicles of the ovaries. However, no alternation of the ovarian architecture supporting the possible role of PRRS infection in porcine female infertility could be demonstrated. In contrast, in gestating sows, the experimental challenge of the PRRS virus caused no consistent or significant microscopic lesions in ovarian tissues (Benson et al. 2001). Moreover, no evidence of PRRS virus antigen was detected in the ovarian tissue of sows at 21 days post infection (Benson et al. 2001). This indicates an inconsistency in the appearance of the PRRS virus in porcine ovarian tissue. In the present study, the PRRS virus was detected in 70.0 % of the ovarian tissues of the replacement gilts culled due to reproductive failure, especially among those culled due to anoestrus. This indicates a possible role of the PRRS virus in ovarian function under field conditions. In general, macrophages in the ovaries are involved in many ovarian functions, e.g. follicular growth, follicular atresia, ovulation, and CL formation (Araki et al. 1996). It was suggested that phagocytic macrophages containing the PRRS virus may deliver the virus to neighbouring cells, such as granulosa cells (Sur et al. 2001). This may subsequently influence granulosa cell function, e.g. steriodogenesis, oocyte maturation, and follicle development. The interruption of these process may influence the oestrus cycle and puberty attainment in gilts. In the present study, the PRRS virus was detected in the gilts with delayed puberty (92.0 %) more than those that had already attained puberty (62.8 %). The age at culling of these delayed puberty gilts averaged 256 days. Normally, Landrace x Yorkshire crossbred gilts in Thailand attain puberty at 195 days of age (Tummaruk et al. 2007). Thus, the age at puberty is delayed for 61 days. PRRS virus infection in the ovary of replacement gilts may, to some extent, interfere with ovarian functions and contribute to anoestrus problems or delayed puberty in replacement gilts. In boars, PRRS virus infection clearly induces the depletion and death of testicular germ cells and results in hypospermatogenesis (Sur et al. 1997). Moreover, PRRS virus infection in the boar causes a significant reduction in semen quality, e.g. distal cytoplasmic droplet and sperm motility (Prieto et al. 1996; Guérin and Pozzi 2005). The virus might have a role in the ovarian tissue of replacement gilts.

In general, the atretic process of the follicles depends on many factors including the physiological condition of the animal (Orisaka et al. 2009). The atretic process has not yet been well described; however, macrophages play a major role by producing pro-inflammatory mediators, e.g. interleukin 1, interleukin 2, and tumour necrosis factor. A previous study has demonstrated that macrophages are involved in the atretic

Table 7 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by oestrus behaviour

Oestrus behaviour	Number of gilts	Number of gilts with PRRS virus detection	Percentage
Anoestrus gilts	25	23	92.0a
Pubertal gilts	75	47	62.8b

Different letters within a column denote data that differ significantly ($P=0.005$)

Table 9 Number and percentage of gilts with porcine reproductive and respiratory syndrome (PRRS) virus detection in the ovarian tissue by number of ovulations

Number of ovulations	Number of gilts	Number of gilts with PRRS virus detection	Percentage
≤13	11	9	81.8a
14–17	33	18	54.6a
≥18	15	10	66.8a

The same letter within a column denotes data that do not differ significantly ($P>0.05$)

process in both the pre-pubertal and pubertal stages, and macrophages are usually absent in healthy developing swine follicles (Sur et al. 2001). The present study demonstrated the presence of the PRRS virus in both cyclic and non-cyclic gilts. Macrophages infected with the PRRS virus may invade the granulosa cell layer and alter apoptosis and/or proliferation of germinal epithelial cells. However, Sur et al. (2001) did not find any significant differences in ovarian architecture in PRRS virus-infected gilts compared with that in non-infected gilts. Under field conditions, the replacement gilts were exposed to the PRRS virus before the first insemination (Tummaruk and Tantilertcharoen 2012). If the ovaries of the gilts were infected with the PRRS virus, granulosa cell proliferation, steriodogenesis, and oocyte quality may be reduced. This may subsequently cause infertility problems in mated gilts, which is commonly seen under field conditions (Tummaruk et al. 2010a, b). In addition, the PRRS virus is frequently detected in the ovarian tissue of gilts with a relatively good body weight (i.e. 150–160 kg), rather than those with a poor body weight. The reason is not known, but this certainly increases the risk of mating the gilts, while the PRRS virus has not been eliminated from their reproductive organs. As a result, fertility of the gilts might be compromised. Based on these findings, it is not recommended to expose gilts to the PRRS virus. Therefore, the acclimatisation of replacement gilts should be omitted or ended for a certain period of time before the first insemination.

In conclusion, the PRRS virus was detected in 70.0 % of ovarian tissues of the replacement gilts culled due to reproductive failure. The frequency of PRRS virus detection depended on the body weight, ADG, and oestrus cycle. Gilts with a body weight of 151–160 kg had a high frequency of PRRS virus detection (89.5 %). This may increase the risk of mating the gilts, while the PRRS virus has not been eliminated from their reproductive organs. The PRRS virus was frequently detected in delayed puberty gilts (92.0 %). The ovulation rate in the ovary containing the PRRS virus tended to be lower than the ovary without the PRRS virus. These findings indicated that the natural exposure to PRRS virus in the replacement gilts cause an existence of the virus in the ovarian tissues and may subsequently influence the ovarian function.

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ORIGINAL ARTICLE

Granulosa cell proliferation in the gilt ovary associated with ovarian status and porcine reproductive and respiratory syndrome virus detection

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Abstract The aims of the present study were to quantify the number of follicles and determine granulosa cell proliferation in the ovaries of gilts in relation to ovarian status and porcine reproductive and respiratory syndrome (PRRS) virus infection. Ovarian tissues were obtained from 37 Landrace × Yorkshire crossbred gilts aged 270.7±24.5 days and weighing 144.5±15.0 kg (27 cycling and 10 prepubertal gilts). PRRS virus was detected using immunohistochemistry, and the ovaries were classified as negative (n=17) or positive (n=20) to PRRS virus. Granulosa cell proliferation was determined by proliferating cell nuclear antigen (PCNA) immunostaining. The proportion of PCNA-expressing granulosa cells in preantral (n=197) and antral follicles (n=76) was determined using Image-Pro® Plus software. The number of follicles in ovarian tissue containing PRRS virus did not differ significantly compared with those without PRRS virus. Granulosa cell proliferation was influenced by the type of follicle (P<0.05), ovarian status (P<0.05), the age of the gilt (P<0.05), and the interaction between ovarian status and PRRS virus detection (P=0.06). Granulosa cell proliferation in cycling ovaries was higher than in prepubertal ovaries (84.0±5.1 and 35.2±8.0 %, P<0.05) in gilts without PRRS virus but not in ovaries containing PRRS virus (68.5±4.3 and 44.4±9.2 %, P>0.05). It can be concluded that the detection of PRRS virus in the gilt's ovarian tissue was not associated with the number of follicles but was associated with the proliferation of granulosa cells in the cycling ovaries. This might subsequently cause delayed follicle development, reduced oocyte quality, and infertility in gilts.

Keywords Gilt · Granulosa cell · Proliferating cell nuclear antigen · PRRS · Reproduction

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Introduction

The selection of high-quality replacement gilts is an important key to improving pig production, since production from individual females contributes to the overall productivity of swine herds (Roongsitthichai et al. 2013). Gilts and sows with a history of poor reproductive performance (e.g., prolonged weaning-to-service interval, repeated service, and abortion) have suboptimal productivity and short longevity (Takai and Koketsu 2007; Tummaruk et al. 2010). In the last decade, the replacement rate of gilts in substitution for culled sows has been 40 to 60 % per year (Engblom et al. 2007). This high replacement rate is because of the high level of unplanned removal and culling of sows at a younger age than in the past (i.e., parities above 6) (Engblom et al. 2007). This has also affected production costs and herd efficiency. In general, the proportion of gilts in commercial swine herds accounts for 20 to 25 % of the sows in production. This reflects the importance of selecting quality gilts to be the production units in a herd. In general, age, body weight, and estrous behavior of gilts are the major criteria to be considered before first mating. Based on economic analysis, gilts should conceive before 220 to 230 days of age (Schukken et al. 1994; Koketsu et al. 1999). Koketsu et al. (1999) revealed that, if the age of first insemination of gilts was increased, their longevity decreased. Gilts that have a low average daily gain (ADG) tend to have a low conception rate and have to be removed from herds due to reproductive disturbances (Sur et al. 1997; Roongsitthichai

et al. 2013). It has been demonstrated that 15 to 20 % of sows are removed from a herd after having produced only one litter, and more than 50 % of them are removed before attaining the fifth parity (Engblom et al. 2007).

Porcine reproductive and respiratory syndrome (PRRS) virus has been isolated from the ovaries of gilts (Sur et al. 2001). PRRS virus is transmitted following an exposure of naïve animals to infected animals or to semen from infected boars. The duration of viral shedding in the boar semen of the infected boars varies from 2 to 92 days (Karniychuk and Nauwynck 2013). PRRS virus reaches the tissue of the boar reproductive tract by migration of infected macrophages (Prieto and Castro 2000). In the female pigs, endometrial tissue can be infected by PRRS virus contaminated semen and thereafter distribute to regional lymph node and other organs (Prieto and Castro 2000). In boars, it has been clearly demonstrated that the virus is able to induce apoptosis of testicular germ cells (Sur et al. 1997); however, in female gonads, only limited information is available about its impact. To our knowledge, only one study has demonstrated that PRRS virus can penetrate the resident macrophages of the ovary, but its involvement in follicle development, ovulation, and corpus luteum formation is not clearly understood (Sur et al. 2001). In the porcine ovary, macrophages are essential cells for cleaning up apoptotic debris during follicular atresia (Miyake et al. 2006). The presence of macrophages containing phagocytosed cells in cytoplasm has been reported in follicles of pig (Sur et al. 2001). Furthermore, macrophages also play many important roles in the porcine ovaries, e.g., phargocytosis and degeneration of foreign antigen, matrix dissolution, and tissue remodeling, production, and secretion of cytokines, chemokines, and growth factors (Miyake et al. 2006). Since majority of the porcine follicles undergo atresia, macrophages are therefore an important cell for eliminating those follicles that will not be ovulated and thus maintain normal function of the porcine ovary. Since macrophage is an important target cell of PRRS virus infection, the association between some ovarian activities, e.g., follicular growth, and the presence of PRRS virus in the ovarian tissue of replacement gilts is therefore of interest.

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase delta that is necessary for DNA synthesis (Kurki et al. 1986; Bravo and McDonald-Bravo 1987). The expression of PCNA increases during the G1 phase, reaches its highest level in the S-phase, and decreases during the G2/M phases of the cell cycle (Kurki et al. 1988). An earlier study has demonstrated that expression of PCNA in the ovaries is involved with follicular development in many species, e.g., rats (Oktay et al. 1995), cows (Wandji et al. 1996), baboons (Wandji et al. 1997), pigs (Phoophitphong et al. 2012), and humans (Kelsey et al. 2010). The aims of the present study were to quantify the number of follicles and determine granulosa cell proliferation in the ovaries of gilts in relation to ovarian status (i.e., prepubertal and cycling ovaries) and PRRS virus detection.

Materials and methods

Animals, data and sample collection

Ovarian tissues of 37 Landrace×Yorkshire crossbred gilts were obtained from slaughter houses. The genital organs and historical data of the gilts were collected from two commercial swine herds in Thailand. The historical data of the gilts consisted of the herd and gilt identity, breed, date of birth, date of entry into the herd, date of first observed estrus, date of culling, and body weight at culling. The age at culling was calculated. The ADG from birth to culling was calculated: ADG (grams per day)=(body weight at culling−1.5/age at culling)×1000. The organs were collected immediately after slaughter and were placed on ice and transported to the laboratory within 24 h. The weight of the ovaries was measured using an electronic balance (BJ 210C, max=210 g/day=0.01 g, Precisa Instruments Ltd., Switzerland). The appearance of the ovaries was used to define the stage of the estrous cycle. The ovarian structures were defined according to a previous study (Tummaruk et al. 2009a). Briefly, corpora lutea (CL) were the structures on the ovaries with a pink, tan, or yellow color and a diameter of between 5 and 15 mm. Corpora albicantia (CA) were regressing and shrunken CL. Follicles were transparent fluid-containing structures in the ovaries (Fig. 1). The number of CL was counted and defined as the number of ovulations. The ovary was defined as 'prepubertal' when it contained only small follicles (diameter ≤3 mm) (Evans and O'Doherty 2001; Tummaruk et al. 2009a) and had no CL or CA (Fig. 1a), and as 'cycling' when it contained CL or CA and follicles (Fig. 1b).

Tissue processing

The ovaries were fixed in 10 % neutral-buffered formalin for 24 to 48 h, processed by an automatic tissue processor (Tissue-Tek VIP 5 Jr., Sakura, Tokyo, Japan), and embedded in a paraffin block (Tissue-Tek TEC, Sakura, Tokyo, Japan). The paraffin blocks were cut into 5-μm-thick sections using a microtome (Shandon, Anglia Scientific Instrument Ltd., Cambridge, UK). For each ovarian tissue, two sections were cut, and each section was placed on a separate slide. One section was used to detect PRRS virus infection using immunohistochemistry, and the other was stained by PCNA immunohistochemistry to determine the number of follicles and granulosa cell proliferation.

Immunohistochemical detection of PRRS virus

PRRS virus detection in the gilt ovaries was carried out by immunohistochemistry according to our previous protocol

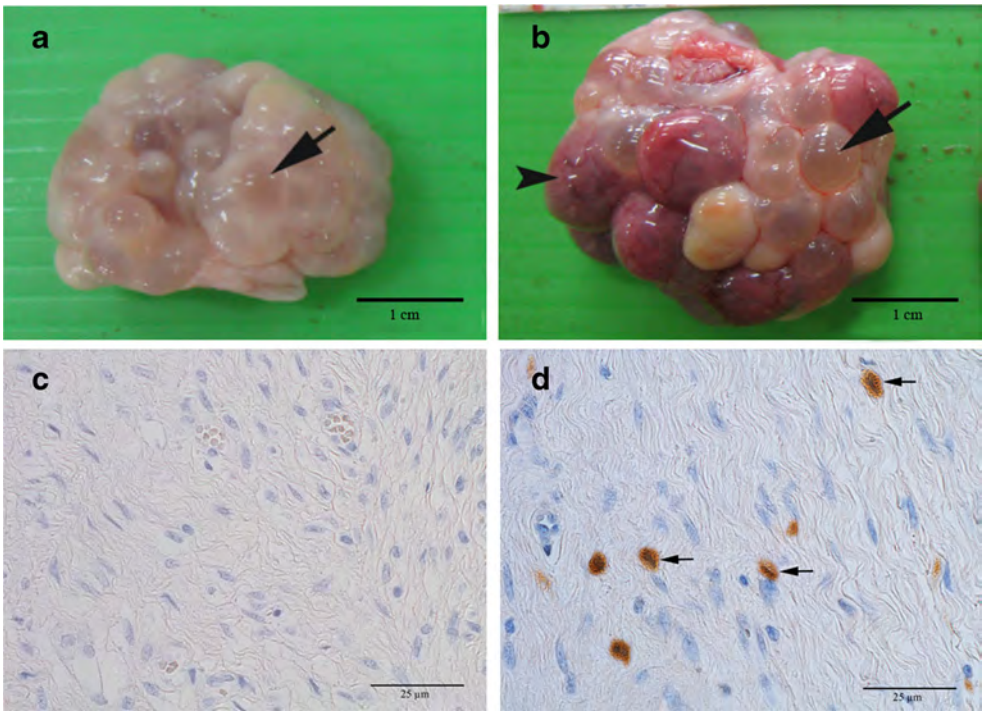

Fig. 1 **a**–**b** Small follicles (*arrow*) in prepubertal ovaries and corpus luteum (*arrowhead*), corpus albicantea, and follicles in cycling ovaries (*bar*=1 cm). **c**–**d** Immunohistochemical expression of negative (**c**) and positive (**d**) PRRSV in macrophages in gilt ovarian tissue

(Olanratmanee et al. 2011). Briefly, the ovarian tissue was cut and placed on 3-aminopropyl-triethoxy silane-coated slides. The sections were deparaffinized in xylene and rehydrated in graded alcohol. A polymer-based non-avidin–biotin technique was applied. An antigen-retrieval technique was used in order to enhance the reaction between antigens and antibodies by enzymatic treatment, using 0.1 % trypsin at 37 °C for 30 min. After washing in phosphate-buffered saline (PBS), endogenous peroxidase activity was inhibited by immersing the sections in 0.3 % hydrogen peroxide in absolute methanol for 30 min at room temperature. Thereafter, the sections were blocked with 1.0 % bovine serum albumin at 37 °C for 30 min and incubated at 4 °C overnight (12 to 15 h) with a diluted (1:1000) primary monoclonal antibody SDOW17 (Rural Technologies, Inc., USA). After washing in PBS, a goat secondary antibody dextran coupled to peroxidase molecules (Dako REALTM EnvisionTM/HRP, Rabbit/Mouse®, Dako, Denmark) was applied to the tissue sections and incubated at 37 °C for 45 min. In the final step, the color of the bound enzyme (brown color) was developed using 0.05 % DAB (3, 3′-diaminobenzidine tetrahydrochloride) for 4 to 15 min. All sections were counterstained with Mayer's hematoxylin, dehydrated, and then mounted for investigation under a light microscope. Negative control procedures included the omission of primary antibody. Known PRRS virus-infected lung and lymph node tissues served as positive controls. The sections were interpreted as positive if they contained at least one positive cell (brown intracytoplasmic staining) (Olanratmanee et al. 2011) (Fig. 1c and d).

PCNA immunohistochemistry

A PCNA immunostaining technique was carried out according to our previous study (Phoophitphong et al. 2012). Briefly, the pig ovarian tissues were deparaffinized and placed in an antigen retrieval solution (0.01 M citrate buffer, pH 6.0) in a microwave oven at 600 watts for 10 min. Endogenous peroxidase was blocked using freshly prepared 3.0 % hydrogen peroxide at room temperature for 10 min. Nonspecific staining was blocked with 1.0 % bovine serum albumin at 37 °C for 30 min. The slides were incubated with mouse monoclonal anti-PCNA (clone PC10, DAKO, Carpinteria, CA, USA) as a primary antibody at a dilution of 1:200 at 4 °C overnight. After incubation with the primary antibody, the sections were incubated with DAKO EnVision™ reagent for 45 min at room temperature. Staining was developed with 3, 3′-diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma, Germany) for 3 min at room temperature. The sections were counterstained with Mayer's hematoxylin for 1 min, dehydrated through a graded series of ethanols, placed into xylene, and mounted with mounting media. During the steps in the staining procedure, the slides were washed three times in PBS for 5 min each time. For the negative control, PBS was used instead of the primary antibody (Fig. 2a).

Follicle categorization

The follicles were classified into three categories as described earlier (Phoophitphong et al. 2012), i.e., (I) "primordial follicles" were follicles having an oocyte surrounded by one or

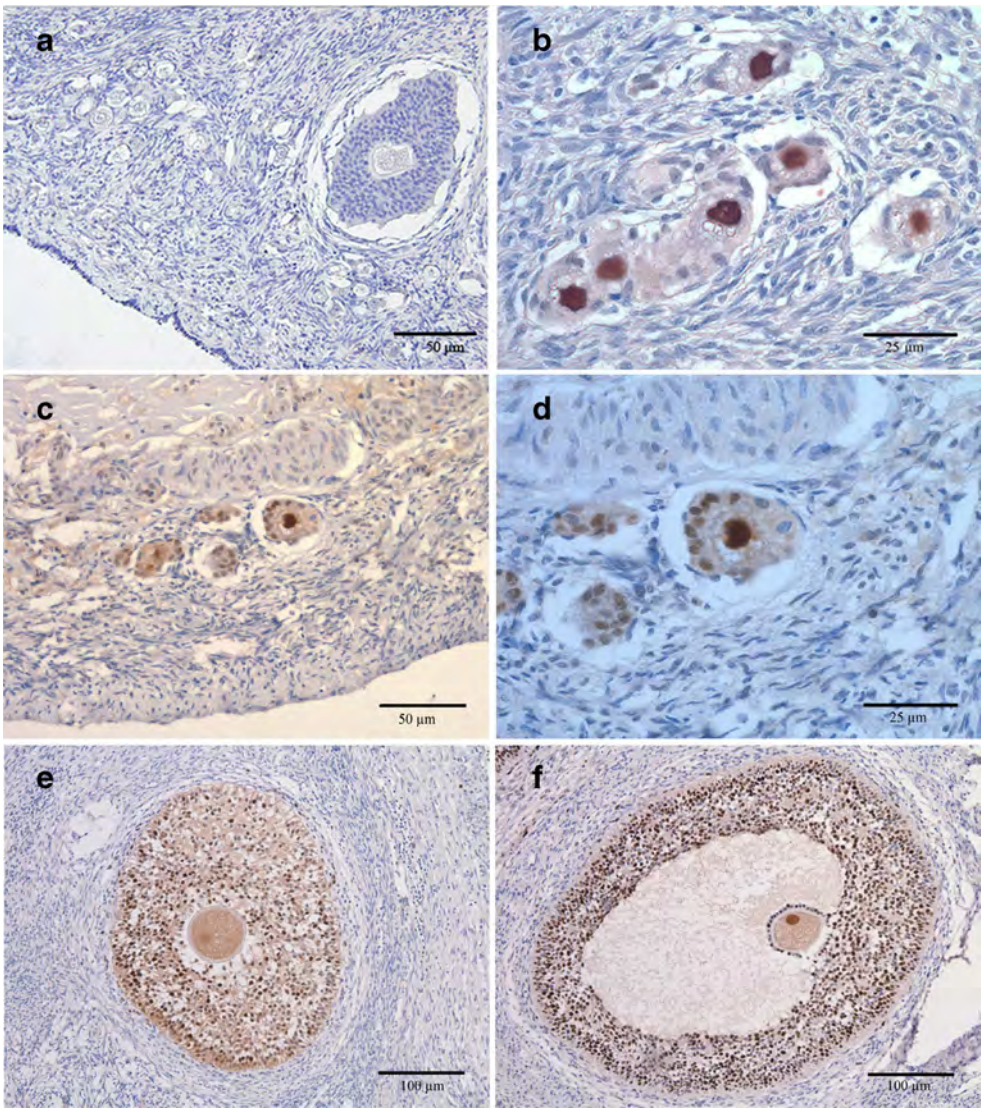

Fig. 2 Hematoxylin staining (**a**, negative control) and PCNA immunostaining of primordial follicles (**b**), primary follicles (**c**–**d**), pre-antral (**e**), and antral (**f**) follicles at magnification: ×20 (**a**, **c**, **e**, and **f**) and×40 (**b** and **d**)

more flattened pre-granulosa cells at the periphery of the follicle and no cuboidal cells (Fig. 2b); (II) "primary follicles" were follicles having a central oocyte surrounded by either a mixture of flattened pre-granulosa cells and plumb cuboidal granulosa cells or a single layer of cuboidal granulosa cells (Fig. 2 c and d); and (III) "growing follicles" including both pre-antral (secondary follicle) and antral follicles (tertiary follicle) and consisting of a visible zona pellucida and/or multiple layers of granulosa cells (Fig. 2e and f). Pre-antral follicles were follicles having a central oocyte and visible zona pellucida surrounded by multiple layers of granulosa cells with no antral formation (Fig. 2e). Antral follicles were follicles having an oocyte and zona pellucida surrounded by multiple layers of granulosa cells with antral formation (Fig. 2f). For each category of follicle, both the number and proportion of follicles were calculated. The number of follicles counted per 100 μm^2 of the real tissue section was calculated by: the number of follicles per 100 μm^2=(number of counted follicles/ tissue area)×100. The proportion of follicles was calculated by: the proportion of each category of follicles=(number of each category of follicles/ total number of all follicles)×100. The total number of follicles counted per 100 μm^2 was the sum of primordial follicles, primary follicles, and growing follicles per 100 μm^2.

Determination of granulosa cell proliferation

Granulosa cell proliferation was determined in pre-antral (n=197) and antral (n=76) follicles from 37 ovaries. All of the visible pre-antral and antral follicles in the ovarian tissue sections of the gilts were evaluated. In total, 273 follicles were evaluated. On average, 7.3 follicles (range 1 to 18) per ovary were identified. Granulosa cells with positive PCNA staining were evaluated using Image-Pro® Plus software under a light microscope (Fig. 3). The percentage of PCNA positive granulosa cells was calculated as:

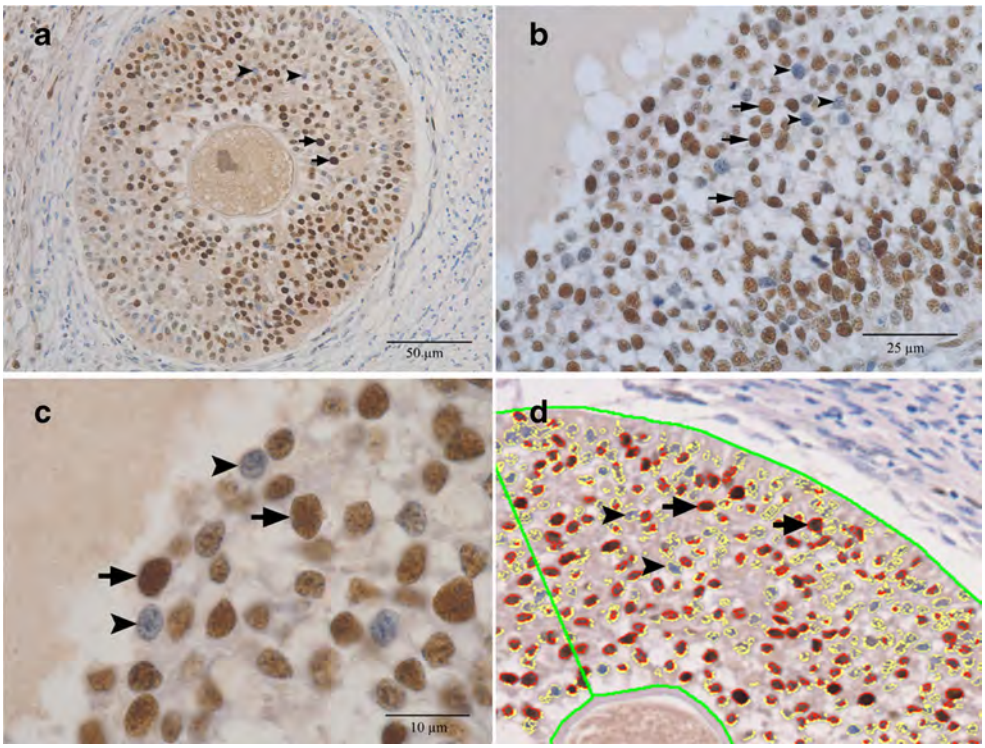

Fig. 3 PCNA immunostaining of granulosa cell proliferation in pre-antral follicle and antral follicles at magnification: ×20 (**a**), ×40 (**b**), and×100 (**c**) and demonstration of image analysis (**d**). Positive immunostaining for PCNA in granulosa cells stained dark brown (*arrow*) and negative immunostaining in granulosa cells stained blue (*arrowhead*)

PCNA-positive cells (%)=[(number of PCNA positive cells/ total number of granulosa cells counted)×100]. The follicles were classified according to their ovarian status, i.e., prepubertal (n=59) and cycling (n=214) ovaries. Also, the gilts were classified into four groups according to age: 229–258 days (n=55), 259–272 days (n=125), 274–284 days (n=33), and 287–345 days (n=60).

Statistical analysis

The statistical analyses were carried out using SAS version 9.0 (SAS institute Inc., Cary, NC, USA). Descriptive statistics including general means, standard deviation (SD), and the range of all the continuous data were calculated. The numbers of ovarian follicles are presented as mean±SD. Multiple analysis of variance (ANOVA) was used to analyze continuous data using the general linear model procedure of SAS. Dependent variables included the numbers of each type of follicle counted per 100 μm^2 [i.e., primordial follicles, primary follicles and growing follicles (both pre-antral and antral follicles)], the proportion of each category of follicle, the total number of follicles counted per 100 μm^2, the weight of the ovary, and ADG. The statistical model included the effect of ovarian status (prepubertal and cycling ovary), age class (229–258, 259–272, 274–284, and 287–345), and PRRS virus detection (positive or negative). The number of CL (i.e., ovulation rate) was analyzed by multiple ANOVA. The statistical model included the effect of age class and PRRS virus detection. Least square means were calculated from the statistical models and compared using the least significant difference test with Tukey-Kramer adjustment for multiple comparisons. Pearson's correlation was performed to determine the associations among the percentage with granulosa cell proliferation (i.e., percentage of positive PCNA immunostaining per follicle) and age of the gilts, body weight, ADG, number of ovulations, and weight of the ovaries. The percentage of positive PCNA immunostaining per follicle was analyzed by multiple ANOVA using the general linear mixed model procedure of SAS. The statistical model included the fixed effect of follicle type (pre-antral and antral), PRRS virus detection, ovarian status, age class, the interaction between follicle type and PRRS virus detection, and the interaction between ovarian status and PRRS virus detection. Other interactions were also tested but excluded from the final statistical models due to insignificance (P>0.1). The gilt's identity was included in the statistical model as a random effect. Least square means were obtained from the statistical models and were compared among each category of factors using a least significant difference test with Tukey-Kramer adjustment for multiple comparisons. P<0.05 was regarded as statistically significant.

Results

Descriptive data

Descriptive statistics on reproductive data and the density of follicles in the gilt ovarian tissues are presented in Table 1. The

Table 1 Descriptive statistics on gilts' historical data, gross morphology, and density of follicles in their ovarian tissue (n=37)

Parameter	Mean±SD	Range
Age at culling (days)	270.7±24.5	229.0–345.0
Body weight at culling (kg)	144.5±15.0	117.0–179.0
Age at first observed oestrus (days)	214.2±17.6	178.0–272.0
Average daily gain (g/day)	530.2±55.1	438.0–665.0
Weight of the ovary (g)	5.7±2.3	2.9–10.1
Ovulation rate	16.7±3.4	9.0–23.0
Total number of follicles per 100 μm^2	22.5±10.9	4.3–54.2
Number of primordial follicles (follicles/100 μm^2)	17.0±10.3	2.0–46.8
Number of primary follicles (follicles/100 μm^2)	5.0±2.9	0.4–14.0
Number of growing follicles (follicles/100 μm^2)	0.5±0.2	0.1–1.0
Granulosa cell proliferation (%)	61.4±33.6	0.2–99.9

average age and live weight at culling of the gilts were 270.7± 24.5 days and 144.5±15.0 kg, respectively. On average, each 100 μm^2 of ovarian tissue consisted of 17.0 primordial follicles, 5.0 primary follicles, and 0.5 growing follicles. The proportion of primordial, primary, and growing follicles was 71.9, 25.4, and 2.6 %, respectively. Of the 37 gilts, 27 gilts (73 %) had cycling ovaries and ten gilts had prepubertal ovaries (27 %). All of the gilts with cycling ovaries had dominant CL (Fig. 1). The ovulation rate was 16.7±3.4 (ranged 9.0 to 23.0).

Effect of PRRS virus

Of these ovaries (n=37), PRRS virus was detected in 20 (54.1 %) and was not detected in 17 (45.9 %). Figure 1 demonstrates positive immunohistochemical expression of PRRS virus in the ovarian tissue of replacement gilts. Reproductive data, follicle density, and granulosa cell proliferation in the ovarian tissue of gilts with and without PRRS virus are presented in Table 2. On average, the total number of follicles per 100 μm^2 of ovarian tissue in gilts without PRRS virus (23.7± 2.8) did not differ significantly compared with those with PRRS virus (24.5±2.8, P>0.05). The density of primordial, primary, and growing follicles per 100 μm^2 of ovarian tissue in gilts with and without PRRS virus is presented in Table 2. As can be seen from the table, neither the number nor the proportion of follicles in the ovarian tissue of gilts with PRRS virus differed significantly compared with those without PRRS virus (Table 2).

PCNA immunostaining of granulosa cells in pre-antral and antral follicles is presented in Fig. 3. Granulosa cell proliferation was influenced by the type of follicle (P<0.05), ovarian status (P<0.05), the age of the gilts (P<0.05), and the interaction between ovarian status and PRRS virus detection (P= 0.06). On average, the proliferation of granulosa cells in follicles without PRRS virus did not differ significantly compared with those with PRRS virus (59.6±4.7 versus 56.4± 5.2 %, P>0.05). The granulosa cell proliferation in antral follicles was significantly higher than in pre-antral follicles in both PRRS virus-positive and PRRS virus-negative ovarian tissues (Table 3).

In ovarian tissue from gilts without PRRS virus, granulosa cell proliferation in cycling ovaries was higher than that in prepubertal ovaries (84.0±5.1 and 35.2±8.0 %, P<0.05). However, in ovarian tissue containing PRRS virus, the proliferation of granulosa cells in cycling ovaries did not differ significantly compared to that in prepubertal ovaries (68.5± 4.3 and 44.4±9.2 %, P>0.05). Furthermore, the highest percentage of granulosa cell proliferation (i.e., 84.0 %) was found in cycling ovaries without PRRS virus (Table 4).

Effect of gilts' age and ovarian status

The gilts' historical data, gross morphology, follicle density, and granulosa cell proliferation by age group are presented in Table 5. It was found that the total number of follicles per 100 μm^2 and the proliferation of granulosa cells in the ovarian tissue of gilts aged 274–284 days were higher than in gilts aged 229–258 days (P<0.05) (Table 5).

Historical data, gross morphology, follicle density, and granulosa cell proliferation in gilts with prepubertal ovaries and cycling ovaries are presented in Table 6. As can be seen from the table, gilts with cycling ovaries had greater ovarian weight, ADG, and body weight than gilts with prepubertal ovaries. Furthermore, the number of primary follicles and growing follicles per 100 μm^2 of ovarian tissue in gilts that had cycling ovaries was higher than in those with prepubertal ovaries (Table 6). Granulosa cell proliferation in gilts with cycling ovaries was also higher than that in gilts with prepubertal ovaries (76.2±3.4 and 39.8± 6.5 %, P<0.05).

Discussion

PRRS virus in gilts' ovaries is mainly found in macrophages within the ovarian tissue. Macrophages containing PRRS virus antigen are commonly found in the ovarian medulla, regressing CL, and degenerating follicles (Sur et al. 2001). Macrophages in the ovary are involved in many ovarian activities, e.g., follicular growth, follicular atresia, ovulation, and CL formation (Araki et al. 1996). In the present study, follicles counted using PCNA immunostaining revealed no differences in follicle density in ovarian tissues with and without PRRS virus. This indicates that PRRS virus does not affect the number of follicles in a gilt's ovaries, in agreement with earlier studies (Benson et al. 2001; Sur et al. 2001). Benson et al.

Table 2 Gilts' historical data, gross morphology, and follicle density in their ovarian tissue with and without porcine reproductive and respiratory syndrome (PRRS) virus detection (least square means±SEM)

Parameter	PRRS virus-negative (n=17)	PRRS virus-positive (n=20)	P value
Gross			
Age at culling (days)	275.8±3.5	268.2±3.6	0.088
Body weight (kg)	141.9±3.8	141.5±3.9	0.929
Average daily gain (g/day)	510.9±13.7	522.1±14.0	0.510
Weight of the ovary (g)	5.1±0.5	5.2±0.5	0.920
Ovulation rate	17.5±1.0	16.5±0.8	0.445
Follicles (per 100 μm^2)			
Total number of follicles	23.7±2.8	24.5±2.8	0.818
Number of primordial follicles	19.3±2.6	19.6±2.6	0.927
Number of primary follicles	4.0±0.8	4.5±0.8	0.620
Number of growing follicles	0.4±0.1	0.4±0.1	0.762
Proportion of primordial follicles	75.1±3.7	75.0±3.8	0.978
Proportion of primary follicles	22.9±3.6	22.5±3.7	0.922
Proportion of growing follicles	1.9±0.5	2.5±0.5	0.383
Granulosa cells			
Granulosa cell proliferation (%)	59.6±4.8	56.4±5.3	0.637

(2001) demonstrated that experimental challenge with PRRS virus in gestating sows caused no consistent or significant microscopic lesions in the ovarian tissues of the sows. Furthermore, no evidence of PRRS virus antigen was detected in the ovarian tissue of sows at day 21 post-infection (Benson et al. 2001). In addition, progesterone levels at 111 days of gestation in PRRS virus-infected sows did not differ significantly compared with those in uninfected sows (Benson et al. 2001). On the other hand, in the testis of boars, PRRS virus infection causes depletion and death of testicular germ cells and results in hypospermatogenesis (Sur et al. 1997). Sur et al. (2001) found that PRRS virus could be isolated from gilts' ovaries at day 7 post-infection. Numerous PRRS virus-positive cells in the ovaries were found in atretic follicles (Sur et al. 2001). However, no alteration in the ovarian architecture supporting a possible role of PRRS infection in porcine female infertility could be demonstrated. It has been suggested that phagocytic macrophages containing PRRS virus may deliver the virus to neighboring cells, such as granulosa cells (Sur et al. 2001). In the present study, it was found that granulosa cell proliferation in cycling ovaries was reduced by 18.4 % (i.e., from 84.0 to 68.5 %, Table 4) in ovarian tissue with PRRS virus compared with ovaries without PRRS virus. The reason might be that PRRS virus infection may interfere with or reduce the synthesis of certain ovarian growth factors, e.g., insulin-like growth factor (IGFs), epidermal growth factor, vascular endothelial growth factor, thrombopoietin, erythropoietin, and members of Notch family (Sirotkin 2011). Hence, this may cause poor follicle development and poor steroidogenesis, and may also impair the qualities of the oocytes. For instance, lack of IGFs decreases the response of granulosa and theca cells and oocytes to gonadotropins (Sirotkin 2011). A complete mechanism on the impact of PRRS virus infection on the ovarian function has not been fully elucidated. It is known that macrophage is a target cell of PRRS virus (Olanratmanee et al. 2011), and macrophage plays a critical role on a variety of cellular behavior, e.g., adhesion, migration, invasion, and survival (Miyake et al. 2006).

Table 3 Granulosa cell proliferation in pre-antral and antral follicles in the ovarian tissue of gilts with and without porcine reproductive and respiratory syndrome (PRRS) virus detection (least-squares means±SEM)

Follicle type	n	Granulosa cell proliferation (%)	
		PRRS virus-negative	PRRS virus-positive
Pre-antral	197	52.5±5.1aA	46.6±5.4aA
Antral	76	66.7±6.0aB	66.3±6.3aB

Small letters indicate significant differences within rows, and capital letters indicate significant differences within columns (P<0.05)

Table 4 Granulosa cell proliferation in the follicles of gilts with prepubertal and cycling ovaries with and without porcine reproductive and respiratory syndrome (PRRS) virus detection (least squares means±SEM)

Ovarian status	n	Granulosa cell proliferation (%)	
		PRRS virus-negative	PRRS virus-positive
Prepubertal	214	35.2±8.0a,A	44.4±9.2a,A
Cycling	59	84.0±5.1a,B	68.5±4.3a,A

Small letters indicate significant differences within rows, and capital letters indicate significant differences within columns (P<0.05)

Table 5 Gilts' historical data, gross morphology, follicle density, and granulosa cell proliferation in ovarian tissue from animals culled at different ages (least square means±SEM)

Parameters	Age at culling (days, range)			
	229–258	259–272	274–284	287–345
Gross				
Age at culling (days)	242.7±4.8a	266.6±3.4b	277.1±6.5bc	301.6±5.1d
Body weight (kg)	132.2±5.2a	143.3±3.6a	146.0±7.1a	145.2±5.5a
Average daily gain (g/day)	540.0±18.0a	532.3±13.1ab	520.1±25.8ab	473.7±20.0b
Weight of the ovary (g)	5.5±0.7a	4.8±0.5a	5.9±0.9a	4.5±0.7a
Ovulation rate	15.6±1.2a	15.1±1.2a	19.5±1.9a	17.9±1.1a
Follicles (per 100 μm^2)				
Total number of follicles	15.5±3.9a	23.5±2.6ab	34.5±5.2b	23.2±4.1ab
Number of primordial follicles	11.3±3.5a	18.2±2.4a	30.3±4.8a	18.2±3.7a
Number of primary follicles	3.9±1.1a	4.8±0.8a	3.6±1.5a	4.8±1.2a
Number of growing follicles	0.3±0.1a	0.5±0.1a	0.4±0.1a	0.3±0.1a
Proportion of primordial follicles	63.8±5.1a	75.9±3.5a	86.8±6.9a	73.8±5.4a
Proportion of primary follicles	33.1±5.0a	21.5±3.5a	11.9±6.8a	24.4±5.3a
Proportion of growing follicles	3.1±0.7a	2.6±0.5a	1.3±0.9a	1.8±0.8a
Granulosa cells				
Granulosa cell proliferation (%)	46.4±6.3a	60.5±4.4ab	74.2±8.2b	60.0±6.6ab

Different superscripts within rows indicate significant differences ($P<0.05$)

This might be explained in part the reduced granulosa cells proliferation in the ovarian tissue infected with PRRS virus.

In general, the atretic process in follicles depends on many factors, including the physiological condition of the animal.

Table 6 Gilts' historical data, gross morphology, follicle density, and granulosa cell proliferation in ovarian tissue with prepubertal ovaries compared with cycling ovaries (least square means±SEM)

Parameters	Ovarian status		*P* value
	Prepubertal	Cycling	
Gross			
Age at culling (days)	270.7±4.9a	273.3±2.7a	0.646
Body weight (kg)	134.7±5.3	148.6±2.9	0.028
Average daily gain (g/day)	492.3±19.3	540.8±10.4	0.034
Weight of the ovary (g)	3.5±0.7	6.8±0.4	<0.001
Ovulation rate	0	16.7±3.4[a]	–
Follicles (per 100 μm^2)			
Total number of follicles	24.4±3.9	23.8±2.1	0.895
Number of primordial follicles	21.2±3.6	17.8±1.9	0.407
Number of primary follicles	3.0±1.1	5.5±0.6	0.053
Number of growing follicles	0.3±0.1	0.5±0.1	0.011
Proportion of primordial follicles	71.3±2.8	78.8±5.2	0.211
Proportion of primary follicles	19.4±5.1	26.0±2.8	0.263
Proportion of growing follicles	1.7±0.7	2.7±0.4	0.268
Granulosa cells			
Granulosa cell proliferation (%)	39.8±6.5	76.2±3.4	<0.001

[a] Mean±SD

The atretic process has not yet been well described; however, macrophages play a major role by producing pro-inflammatory mediators, e.g., interleukin 1, interleukin 2, and tumor necrosis factor. A previous study has demonstrated that macrophages are involved in the atretic process in both the prepubertal and pubertal stages, and are usually absent in healthy developing swine follicles (Sur et al. 2001). We found that PRRS virus was detected in both prepubertal and pubertal gilts. Therefore, PRRS virus infected macrophages may invade the granulosa cell layer and contribute to the apoptosis of germinal epithelial cells (Sur et al. 2001). However, Sur et al. (2001) did not find any significant differences in ovarian architecture in PRRS virus-infected gilts compared with that in non-infected gilts. In the present study, granulosa cell proliferation tended to be reduced in ovarian tissues containing PRRS virus compared with ovaries without PRRS virus. The highest granulosa cell proliferation was found in the follicles of cycling ovaries without PRRS virus detection. A previous study demonstrated that insemination of either seronegative or preimmunized gilts with boar semen containing PRRS virus had no effect on conception or fertilization rates (Prieto et al. 1997a). Furthermore, PRRS virus infection does not appear to affect the embryo before attachment but may cause embryonic death due to transplacental infection (Prieto et al. 1997b). However, in practice, most replacement gilts were exposed to PRRS virus during the acclimatization period, which takes place sometime before first insemination (Tummaruk and Tantilertcharoen 2012). As indicated by the present results, when the ovaries of the gilts are cycling (puberty), PRRS virus infection may decrease granulosa cell proliferation and

possibly cause poor steroidogenesis and compromised oocyte quality. This may subsequently cause infertility problems in mated gilts, which are commonly seen under field conditions (Tummaruk et al. 2010). Based on the information of these findings, pubertal gilts are not recommended to be exposed to PRRS virus. Therefore, the acclimatization process should be done (ended) before the gilts' exhibit first standing heat. On average, Landrace × Yorkshire crossbred gilts in Thailand show first standing heat at 200 days of age (Tummaruk et al. 2009b). Pubertal gilts should be kept in individual stalls for some time (the so-called cool down period) before first insemination takes place.

The limitation of the present study is that all of the reproductive organs were obtained from only PRRS virus seropositive herds. Of these gilts, PRRS virus was detected in the ovarian tissues of one group, but not in the other. It was found that the presence of PRRS virus in the ovarian tissues of the gilts reduced granulosa cell proliferation to some extent, especially in cycling ovaries. However, it should be remarked that no ovarian tissue from PRRS virus seronegative herd could be obtained. The reason is that PRRS virus seronegative herds are rarely found under field conditions in commercial Thai swine herds (Tummaruk et al. 2013).

In the present study, ovarian status also influenced granulosa cell proliferation. It was clearly demonstrated that granulosa cell proliferation in cycling ovaries was higher than that in prepubertal ovaries. In fact, the ages of the gilts with cycling and prepubertal ovaries did not differ significantly, while the growth rate and body weight in gilts with cycling ovaries were higher than in those with prepubertal ovaries. This indicated that the physical maturity of gilts with cycling ovaries might be somewhat more advanced than those with prepubertal ovaries. Nevertheless, the total number of ovarian follicles per 100 μm^2 of ovarian tissue as well as the proportion of each category of follicle did not differ significantly between cycling and prepubertal ovaries (Table 6). This indicated that gilts with prepubertal ovaries certainly had a sufficient number of ovarian follicles but failed to accomplish ovulation. The reason might possibly be a lack of steriodogenesis, suboptimal luteinizing hormone (LH) secretion, or impaired LH receptors at the ovarian level. The present study demonstrated that granulosa cell proliferation in growing follicles was much lower in prepubertal ovaries than in cycling ovaries (39.8 versus 76.2 %, Table 6). In general, it is widely accepted that the quality of granulosa cells is closely associated with oocyte development (Pandy et al. 2010). Therefore, it can be speculated that the poorer granulosa cell proliferation is, the more impaired oocyte development will be. Furthermore, poor granulosa cell development may also subsequently cause poor steroid production and impair ovarian function. Thus, ovulation and puberty attainment will not occur.

It can be concluded that the detection of PRRS virus in the gilt's ovarian tissue was not associated with the number or type of follicles, but it was associated with the proliferation of granulosa cells in cycling ovaries. Cycling ovaries had greater granulosa cell proliferation than prepubertal ovaries. These findings imply that PRRS virus detection in gilt ovarian tissue may be one factor that can impair follicle development and oocyte quality, and subsequently lead to abnormal estrous behavior and infertility in replacement gilts.

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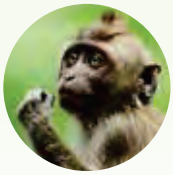



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A051-SW013 Effect of PRRS virus infection in the ovarian tissue on follicle growth in prepubertal and pubertal gilts

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Keywords: gilts, granulosa cells, ovary, proliferating cell nuclear antigen, PRRS

Introduction

Porcine reproductive and respiratory syndrome (PRRS) virus has been isolated from ovary of the gilts (1). However, the involvement of PRRS virus in the follicle growth has not been clearly determined. Proliferating cell nuclear antigen (PCNA) is an auxiliary protein that necessary for DNA synthesis. It has been demonstrated that PCNA expression in the ovary involves with follicle growth in many species (2, 3). The purpose of the present study was to evaluate the effect of PRRS virus infection in the porcine ovarian tissue on follicle growth in prepubertal and pubertal gilts.

Materials and Methods

Ovarian tissue sections were obtained from 37 Landrace x Yorkshire crossbred gilts. For each gilt, two sections were cut and each section was placed on a separated slide. One section was used to determine PRRS virus infection by using immunohistochemistry and another was stained by PCNA immuno-histochemistry (3). The gilts were classified on the criterion of an immunohistochemical expression of PRRS virus in the ovarian tissues as positive (n=20) and negative (n=17) groups (Figure 1A and 1B). Stages of the reproductive cycle were classified according to the ovarian appearance and structures that is corpus luteum (CL) and follicles (4). Gilts with ovaries having only small follicles (<5 mm) were defined as a prepubertal (n=10) and those with ovaries having CL were defined as a pubertal (n=27) (Figure 1C and 1D). For the PCNA sections, a total of 273 follicles (197 pre-antral and 76 antral follicles) were determined for the proportion of PCNA expression under light microscope. The positive areas of granulosa cells with positive PCNA staining were calculated using Image-Pro® Plus software.

The statistical analyses were carried out using multiple ANOVA. The statistical model include effect of follicle type (preantral and antral), PRRS virus infection (positive and negative), ovarian status (prepubertal and pubertal), reason for culling (abortion, anestrus, repeat service, vaginal discharge and non-reproductive causes) and interaction between follicle type and PRRSV, follicle type and ovarian status and PRRSV and ovarian status. Least square means were obtained from each class of the factor and were compared by using least significant difference test. $P<0.05$ were regarded to be statistically significant.

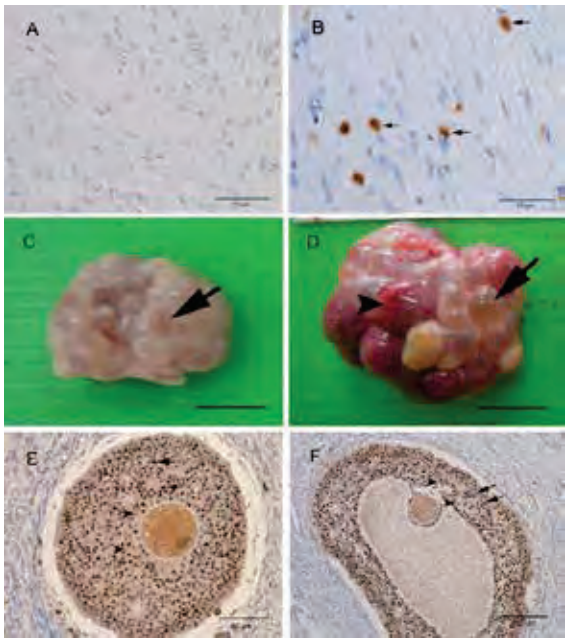

Figure 1. (A-B) Immunohistochemical expression of negative (A) and positive (B) PRRSV in macrophages of the gilt's ovarian tissue. (C-D) Small follicles (arrow) of ovary in prepubertal gilt and corpus luteum (arrow-head) in the ovary of pubertal gilt (bar = 1 cm). (E-F) PCNA immuno-staining of growing follicle demonstrate positively stained granulosa cells (arrow) and negatively stained granulosa cells (arrow-head) in pre-antral (E) and antral (F) follicles.

Result and Discussion

The follicle growth was demonstrated by the percentage of granulosa cells proliferation stained by PCNA (Figure 1E and 1F). The proportion of PCNA expression in the growing follicles of PRRSV negative and positive ovarian tissue is presented in Table 1. The present study demonstrated that the growth of the ovarian follicle of pubertal gilt was higher than pre-pubertal gilt in negative PRRS group but not in PRRS positive group (Table 1).

Table 1. Percentage of granulosa cells proliferation (least-squares means±SEM) in the ovarian tissue of gilts infected with porcine reproductive and respiratory syndrome virus.

Gilt status	PRRS virus infection	
	negative	positive
Prepubertal	$36.6\pm6.5^{a,A}$ (n=33)	$49.8\pm7.6^{a,A}$ (n=26)
Pubertal	$76.6\pm3.7^{a,B}$ (n=82)	$59.9\pm4.6^{b,A}$ (n=132)

n=number of follicle, ^{a,b,} Different letters within row differed significantly ($P<0.05$), ^{A,B} Different capital letters within column differed significantly ($P<0.05$).

This indicated that PRRS infection in the ovarian tissue of gilt influence the proliferation of granulosa cells and may lead to poor follicle growth as well as poor quality of oocyte. However, infection of PRRS virus in the ovarian tissue of prepubertal gilt did not influence the proliferation of granulosa cell of the follicle. This indicated that the acclimatization and/or vaccination should be introduced to the replacement gilt during prepubertal stage. The introduction of PRRS live virus to pubertal gilt is therefore not recommended. Additionally, the immunohistochemical expression of PCNA of granulosa cells in gilt can be applied to study the folliculogenesis in pig.

It could be conclude that PRRS virus infection effect the granulosa cell proliferation of pubertal gilts but not in pre-pubertal gilts.

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Follicle development and number of ovulation in the ovarian tissue of gilts infected by porcine reproductive and respiratory syndrome virus

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Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) has been isolated from the ovary of the female pigs and may be involve with the reproductive failure in gilts and sows[5]. PRRSV can induce apoptosis in germ cells from testes, alveolar macrophage and mononuclear cells from lymphoid tissues[3,4]. Nevertheless, little information about the pathogenesis of PRRSV infection in the gilts ovary has been done[5]. Furthermore, the influence of PRRSV infection in the ovary on follicle development has not been elucidated. The aim of the present study was to determine follicles development and number of ovulation in the ovarian tissue of gilts infected by PRRSV.

Materials and Methods

Ovarian tissue sections were obtained from 19 Landrace x Yorkshire crossbred gilts aged 267.8±19.2 days and weighted 145.7±11.8 kg. The genital organs were collected from slaughterhouses, placed on ice and transported to the laboratory within 24 h of culling. Ovulation rate was defined as the total number of corpora lutea (CL) from both ovaries. The ovaries were fixed in 10% neutral-buffered formalin for 24-48 h, processed by an automatic tissue processor and embedded in paraffin block. The paraffin embeddings were cut into 5 μm thick by using microtome. At each ovarian tissue, two sections were cut and each section was placed on a separate slide. One section was used to determine PRRSV infection using immunohistochemistry[2], while another was stained by PCNA immuno-histochemistry. For the PCNA sections, the follicles were categorized as primordial, primary and growing follicles and were quantified under light microscope. The number of follicles was expressed as the total number of follicles per 100 μm^2 of the tissue section. The gilts were classified on the criterion of body weight (≥150 kg, n=7 versus <150 kg, n=12) and the present or absent of PRRSV in the ovarian tissue (positive, n=10 versus negative, n=9). Multiple analysis of variance was used to analyze the effect of body weight and PRRSV infection on the number of follicles. $P<0.05$ were regarded to be statistically significant.

Results

On average, the total number of follicles in negative and positive PRRSV ovarian tissue was 21.6±3.1 and 19.2±2.7, respectively ($P=0.56$). Number of primordial, primary and growing follicles and ovulation rate in PRRSV positive and negative ovarian tissues are presented in Table 1. The number of primary follicles in gilts with a body weight of ≥150 kg was higher than gilts with a body weight of <150 kg (8.9±1.1 *versus* 4.6±0.8, $P=0.007$).

Table 1. Number of primordial, primary and growing follicles in PRRSV positive and negative ovarian tissue

Follicle	PRRSV	
	Positive	Negative
Primordial	12.2±6.7[a]	14.0±7.4[a]
Primary	6.4±4.5[a]	6.0±1.3[a]
Growing	0.6±0.2[a]	0.5±0.2[a]
Ovulation rate	15.0±2.9[a]	16.7±5.3[a]

[a] The same superscript within a row do not differ significantly (P>0.05)

Conclusions and Discussion

PRRSV in the gilt ovary is mainly found in the macrophages in the ovarian tissue[5]. Follicular count using PCNA immunohistochemistry revealed that no differences between number of follicles in negative and positive PRRSV ovarian tissues. This indicated that PRRSV might not affect the number and type of follicles in the gilts ovarian tissue. This is in agreement with earlier studies[1, 5]. However, a high variation on the number of primary follicles in the PRRSV positive ovarian tissues was remarked (Table 1). This indicated that abnormal follicles development may occurred only in some gilts. Additional works will be carried to determine the occurrence of apoptosis and also some additional number of gilts will be added.

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The Proceeding

Thai Agricultural Path Advances toward ASEAN for Sustainable Development

51st

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เล่มที่ 2

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ผลกระทบของการตรวจพบเชื้อไวรัสพีอาร์อาร์เอสในเนื้อเยื่อรังไข่ของสุกรสาวทดแทนต่อการงอกขยายของแกรนูโลซาเซลล์ในฟอลลิเคิลที่กำลังเจริญเติบโต

Impact of PRRS virus detection in the ovarian tissue of replacement gilts on granulosa cells proliferation in the developing follicles

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บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อประเมินผลกระทบของการตรวจพบเชื้อไวรัสพีอาร์อาร์เอส (PRRS) ในเนื้อเยื่อรังไข่ของสุกรสาวทดแทนต่อการงอกขยายของแกรนูโลซาเซลล์ในฟอลลิเคิลที่กำลังเจริญเติบโต เก็บรังไข่จากสุกรสาวพันธุ์ผสมแลนด์เรซและยอร์กเชียร์ จำนวน 12 ตัว แบ่งเป็นกลุ่มที่ให้ผลบวก (n=6) และลบ (n=6) ต่อการตรวจพบเชื้อไวรัสพีอาร์อาร์เอส บนเนื้อเยื่อรังไข่จากการตรวจด้วยวิธีอิมมูโนฮิสโตเคมี นำเนื้อเยื่อรังไข่จากสุกรสาวทั้งหมดมาผ่านกระบวนการอิมมูนโนฮิสโตเคมี เพื่อตรวจหาการงอกขยายของกรานูโลซาเซลล์บนฟอลลิเคิลที่กำลังเจริญเติบโตจำนวน 56 ใบ โดยฟอลลิเคิลที่ทำการศึกษาประกอบด้วย 2 ระยะ ได้แก่ ระยะพรีแอนทรัม 41 ใบ และระยะแอนทรัม 15 ใบ ตรวจวัดสัดส่วนของกรานูโลซาเซลล์ที่พบโปรตีนพีซีเอ็นเอภายใต้กล้องจุลทรรศน์แสงสว่างแล้วทำการประเมินด้วยโปรแกรมวิเคราะห์ภาพ แล้วเปรียบเทียบสัดส่วนของกรานูโลซาเซลล์ที่กำลังมีการงอกขยาย (ติดสีพีซีเอ็นเอ) ในฟอลลิเคิลทั้ง 2 ระยะ บนรังไข่ที่ติดเชื้อไวรัสพีอาร์อาร์เอส เปรียบเทียบกับรังไข่ที่ไม่ติดเชื้อไวรัสพีอาร์อาร์เอส ผลการทดลองพบว่าในฟอลลิเคิลระยะพรีแอนทรัม กลุ่มเนื้อเยื่อรังไข่ที่ตรวจไม่พบเชื้อไวรัสพีอาร์อาร์เอส มีสัดส่วนของแกรนูโลซาเซลล์ที่กำลังมีการงอกขยายจำนวนสูงกว่ากลุ่มเนื้อเยื่อรังไข่ที่ตรวจพบเชื้อไวรัส พีอาร์อาร์เอส (57.4±5.8% และ 36.6±5.7% ตามลำดับ P=0.01) ส่วนในฟอลลิเคิลระยะแอนทรัมพบว่ากลุ่มเนื้อเยื่อรังไข่ที่ตรวจไม่พบเชื้อไวรัสพีอาร์อาร์เอส มีสัดส่วนของแกรนูโลซาเซลล์ที่กำลังมีการงอกขยายไม่แตกต่างจากกลุ่มที่เนื้อเยื่อรังไข่ที่ตรวจพบเชื้อไวรัสพีอาร์อาร์เอส (P=0.800) การศึกษานี้สรุปว่าการติดเชื้อไวรัสพีอาร์อาร์เอส ในเนื้อเยื่อรังไข่ของสุกรสาวทดแทนทำให้การเกิดการงอกขยายของแกรนูโลซาเซลล์ลดลงในฟอลลิเคิลระยะพรีแอนทรัม แต่ไม่มีผลต่อฟอลลิเคิลระยะแอนทรัม ผลกระทบนี้อาจทำให้ฟอลลิเคิลระยะพรีแอนทรัมมีการพัฒนาช้าลงและทำให้คุณภาพของโอโอไซต์ต่ำลง ซึ่งอาจเป็นสาเหตุของปัญหาการไม่เป็นสัดและความไม่สมบูรณ์พันธุ์ในสุกรสาวได้

Keywords: granulosa cell, PRRSV, Proliferating cell nuclear antigen, gilts

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ABSTRACT

The purpose of this study was to evaluate the influence of PRRSV detection on the granulosa cells proliferation during follicle development in replacement gilts. The gilts were classified on the criterion of the PRRSV immunohistochemical expression in the ovarian tissues as positive (n=6) and negative (n=6) groups. Ovarian sections were evaluated for PCNA by use of immuno-histochemistry and categorized as pre-antral (n=41) and antral follicles (n=15). The proportion of PCNA expression in granulosa cells were determined under light microscope and calculated using Image-Pro® Plus software. The proportion of proliferating granulosa cells in either preantral and antral follicles were compared between PRRS positive and PRRS negative ovarian tissue. The results revealed that pre-antral follicle with negative PRRSV had a higher percentage of proliferative marker than those with positive PRRSV (57.4±5.8% and 36.6±5.7%, respectively, *P*=0.01). For antral follicles, the negative PRRSV had no difference in PCNA expression of granulosa cells compared with the positive PRRSV (*P*=0.800). It could be concluded that PRRSV infection in the gilt ovarian tissues reduced the proliferation of granulose cells in pre-antral follicles, but did not influence antral follicles. This impact may result in a delay follicle development and reduce the oocytes quality and subsequently caused abnormal estrus behavior and infertility problem in replacement gilts.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) has been isolated from the ovary of the gilts (Sur *et a*l., 2001; Olanratmanee *et al*., 2011b). In boar, it has been clearly demonstrated that the virus is able to induces apoptosis of the testicular germ cells (Sur *et al*., 2001), while in the female's gonad, only limited information is known. To our knowledge, only one study has demonstrated that PRRS virus can penetrate the resident macrophages of the ovary, but its involvement in the follicle development, ovulation and corpus luteum formation has not been clearly determined (Sur *et al*., 2001).

Proliferating cell nuclear antigen (PCNA) is an auxillary protein of DNA polymerase delta that necessary for DNA synthesis (Kurki *et al*., 1986; Bravo *et al*., 1987). The expression of PCNA increases during G1 phase, gets to the highest level in S-phase and decreases during G2/M phases of the cell cycle (Kurki *et al*., 1988). An earlier study has demonstrated that the expression of PCNA in the ovary involve with follicular development in many species, e.g, rat (Oktay *et al.,* 1995), cow (Wandji *et al.,* 1996), baboon (Wandji *et al.,* 1995), pig (Tománek and Chronowska, 2006; Phoophitphong *et al.,*

2012) and human (Kelsey *et al.,* 2010). The purpose of the present study was to evaluate the granulosa cells proliferation during follicle development (pre-antral and antral stages) in the ovarian tissue of replacement gilts with and without PRRSV detection.

Material and methods

Animals and management

Ovarian tissue sections were obtained from 12 Landrace x Yorkshire crossbred gilts aged 275.2±34.2 days and weighted 142.7±19.1 kg. The gilts were obtained from two commercial swine herd in the middle and northern parts of Thailand. The replacement gilts were kept in the gilt pools for at least 60 days before sending to the breeding unit. All the replacement gilts were vaccinated against foot-and-mouth disease virus (FMDV), classical swine fever virus (CSFV), Aujeszky's disease virus (ADV), porcine parvovirus (PPV) and porcine reproductive and respiratory syndrome virus (PRRSV) between 154 and 210 days of age. Moreover, an acclimatization was conducted before service, by grouping the replacement gilts with the weaned sows selected for removal for about 28 days. The acclimatization process was initiated by introducing such weaned sows to the gilts' pen at 154–196 days of age with a ratio of one sow per 6-10 gilts. The sows used for acclimatization were rotated on a weekly basis and were removed from the herd after acclimatization. Using this acclimatization process, the gilts were exposed to many types of viral pathogens circulating within the herds (e.g., PRRSV, PPV and enterovirus) before sending to the breeding unit.

Sample collection and immunohistochemistry

The genital organs were collected from slaughterhouses, placed on ice and transported to the laboratory within 24 h of culling. Ovulation rate was defined as the total number of corpora lutea (CL) from both ovaries. The ovaries were fixed in 10% neutral-buffered formalin for 24-48 h, processed by an automatic tissue processor and embedded in paraffin block. At each ovarian tissue, two sections were cut and each section was placed on a separated slide. One section was used to determine PRRS virus infection using immunohistochemistry (Olanratmanee *et al*., 2011b), while another was stained by PCNA immuno-histochemistry. PCNA immuno-staining technique has been modified after previous studies in the rat's ovarian tissue (Mushkelishvili *et al*., 2005; Picut *et al*., 2008). The slides were incubated with mouse monoclonal anti-PCNA (clone PC10, DAKO, Carpinteria, CA, USA) as a primary antibody at a dilution of 1:200 overnight at 4°C. After incubation with the primary antibody, the sections were incubated with DAKO EnVision™ reagent for 45 min at room temperature. The primary antibody was visualized by 3,3' -diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma, Germany) for 3 min at room temperature. For negative control, PBS was used instead of the primary antibody.

Follicles categorization

The gilts were classified on the criterion of an immunohistochemical expression of PRRS virus in the ovarian tissues as positive (n=6) (Figure 1) and negative (n=6) groups. The follicles were classified into 2 categories as described earlier (Oktay *et al*., 1995; Picut *et al*., 2008). Pre-antral follicles were follicles having a central oocyte and visible zona pellucida surrounded by multiple layers of granulosa cells with no antral formation (Figure 2). Antral follicles were follicles having an oocyte and zona pellucida surrounded by multiple layers of granulosa cells with antral formation. For the PCNA sections, a total of 56 follicles were categorized as pre-antral (n=41) and antral follicles (n=15) and were determined for the proportion of PCNA expression under light microscope. The positive areas of granulosa cells with positive PCNA staining were calculated using Image-Pro® Plus software.

Statistical analysis

The statistical analyses were carried out using SAS version 9.0 (SAS institute Inc., Cary, NC, USA). The proportion of PCNA positive area was analyzed by using general linear model (GLM) procedure. The statistical model included follicle type (pre-antral and antral), PRRSV infection (positive and negative) and interaction between follicle type and PRRSV infection. Least squared means were obtained from the statistical models and were compared by using least significant different (LSD) test. $P<0.05$ were regarded to be statistically significant.

Result and Discussion

Figure 1 demonstrated PRRS virus detection in the ovarian tissue of replacement gilts and Figure 2 demonstrated the PCNA immnostaining of granulosa cells in a pre-antral and antral follicles. Regardless to the follicle types, follicles in the negative PRRS virus ovarian tissue tended to have a higher percentage of proliferative marker than those with positive PRRS virus ovarian tissue (57.0±5.2% versus 44.9±6.0%, $P=0.132$). For pre-antral follicles, negative PRRS virus ovarian tissue had 20.8% higher PCNA expression granulosa cells than the positive PRRS virus ovarian tissues (57.4±5.8% versus 36.6±5.7%, $P=0.01$). For antral follicles, the proliferation of granulosa cells in PRRS virus negative and PRRS virus positive ovarian tissue did not differ significantly (56.6±8.6% versus 53.2±10.5%, $P=0.800$) (Table 1). The proliferation of granulosa cells in antral and pre-antral follicles did not differ significantly (54.9±6.8% versus 47.0±4.1%, respectively, $P=0.324$).

In the present study, based on immunological detection of PRRSV in the ovarian tissue, 6 gilts were classified as positive and other 6 gilts were classified as negative. However, all of the reproductive organs were obtained from PRRSV sero-positive herds and PRRSV vaccination was

also performed in all replacement gilts. Thus, all of the gilts had been sero-positive gilts. Of these gilts, one group (n=6) detected PRRSV in the ovarian tissue, while another group (n=6) did not detected PRRSV in their ovarian tissue. Our hypothesis is that, if the gilts expose to PRRSV and the virus remains in their ovary until the time of insemination (i.e., at age >220 days). Would it be any significant impact on the follicles development and/or the oocyte qualities? It was found that the presence of PRRSV in the ovarian tissue of gilts significantly reduce granulosa cells proliferation of pre-antral follicles for 20% (36.6 versus 57.4%). This may reduce some of the ovarian growth factors (Sirotkin, 2011) and subsequently cause poor follicles development, reduced steroidogenesis and impair the oocyte's qualities. However, additional research should be carried to determine some more parameters indicating the ovarian function (e.g., apoptosis, expression of steroid receptor) as well as some ovarian growth factors.

Table 1 Granulosa cells proliferation (least-squares means±SEM) in the ovarian tissue of gilts infected with porcine reproductive and respiratory syndrome virus

	PRRS virus negative	PRRS virus positive
Pre-antral follicle	57.4±5.8%[a]	36.6±5.7%[b]
Antral follicle	56.6±8.6%[a]	53.2±10.5%[a]

[a,b] Different letters within row differed significantly ($P<0.05$)

Conclusions

It could be concluded that PRRS virus infection in the ovarian tissue of gilts significantly reduced proliferation of granulose cells of pre-antral follicles. This may subsequently resulted in an increase in the number of poor quality oocytes, poor steroidogenesis and may cause poor estrus behavior and/or infertility problems in replacement gilts.

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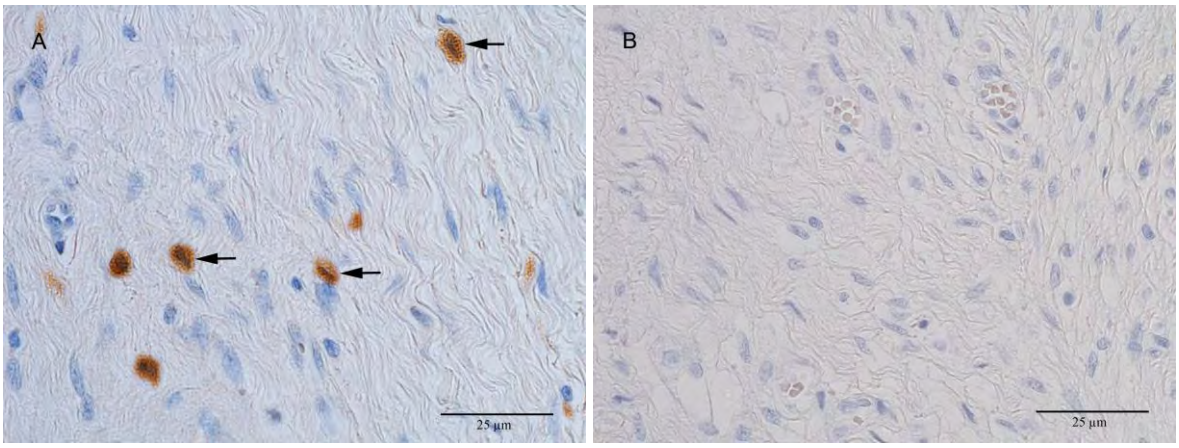

Figure 1 Porcine reproductive and respiratory syndrome virus (PRRSV) infection in macrophages of ovarian medulla (A) and negative PRRS infection (B) (magnification 400x).

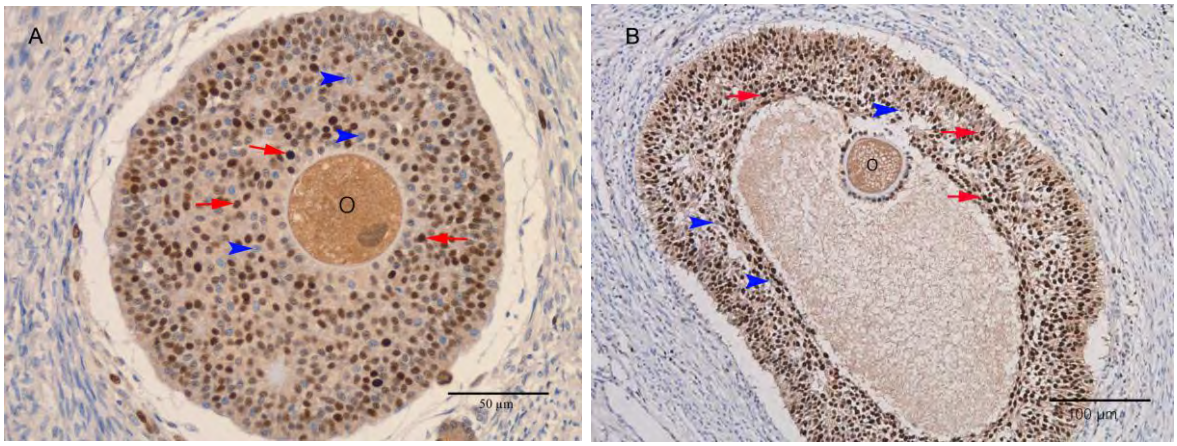

Figure 2 Proliferating cell nuclear antigen (PCNA) immuno-staining of growing follicles. Positive stained granulosa cells (arrow) and negative stained granulosa cells (arrow-head) in pre-antral (A) and antral (B) follicles. (magnification 200x and 100x).

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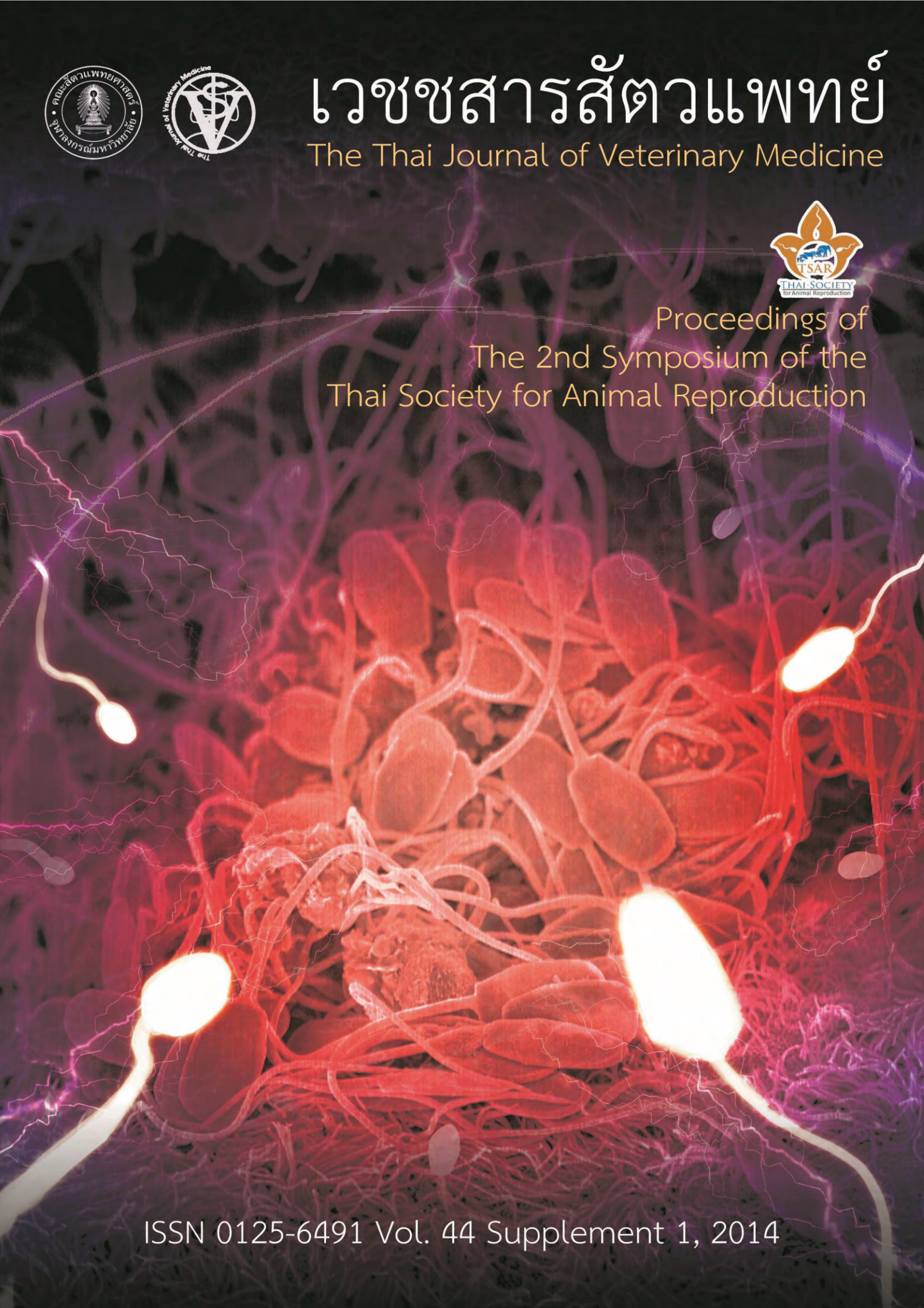

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Expression of Estrogen Receptor Alpha in the Endometrium of Gilts Infected with Porcine Reproductive and Respiratory Syndrome Virus

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Keywords: ERα, immunohistochemistry, PRRS, uterus, gilt

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease in pig industry (1). The target cells of PRRS virus (i.e., macrophage) are presented in the endometrium of pig (2). Karniychuk et al. (3) found that PRRS virus can replicate and induce apoptosis in the fetal implantation sites at the last stage of gestation. These findings indicate that PRRS virus infections may cause pathological changes in the endometrium and lead to infertility. However, to our knowledge, the impact of PRRS virus infection on the endometrial function has not been fully elucidated. Thus, the objective of the present study was to determine the effect of PRRS virus infection on the expression of estrogen receptor α (ERα) in the endometrium of gilts using immunohistochemistry.

Materials and Methods

Uterine tissues were collected from 56 Landrace × Yorkshire crossbred gilts (4). The uterus were fixed in 10% neutral-buffered formalin and processed histologically. The expression of ERα was determined using immunohistochemistry on paraffin sections using polyclonal antibodies against ERα (5). ERα staining was evaluated in epithelium, subepithelium, and glandular layers of the uterus. Five objective fields of each uterine section were randomly selected to evaluate using Image-Pro® Plus software under a light microscope. The percentage of ERα positive cells was calculated as: ERα positive cells (%) = [(number of ERα positive cells/ total number of cells counted) × 100]. The gilts were classified according to PRRS virus detection as positive (n=26) and negative (n=30). The reproductive status of the gilts was defined according to the ovarian appearance into three groups, i.e., prepubertal (n=12), luteal phase (n=26) and follicular phase (n=16) (4). The statistical analyses were carried out using SAS. Multiple ANOVA was used to analyze the data. The statistical model included the effect of reproductive status (prepubertal, luteal and follicular phases), tissue layers (epithelium, subepithelium, and glandular layers), PRRS virus detection (positive and negative), and the interaction between reproductive status and PRRS virus detection. Least-square means were calculated and compared using Tukey-Kramer test. $p<0.05$ was regarded as statistically significant.

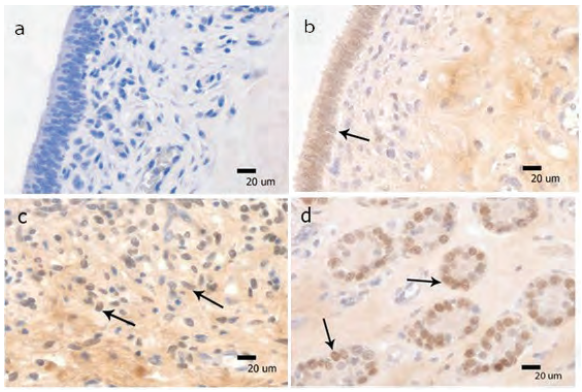

Figure 1 Expression of ERα in endometrium of PRRSV infected gilts: a) negative control; b) positive control; c, d) immunostaining of ERα in subepithelial and glandular layers. Black arrows indicate positive staining cell, 200× magnification.

Results and Discussion

The result showed that the immuno-staining of ERα in the endometrium of gilts are presented in epithelial, sub-epithelial and glandular layers of the uterus (Fig. 1). The percentage of ERα immuno-staining did not differ significantly between the PRRS virus detection and those without PRRS virus detection in the endometrium of gilts (Table 1). The difference of ERα immuno-staining was found between prepubertal and follicular

phase gilts in subepithelial layer (17.9 and 30.4%, respectively, p=0.014) and glandular layer (15.1 and 46.1%, respectively, p<0.001) of the gilt's endometrium. The difference in ERα immuno-staining between prepubertal and follicular phase gilts indicated that the ovarian steroid hormones in gilts influenced the expression of ERα rather than the presence of PRRS virus. In conclusions, PRRS virus did not affect the expression of the ERα in endometrium.

Table 1 The percentage of ERα positive cells in the epithelium, subepithelial and glandular tissues layers of the endometrium in gilts with and without PRRS virus detection in the uterus (LS means±SEM.)

Tissue layer	PRRS virus detection	
	Negative	Positive
Epithelium	22.8±2.6[a]	25.2±2.8[a]
Subepithelium	12.4±1.8[a]	15.3±1.9[a]
Glandular	32.8±2.9[a]	31.2±3.1[a]

[a]Common superscript within row did not differ significantly (p>0.05)

Acknowledgements

Financial support for the present study was provided by Ratchadaphiseksomphot Endowment Fund.

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Impact of Porcine Reproductive and Respiratory Syndrome Virus Infection on the Expression of Estrogen Receptor Beta (ERβ) in the Ovary of Gilts

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Keywords: ERβ, estradiol, ovary, PRRS, gilt

Introduction

Estrogens play the important roles in growth and differentiation of the reproductive system in gilts and sows. The most active estrogen synthesized by antral follicles is estradiol-17β. Theca interna and granulosa cells are major component of the antral follicles that synthesize estrogen. Estrogen regulates reproductive function by binding to specific receptor protein, estrogen receptor (ER). In general, ER is expressed as two isoforms, i.e., ERα and ERβ (1). ERα is the classical ER that was identified since 1986, while ERβ has been identified later (3). Although ERα and ERβ are encoded on different chromosome, they have a considerable sequence homology in their domain (3). Therefore, it can be speculated that the presence of any of the ER subtypes indicate the tissue action of estrogen. The objective of the study was to determine the effect of porcine reproductive and respiratory syndrome (PRRS) virus detection on the expression of ERβ in ovarian follicles of gilt.

Materials and Methods

Ovarian tissues were collected from 62 Landrace x Yorkshire crossbred gilts (4). The ovaries were fixed in 10% neutral-buffered formalin and processed histologically. PRRS virus detection in the gilt ovaries was carried out by immunohistochemistry (2). The ovarian tissues sections were incubated with rabbit polyclonal anti-ERβ as a primary antibody, biotinylated secondary antibody-horse anti-mouse anti-rabbit IgG and avidin-biotin-peroxidase complex. Five follicles per ovarian tissue sections were evaluated using Image-Pro® Plus software. The percentage of ERβ positive cells was calculated. The gilts were classified according to PRRS virus detection as positive (n=38) and negative (n=24).

The statistical analyses were carried out using SAS (SAS Cary, NC, USA). Multiple ANOVA was used to analyse the ERβ positive cells using the general linear model procedure of SAS. The statistical model included the effect of reproductive status (prepubertal and cycling gilts), PRRS virus detection (positive and negative) and the interaction between reproductive status and PRRS virus detection. Least-square means were compared using Tukey-Kramer test. $p<0.05$ was regarded as statistically significant.

Results and Discussion

The result showed that ERβ immunoreaction was found in both pre-antral and antral follicles (Fig. 1).

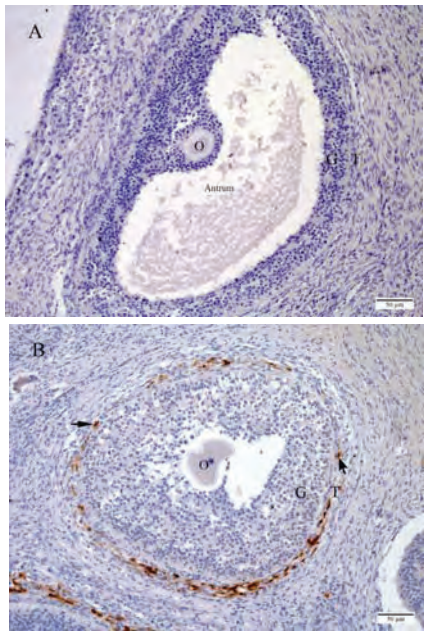

Figure 1 Localization of ERβ protein in the representative paraffin sections of gilt ovarian follicles. Positive immunostaining in the ovarian theca interna cells (B) compared with negative

control (A). Bar = 50 µm. theca interna cells (B) compared with negative control (A). Bar = 50 µm.

On average, ERβ was detected in 23.7% of the theca interna cells surrounding the follicles. The ERβ expression was not detected in the granulosa cells of the porcine ovarian follicles (Fig. 2).

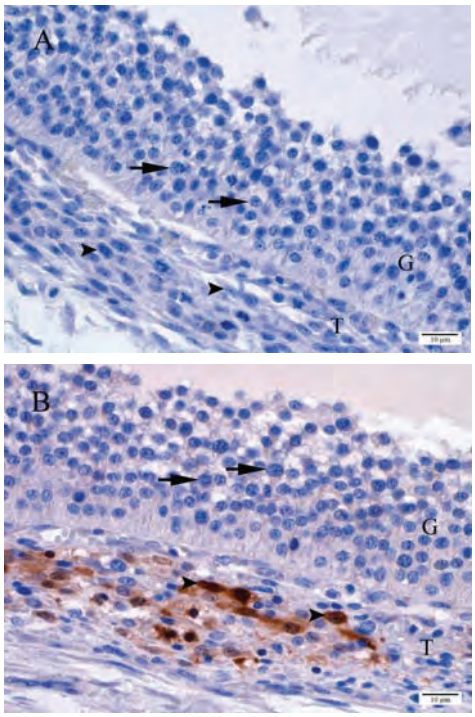

Figure 2 ERβ immunostaining expressed mainly in the nucleus and cytoplasm of theca cells (arrow head, B) compared with negative control (A). No ERβ immunostaing was found in granulosa cells (arrow). Bar = 10 µm.

The percentage of ERβ immuno-staining did not differ significantly between the ovarian tissues detected PRRS virus and those without PRRS virus detection (p>0.05). This indicated that although PRRS virus has an effect on reproductive system, the mechanism may not be mediated through the expression of ERβ in the ovary. In conclusions, PRRS virus detection did not significantly influence the ERβ expression in the ovary of gilt.

Acknowledgements

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Serum Leptin Concentration in Relation to Breed, Body Weight, Backfat Thickness and Age at First Observed Estrus in Gilts

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Keywords: leptin, puberty, reproduction, performance, gilt

Introduction

Leptin is a protein hormone mainly produced from adipose tissue and playing an important role in feed intake regulation and energy balance (1, 2). This hormone has been suggested to control various aspect of reproduction (1, 2, 3). The objective of the present study was to investigate the concentration of serum leptin in Landrace and Yorkshire replacement gilts in relation to their breed, bodyweight, backfat thickness and age at first observed estrus.

Materials and Methods

In total, 80 serum samples from 48 Landrace and 32 Yorkshire gilts were included. The gilts were categorized by bodyweight into 4 groups: <121 kg (n=20), 121-130 kg (n=20), 131-140 kg (n=18), >140 kg (n=9) and grouping by backfat thickness into 4 groups: 10.0-13.0 mm (n=7), 13.5-16.0 mm (n=24), 16.5-19.0 mm (n=14), 19.5-26.0 mm (n=22) and by age of first estrus into 3 groups: ≤180 days of age (n=18), 181-200 days of age (n=17), >200 days of ages (n=32). Serum leptin was investigated by an enzyme immunoassay (Porcine Leptin ELISA kit, MyBioSource Ltd., CA., USA.). The data were analyzed by using Pearson's correlation, multiple ANOVA and least-significant difference test. Values with $p<0.05$ were considered statistically significant.

Results and Discussion

On average, serum leptin concentration of the gilts was 0.9±0.8 ng/ml. The gilts exhibited first standing estrus at 201.5±28.9 days of age and were mated at 253.6±19.2 days of age. Body weight and backfat thickness of the replacement gilts were 136.6±12.3 kg and 16.9±3.5 mm, respectively. The serum leptin concentration did not differ significantly between Landrace and Yorkshire gilts (Table 1). Likewise, the serum leptin concentration did not correlate with the bodyweight, backfat thickness and age at first observed estrus of the gilts (Table 2). The serum leptin concentration did not differ significantly among different body weight classes of the gilts (Table 3). The gilts with backfat thickness (Table 4, 5) of 16.5-19.0 mm had a higher serum leptin concentration than those with backfat thickness of 10.0-13.0 mm (1.31±0.2 vs 0.51±0.3 ng/ml, $p=0.034$) and 13.5-16.0 mm (0.70±0.2 ng/ml, $p=0.028$).

Table 1 Serum leptin concentration in Landrace and Yorkshire gilts

Breed	N	Leptin (ng/ml)
Landrace	41	0.96 ± 0.11[a]
Yorkshire	26	0.60 ± 0.19[a]

Table 2 Pearson's correlation between serum leptin concentration and gilt's reproductive data

Parameters	Correlation
Bodyweight (kg)	r=-0.08, *P*=0.53, n=67
Backfat thickness (mm)	r=0.09, *P*=0.42, n=79
Age at first estrus (d)	r=0.21, *P*=0.07, n=76
Age at first mating (d)	r=-0.08, *P*=0.49, n=79
Non-productive days	r=-0.10, *P*=0.37, n=79

Table 3 Serum leptin concentration by body weight classes

Bodyweight (kg)	N	Leptin (ng/ml)
≤120	20	0.86±0.18[a]
121-130	20	0.87±0.18[a]
131-140	18	0.62±0.20[a]
>140	9	0.76±0.27[a]

Table 4 Serum leptin concentration by backfat thickness classes

Backfat (mm)	N	Leptin (ng/ml)
10.0-13.0	7	0.51±0.3[a]
13.5-16.0	24	0.70±0.2[a]
16.5-19.0	14	1.31±0.2[b]
19.5-26.0	22	0.82±0.2[ab]

Table 5 Serum leptin concentration by age at first observed estrus classes

Age at estrus (d)	N	Leptin (ng/ml)
≤180	18	0.58±0.21[a]
181-200	17	0.85±0.21[a]
>200	32	0.91±0.14[a]

This is the first report on the serum leptin concentration of replacement gilts in Thailand. Furthermore, it was found that the gilts with a backfat thickness of 16.5-19.0 mm had a higher level of serum leptin concentration than those with a lower backfat thickness. This implies that the gilts with high level of backfat thickness might have had a better energy balance and feed intake than those with a lower backfat thickness. This may subsequently contributed to a better reproductive function in the gilts with high backfat and high leptin level.

In conclusion, the serum leptin concentrations did not differ between Landrace and Yorkshire and did not correlate with bodyweight and age at first observed estrus. However, gilts with a backfat thickness of 16.5-19.0 mm had a significantly higher serum leptin concentration than gilts with a backfat thickness of below 16.0 mm.

Acknowledgements

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Localization of Estrogen Receptor β in the Porcine Ovary

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Keywords: estrogen receptor, immunohistochemistry, ovary, gilt

Introduction

Our previous study has demonstrated that a certain number of gilts were culled from the herd due to reproductive failure, especially anestrus and/or abnormal estrus behavior (1). The mechanism associated with anestrus or poor estrus behavior in gilts is needed to be explored. It is well established that estrogen plays an important roles in the growth and differentiation of reproductive system (2). In general, estradiol-17β is the most active form of estrogen. This hormone is secreted by antral follicles of the ovarian tissue (3). Furthermore, corpus luteum (CL) in pig also produces this hormone that performs as a paracrine/autocrine regulator (4). Estrogen receptor beta (ERβ) is the remarkable subtype of ERs in the porcine ovary (5). The ERβ plays a vital role in follicular maturation and ovulation (3). Therefore, the present study aims to explore ERβ expression in different locations of the porcine ovary using immunohistochemical method.

Materials and Methods

Ovarian tissues were obtained from 25 Landrace x Yorkshire pubertal gilts (1). The tissue sections were deparaffinized in xylene, rehydrated through graded ethanol dilutions. Thereafter, the tissue sections were placed in 0.01 M citrate buffer (pH 6.0) in a microwave oven at 750 watts for 15 min to retrieve antigenicity. Endogenous peroxidase was blocked by freshly prepared 3.0% hydrogen peroxide at room temperature (RT) for 10 min. Nonspecific staining was blocked with normal horse serum at RT for 30 min. The slides were incubated with primary antibody, rabbit polyclonal anti-ERβ (Santa Cruz Biotechnology, Inc., Texas, USA) at a dilution 1:100, overnight at 4°C. Thereafter, secondary antibody, biotinylated-horse anti-mouse anti-rabbit IgG (Vector Laboratories, CA, USA) was applied to the samples at RT for 30 min, followed with avidin-biotin-peroxidase complex at RT for 30 min. Staining was developed with DAB substrate. The sections were counterstained with haematoxylin stain and mounted with mounting medium. Negative control section was subjected to the same immuno-histochemical procedure replacing primary antibody by using PBS. Immuno-staining of ERβ in follicles and corpus luteum were determined by Image-Pro® plus software. The percentage of ERβ positive cells was calculated as: ERβ positive cells (%) = [(number of ERβ positive cells/ total number of cells counted) x 100]. For each tissue section, 5 microscopic areas were randomly evaluated. The results were expressed as mean±SD. The percentages of ERβ positive cells were compared among different region of the ovaries including follicles and corpus luteum by using ANOVA. The value of $p<0.05$ was regarded to be statistically significance.

Results and Discussions

The immuno-staining of ERβ in the porcine ovaries are demonstrated in Figure 1 and 2. The immuno-staining of ERβ in the porcine corpus luteum are demonstrated in Figure 3.

The ERβ immuno-expression was found in both follicles and corpus luteum of the porcine ovary. The ERβ immuno-staining was mainly detected in the theca cells of the follicles (Fig. 2) and in the luteal cells in the CL (Fig. 3). In the CL, ERβ immuno-staining located in the cytoplasm of the luteal cells (Fig. 3).

Table 1 ERβ immuno-expression in follicles and corpus luteum (CL) in the porcine ovary (mean±SD)

Part of ovary	Number of observation	ERβ expression (%)
Follicles	25	22.6±19.0[a]
CL	25	87.2±9.0[b]

[a,b]different letters within column differ significantly ($p<0.05$)

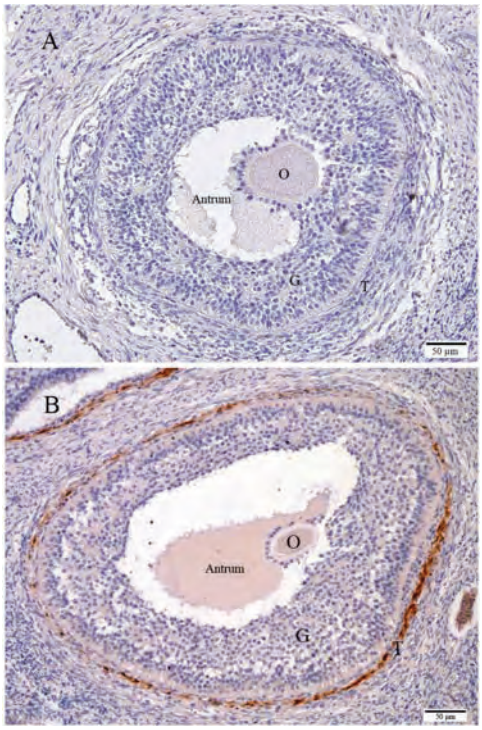

Figure 1 The ERβ immuno-staining in the ovary of pubertal gilts (B) compared with negative control (A). 100× magnification.

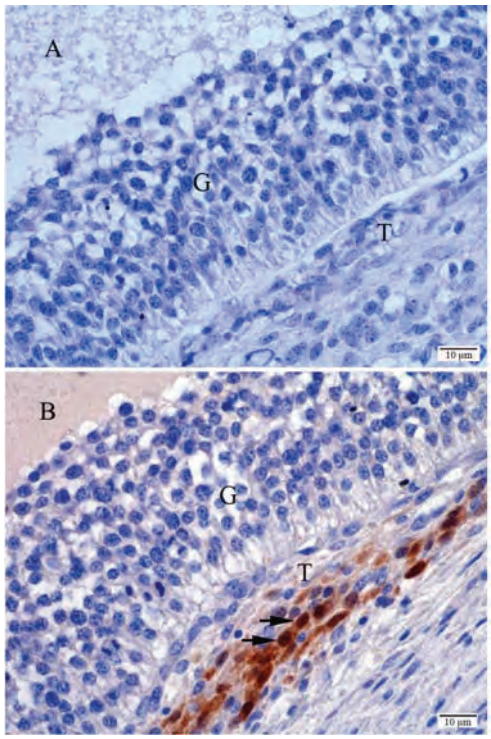

Figure 2 Immuno-staining was found in nuclei and cytoplasm (arrow) of theca cells in follicles (B). Negative control (A). 400× magnification.

In the ovarian follicles, most of the ERβ immunoreaction was found in the cellular nuclei and cytoplasm of the theca cells. This is in accordance with a previous that ERβ protein was exhibited mostly in theca interna cells of the follicle (3). However, the ERβ immunoreaction was rarely expressed in granulosa cells of the follicle (Fig. 1). In the CL, the ERβ immunoreaction was detected in the cytoplasm but not in the nuclei of the luteal cells (Fig. 3). The positive staining in CL indicated that CL could be a source of estradiol production in the porcine species and also in early luteal phase of bovine species (4).

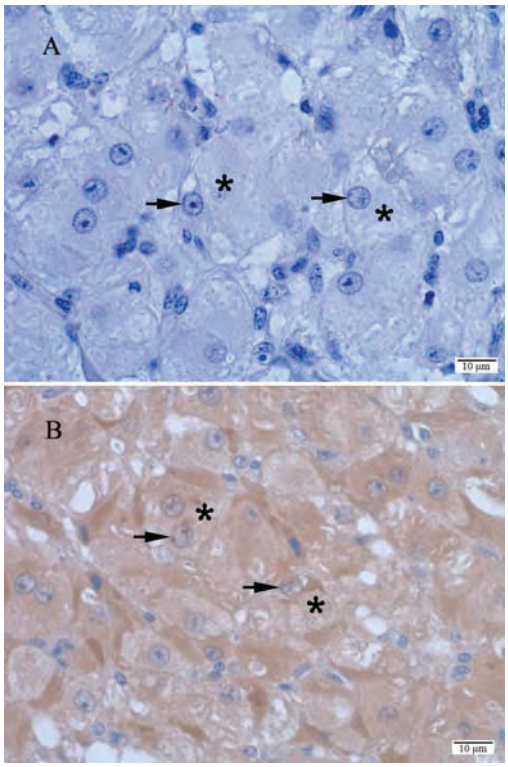

Figure 3 ERβ immunostained was detected in cytoplasm (star) of luteal cells (B) compared with negative control (A) in CL. 400× magnification

The proportion of ERβ expression in the follicles and CL are presented in Table 1. On average, ERβ immunoreaction was detected in 22.6% of the theca cells and in 87.2% of the luteal cells. In conclusion, ERβ immuno-staining was detected in the theca cells and luteal cells in the porcine ovary. This infers that ERβ might play an important role in the porcine ovarian function. Additional study on the variation of ERβ immunoreaction associated with reproductive status will be further investigated.

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