การงอกขยายและการตายของเซลล์กรานูโลซาในฟอลลิเคิลและการแสดงออกของตัวรับฮอร์โมนลูทีไนซิงและฮอร์โมนเอสโตรเจนบนรังไข่ของสุกรสาวสัมพันธ์กับสมรรถภาพทางการสืบพันธุ์

นางสาวดวงกมล ภูพิชญ์พงษ์

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PROLIFERATION AND APOPTOSIS OF GRANULOSA CELLS IN FOLLICLES AND THE EXPRESSION OF LUTEINIZING HORMONE AND OESTROGEN RECEPTORS IN THE OVARIAN TISSUE OF GILT ASSOCIATED WITH REPRODUCTIVE PERFORMANCES

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ดวงกมล ภูพิชญ์พงษ์ : การงอกขยายและการตายของเซลล์กรานูโลซาในฟอลลิเคิลและการแสดงออกของตัวรับฮอร์โมนลูทิไนซิงและฮอร์โมนเอสโตรเจนบนรังไข่ของสุกรสาวสัมพันธ์กับสมรรถภาพทางการสืบพันธุ์ (PROLIFERATION AND APOPTOSIS OF GRANULOSA CELLS IN FOLLICLES AND THE EXPRESSION OF LUTEINIZING HORMONE AND OESTROGEN RECEPTORS IN THE OVARIAN TISSUE OF GILT ASSOCIATED WITH REPRODUCTIVE PERFORMANCES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร. เผด็จ ธรรมรักษ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. สพ.ญ. ดร. ศยามณ ศรีสุวัฒนาสกุล, อ. น.สพ. ดร. เสรี กุญแจนาค, 90 หน้า.

ในภาคสนาม สุกรสาวทดแทนจำนวนมากถูกคัดทิ้งเนื่องจากภาวะไม่เป็นสัด รังไข่ของสุกรสาวกลุ่มนี้ประกอบด้วยฟอลลิเคิลขนาดเล็กและขนาดกลางซึ่งไม่สามารถตกไข่และเข้าสู่วัยเจริญพันธุ์ได้ ความแปรปรวนของการผลิตฮอร์โมนสเตียรอยด์ซึ่งควบคุมการทำงานของระบบสืบพันธ์อาจเป็นสาเหตุของภาวะไม่เป็นสัด เพื่อให้เข้าใจกลไกที่เกี่ยวข้องกับความสมบูรณ์ฟอลลิเคิล ตัวบ่งชี้การงอกขยาย และการตายของเซลล์ การแสดงออกของตัวรับฮอร์โมนเอสโตรเจน และตัวรับฮอร์โมนลูทิไนซิงจึงถูกนำมาศึกษา กระบวนการทางอิมมูโนฮิสโตเคมีต่อแอนติเจนของการงอกขยายของเซลล์ถูกนำมาใช้เพื่อศึกษาจำนวนและชนิดของฟอลลิเคิลในเนื้อเยื่อรังไข่สุกรสาวที่สัมพันธ์กับจำนวนของฟอลลิเคิลและสมรรถภาพทางการสืบพันธุ์ เปอร์เซนต์การตายของเซลล์และการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนในรังไข่ของสุกรสาวถูกศึกษาโดยใช้วิธีเทอร์มินัล ดีออกซีนิวคลิโอทิดิล ทรานเฟอร์เรสมิดีเอต ดียูทีพี นิค เอ็น ลาเบลลิ่ง (ทันแนล) และ วิธีอิมมูโนฮิสโตเคมีต่อตัวรับฮอร์โมนเอสโตรเจนเบต้า ตามลำดับ นอกจากนี้ยังศึกษาการแสดงออกของตัวรับฮอร์โมนลูทิไนซิงในฟอลลิเคิลระยะพรีแอนทรัมและระยะแอนทรัม ผลการทดลองพบว่าสัดส่วนของฟอลลิเคิลระยะไพรมอเดียล ระยะไพรแมรี และระยะที่กำลังเจริญเติบโต คือ 64.2% 32.7% และ 3.1% ตามลำดับ จำนวนของฟอลลิเคิลระยะไพรแมรีมีความสัมพันธ์เชิงบวกกับน้ำหนักตัวแต่สัมพันธ์เชิงลบกับอายุที่สุกรสาวเป็นสัดครั้งแรก ($P < 0.05$) สุกรสาวที่มีน้ำหนักตัวมากและเป็นสัดเร็วจะมีความหนาแน่นของฟอลลิเคิลระยะไพรแมรีมาก สัดส่วนการตายของเซลล์ในฟอลลิเคิลระยะพรีแอนทรัมสูงกว่าฟอลลิเคิลระยะแอนทรัม นอกจากนั้นสัดส่วนการตายของเซลล์ในรังไข่สุกรสาวที่ยังไม่มีวงรอบสูงกว่ารังไข่ที่มีวงรอบปกติทั้งในชั้นกรานูโลซาและทีคา ($P < 0.01$) การตายของเซลล์สัมพันธ์กับภาวะไม่เป็นสัดหรือสุกรสาวที่รังไข่ยังไม่มีวงรอบ การแสดงออกของตัวรับฮอร์โมนเอสโตรเจนเบต้าในชั้นทีคาของฟอลลิเคิลระยะแอนทรัมของรังไข่สุกรสาวที่ยังไม่มีวงรอบต่ำกว่ารังไข่ของสุกรสาวที่มีวงรอบปกติ ($P < 0.01$) การแสดงออกของตัวรับฮอร์โมนลูทิไนซิงในชั้นทีคา อินเทอร์นาของฟอลลิเคิลระยะแอนทรัมสูงกว่าระยะพรีแอนทรัม (65.4% และ 38.3% $P < 0.01$) และในระยะฟอลลิคูลาร์สูงกว่าระยะลูเทียล (58.6% และ 45.2% $P < 0.05$) สรุปว่ารังไข่ที่ไม่มีวงรอบของสุกรสาวคัดทิ้งเนื่องจากการไม่เป็นสัดมีความสัมพันธ์กับความหนาแน่นต่ำของฟอลลิเคิลในระยะไพรแมรี สัดส่วนที่เพิ่มขึ้นของการตายของเซลล์ และการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนเบต้าและตัวรับฮอร์โมนลูทิไนซิงที่น้อยลง

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KEYWORDS: APOPTOSIS / FOLLICLE / GILT / GRANULOSA CELL / LUTEINIZING HORMONE

DUANGKAMOL PHOOPHITPHONG: PROLIFERATION AND APOPTOSIS OF GRANULOSA CELLS IN FOLLICLES AND THE EXPRESSION OF LUTEINIZING HORMONE AND OESTROGEN RECEPTORS IN THE OVARIAN TISSUE OF GILT ASSOCIATED WITH REPRODUCTIVE PERFORMANCES. ADVISOR: ASSOC. PROF. PADET TUMMARUK, D.V.M., M.Sc., Ph.D, CO-ADVISOR: ASST. PROF. SAYAMON SRISUWATANASAGUL, D.V.M., M.Sc., Ph.D, SERI KOONJAENAK, D.V.M., M.Sc., Ph.D, 90 pp.

Under field conditions, majority of the replacement gilts are culled due to anoestrus. The ovaries in most gilt are non-cyclic and contain numerous small and medium size follicles, but fail to ovulate and attain puberty. Alteration of ovarian steroids production which regulates reproductive function may be the cause. To understand the mechanisms underlying follicular maturation, proliferating and apoptotic cell markers, estrogen receptor β (ERβ) and luteinizing hormone receptor (LHR) were investigated. Proliferating cell nuclear antigen (PCNA) was used to determine the number and type of follicles in gilt ovarian tissues associated with the number of follicles and reproductive performances. The percentage of apoptotic cells and the immunoexpression of ERβ were examined using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and anti-ERβ immunohistochemistry, respectively. Additionally, the LHR immuno-staining in preantral and antral follicles was investigated. It was found that the proportions of primordial, primary and growing follicles were 64.2%, 32.7% and 3.1%, respectively. The number of primary follicles was positively correlated with body weight but negatively correlated with age at first observed oestrus ($P < 0.05$). Gilts with a heavy body weight and attain puberty early have a higher density of primary follicles. The proportion of apoptotic cells in preantral follicles was higher than antral follicles. Moreover, the proportion of apoptotic cells in non-cyclic gilts was higher than cyclic gilts in both granulosa and theca cell layers ($P < 0.001$). Apoptosis associated with anoestrus or non-cyclic gilts. In addition, the ERβ immunoexpression in the theca cells of antral follicles in non-cyclic gilts was lower than that in cyclic gilts ($P < 0.001$). The LHR immuno-staining in the theca interna layer of antral follicles was higher than preantral follicles (65.4% vs 38.3%, $P < 0.01$) and it was higher in follicular phase than the luteal phase (58.6% vs 45.2%, $P < 0.05$). In conclusion, inactive ovaries in the anoestrus culled gilts were associated with low density of primary follicles, high proportion of apoptotic cells and poor immmunoexpression of ERβ and LHR.

Department:	Obstetrics Gynaecology and Reproduction	Student's Signature	.....
		Advisor's Signature	.....
Field of Study:	Theriogenology	Co-Advisor's Signature	.....
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LIST OF ABBREVIATIONS

℃	degree Celsius
μm	micrometer
μm^2	micrometer square
ABC	avidin-biotin complex
ADG	average daily gain
ANOVA	analysis of variance
BF	backfat thickness
CA	corpus albican
CL	corpus luteum
cm	centimeter
CORR	correlation analysis
dUTP	deoxyuridine triphosphate
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
ER	estrogen receptor
FREQ	frequency
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
g	gram
g/d	gram/day
GnRH	gonadotropin releasing hormone
h	hour
hCG	human chorionic gonadotropin
H_2O_2	hydrogen peroxide
H&E	hematoxylin and eosin
HIER	heat induced epitope retrieval
Ig	immunoglobulin
IGF-1	insulin-like growth factor 1

kg	kilogram
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LY	landrace and yorkshire
m	meter
min	minute
mm	millimeter
mRNA	messenger ribonucleic acid
M	molar
NPD	non productive day
NPAR1WAY	nonparametric one-way analysis of variance
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
pH	potential of hydrogen ion
PROC	procedure
RT	room temperature
SAS	Statistical Analysis System
SD	standard deviation
sec	second
SEM	standard error of mean
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
TdT	terminal deoxynucleotidyl transferase
TB	total born
UNIVARIARE	univariate analysis

CHAPTER I

Introduction

1.1 Importance and rationale

Swine industry in Thailand is increasing continuously during the last two decades. Crossbred Landrace and Yorkshire (LY) gilt is the main pig breed used most among the swine breeders in Thailand. In general, the gilts productivity account for 15-25% of the whole productivity in the swine herd (Gordon, 1997). Furthermore, 40-60% of the sows on production in the herd are replaced by gilts annually (Lucia et al., 2000; Stalder et al., 2005; Engblom et al., 2007; Tummaruk et al., 2010b). Since the proportion of the gilts in the herd is high, the inferior reproductive performances of gilts affect the whole herd productivity. In practice, the common reproductive problems in gilts consists of anoestrus, repeat breeding, not being pregnant, vaginal discharge, abortion and farrowing problems during the first parity (Heinonen et al., 1998; Tummaruk et al., 2009a). Moreover, up to 65% of the culled gilts had reproductive disturbances (Lucia et al., 2000). In Thailand, it has been reported that 47% of culled gilts had reproductive disturbances, e.g., anoestrus, vaginal discharge, repeat breeding, abortion and not being pregnant (Tummaruk et al., 2006). Moreover, the main reproductive disturbance in replacement gilts in Thailand is anoestrus (Tummaruk et al., 2006; Tummaruk et al., 2007; Roongsitthichai et al., 2013). Of the culled gilts, 52.2% of the anoestrus gilts are identified as prepubertal (i.e., having non-cyclic ovaries, Figure 1) (Tummaruk et al., 2009a). Interestingly, most of the gilts culled due to anoestrus had a numerous of medium size follicles (i.e., diameter > 5 mm) but failure to accomplish ovulation and complete the puberty attainment process (Tummaruk et al., 2009a).

To improve the herd productivity, the desirable replacement gilts are being intensively considered. Earlier studies indicate that the longevity of gilts is related with some of their reproductive traits, e.g, age at first observed oestrus and age at first mating (Koketsu et al., 1999; Tummaruk et al., 2001; Tummaruk et al., 2007;

Roongsitthichai et al., 2013). In Thailand, the LY gilts reach puberty at 195 days of age with an average body weight of 106 kg (Tummaruk et al., 2007). However, the range of age which attained to puberty is highly variable (i.e., from 152 to 224 days) due to various factors. Factors influencing the puberty attainment of gilts and the oestrus behaviour have been comprehensively reviewed (Evans and O'Doherty, 2001). In general, external factors including oestrus detection, season, environment, boar exposure, nutrition and diseases are intensively investigated (Tummaruk et al., 2004; Tummaruk et al., 2007; Tummaruk et al., 2009b). Furthermore, internal factors like breed, body weight and backfat thickness are also significantly influence age at puberty in gilts (Tummaruk et al., 2009b). Both of internal and external factors are closely related with the reproductive hormones and the ovarian function (Evans and O'Doherty, 2001). To our knowledge, the follicle development in gilts culled due to reproductive disturbance, particularly anoestrus, in relation to the expression of luteinizing hormone (LH) receptor as well as oestrogen receptors (ER) has never been comprehensively investigated.

The main function of the porcine ovary is to produce good qualities of ovarian follicles that initiate the female reproductive function. The first ovulation can be observed once the puberty attainment occurs. Oogenesis is closely related with the follicle development as well as the granulosa cell proliferation (Caárdenas and Pope, 2002; Tatone and Amicarelli, 2013). Folliculogenesis is an important process initiating the female reproductive function. In general, the follicles include primordial follicles, primary follicles, secondary (preantral) follicles and growing (antral) follicles (Figure 2). Follicle growth initiates from primary follicles to antral follicles and ends with either ovulation or atresia (Quesnel et al., 1998). These events are being comprehensively investigated by many researchers concerning the development mechanisms.

In gilts, ovarian follicle development initials attentively between 60 - 100 days of age (Schwarz et al., 2008). The pool of primordial follicles are selected and begin to increases in size from small (< 3 mm) to medium (3 - 6.9 mm) follicles (Schwarz et al., 2008). Follicular growth is recognized by an increase in the number of granulosa cells. Both growth factors and steroid hormone regulate ovarian follicular

development (Sirotkin, 2011). Successful follicle development depends on the presence of a number of factors that promote follicular growth and minimize apoptosis (Quirk et al., 2004). However, the percentage of atretic follicles during days 7 - 15 of the oestrous cycle varies from 12% to 73% (Guthrie et al., 1995b). The ovulation rate and the quality of the ovulated oocyte are closely related with the follicle development especially during the last period of the oestrous cycle (Soede et al., 2011). The number of large follicles and the ovulation rate is the primary limiting factor for litter size at birth in pig (Knox et al., 2003). Moreover, the number of ovulated follicles is limited by the capability of recruited follicles to proceed until ovulation and reduce follicular atresia (Caárdenas and Pope, 2002).

During follicular maturation, granulosa cell of antral follicle become gradually more responsive to LH by increasing LH receptor (LHR) in response to both follicle-stimulating hormone (FSH) and oestradiol (Bukovsky et al., 1993). In the final stage of the follicular maturation, both theca and granulosa cells synthesize and secrete several hormones that take part and coordinate in ovarian function (Caárdenas and Pope, 2002). The absence of survival factors, such as, LH, FSH and factors within the ovary, induce apoptotic pathway in ovarian follicle and cause follicular atresia.

Oestrogen promotes the proliferation of granulosa cell and decrease apoptosis (Quirk et al., 2004). The most active form of oestrogen, i.e., oestradiol-17β, is synthesized and excreted from antral follicles of the ovary in mammal species (Pineda, 2003; Knapczyk et al., 2008). Theca cell in the porcine ovary can synthesize oestrogen itself without granulosa cells (Shores et al., 2000; Knapczyk et al., 2008). Oestrogen is activated by binding to the steroid hormone receptor called oestrogen receptors (ERs). Slomczynska et al. (2001) found that ERβ expressed in both granulosa and theca cells of antral follicles by using in situ hybridization and found the highest level of ERβ in granulosa cells of preovulatory follicles. Moreover, in pregnant swine, ERβ is found mostly in theca interna, while ERβ is expressed in some of granulosa cells (Knapczyk et al., 2008). Immmunohistochemical localisation of ERβ in non-cyclic ovaries gives a closer insight into the follicular growth in anoestrus gilts.

Anoestrus is the most important reproductive disorder leads to the main reason for culling of replacement gilts under tropical climates (Tummaruk et al.,

2009a). Therefore, the mechanism of follicular growth and atresia in anoestrus gilts should be emphasized and studied. Nevertheless, many aspects about the premature follicles are still unclear. The use of immunohistochemical staining with some specific growth factors associated with the follicles growth and ovarian functions, may give a better understanding on the developmental process of the follicular growth in relation with the expression of hormone receptors in the ovarian tissues.

1.2 Keywords

Apoptosis, Gilt, Granulosa cell, Luteinizing hormone receptor (LHR), Oestrogen receptor β (ERβ), Proliferation

1.3 Research coherence

Replacement gilts in Thailand, raised under hot and humid climate are removed from the gilt pool due to the reproductive disorders, especially anoestrus and delayed puberty. However, the associated factors lead to these disturbances in gilts are still ambiguous. Moreover, research studies on anoestrus and delayed puberty related with follicle development and hormone receptors is limited.

The present research included 2 parts; the first part (Chapter 2 and 3) examine the proliferation of each follicle type associated with reproductive performances and apoptosis in relation to the reproductive status of gilts. The other part (Chapter 4 and 5) evaluate the reproductive hormone receptors (i.e., LHR and ERβ) involved in the follicle maturation and preovulatory development associated with ovarian status of the gilts.

The quantity and type of ovarian follicles are used as a folliculogenesis predictor, which is related to various hormones and growth factors (Richards, 2001; Fortune, 2003). The proliferating cell nuclear antigen (PCNA) has been used and reported as a method enhancing the visibility of early stage of follicle development in rats (Muskhelishvili et al., 2005; Picut et al., 2008) and pigs ovaries (Tomanek and Chronowska, 2006). However, the association of growth performances and density of

ovarian follicles has not been reported. For that reason, the first study is determine the number and type of follicles and investigated the association between the amount of ovarian follicles and growth performance of gilts (Chapter 2).

Ovarian follicular growth is activated from FSH and LH. While the receptor of FSH and LH on the follicle are produced and function to avoid follicular atresia but growing further until become a preovulatory follicle. The transitional period from preantral to early antral follicle is the sensitive stage of follicle development as it depends on gonadotropin and intraovarian regulators to growth or atresia. At this stage, follicle is strongly coordinated by interactions of intra-ovarian oocyte-granulosa-theca cell (Orisaka et al., 2009). Granulosa and theca cells are the follicular cells associated in folliculogenesis through the cell proliferation and differentiation to enhance the responsiveness to FSH and LH. Thus, apoptosis cell localisation in preantral and antral follicles of non-cyclic gilts are examined (Chapter 3).

During the folliculogenesis, granulosa and theca cells are susceptible to FSH and LH, consequently, FSH and LH receptors are found and interacted during follicle maturation. Moreover, the shift from FSH to LH dependence in follicle is the main action related to the ovulatory follicle selection. LHR are detected on theca cells, which induced androgen and growth factors productions. LHR mRNA expression is increased during day 1 and 3, and was highest on day 5 of follicular phase of oestrous cycle (Guthrie, 2005). Inadequate of LHR in follicle may consequence to follicular atresia and ovulation failure.

Oestrogen, the main female steroid hormone, involves in reproductive function and folliculogenesis by binding to the oestrogen receptor (ER). ERβ, the main receptor expressed on the fully grown follicles, is necessary for granulosa cell differentiation and reaction to gonadotropins. However, the alteration of ERβ expression might influences the reproductive behaviour and ovulation (Drummond et al., 2002). Therefore, the ERβ and LHR immuno-staining in ovarian follicles during reproductive phase and ovarian appearance are investigated (Chapter 4 and 5).

According to the reasons above, the studies of immunohistochemical expression of ERβ and LH in ovarian follicles are evaluated (Chapter 4 and 5). Considering the cellular mechanism and hormone receptors over preantral and

antral follicles and also reproductive stage may contribute to the reasons of anoestrus and delay puberty in gilts.

1.4 Literature review

1.4.1 Gilt reproduction

The selection of high quality replacement gilts is an important key to improve the pig production, since the production from individual female contributes to an overall productivity of the swine herd where also in tropical country as Thailand (Tummaruk, 2012; Roongsitthichai et al., 2013). Gilts and sows with history of poor reproductive performances (e.g., prolonged weaning-to-service interval, repeated service and abortion) had suboptimal productivity and short longevity (Takai and Koketsu, 2007; Tummaruk et al., 2010b). In the latest decade, the replacement rate of gilts to substitute the culled sows from swine herd account for 40–60% per year (Engblom et al., 2007; Tummaruk et al., 2010b). The high replacement rate is because the high unplanned removal and the culling of sows at a younger age than the past (i.e., parities above 6) (Engblom et al., 2007). In general, the proportion of the gilts in commercial swine herd account for 20–25% of the sows on production. This reflects the importance of selecting quality gilts to be the production unit in the herd. Moreover, age, body weight, and oestrus behaviour of the gilts are the major criteria to be considered before first mating. However, based on economic analysis, the gilts should be conceived before 220–230 days of age (Schukken et al., 1994; Koketsu et al., 1999). Koketsu et al. (1999) revealed that if age at first insemination of gilts increased, their longevity would decrease. The gilts that have low average daily gain (ADG) tend to have low conception rate and to be removed from herd due to reproductive disturbance (Tarrés et al., 2006; Tummaruk et al., 2009a; Roongsitthichai et al., 2013).

1.4.2 Puberty attainment

Puberty in gilts is generally defined as the first time that the gilts shows standing oestrus and ovulation. Under field conditions, ovulation is difficult to

determine and, in most case, the ovulation take place in nearly all of the gilts that present standing oestrus. Thus the first observed oestrus is usually defined as puberty attainment in gilts (Tummaruk et al., 2009b). Nevertheless, the age at puberty in gilts varied considerably ranging from 152 to 224 days of ages with average body weight of 106 kg (Tummaruk et al., 2007). Moreover, growth rate was also influences the puberty attainment in gilts (Beltranena et al., 1991; Tummaruk et al., 2009b). Kummer et al. (2006) reported that gilts with high growth rate (> 700 g/day) had a greater total number of piglets born per litter (TB) in the first parity than those with low growth rate (< 700 g/day). A previous report of Young (2004) also support that gilts with superior growth rate (> 860 g/day) produced a larger TB than those with inferior growth rate (460 - 860 g/day). In addition, backfat thickness (BF) is one of the parameter used to determine puberty attainment in gilts. In Thailand, Tummaruk et al. (2007) reported that gilts with higher BF were younger at first insemination and had a higher farrowing rate, larger litter size and shorter wean-to-service interval than those with a lower BF.

1.4.3 Reproductive hormones and oestrous cycle in gilt

After puberty, the gilt normally shows standing oestrus behaviour every 18 to 24 days. The ovarian activities can be divided into 2 phases, i.e., a follicular phase (5 - 7 days) and a luteal phase (13 - 15 days) (Soede et al., 2011). Follicular phase is the period of follicle recruitment and development, which is characterized by a high level of oestrogen (Evans, 2003; Sukjumlong et al., 2003). During this period, most of the small and medium size follicles are restored, which nonatretic and steroidogenesis progress. However, the number of atretic follicles is raised to 50% and granulosa cell proliferation and steroidogenesis are decreased (Knox, 2005). Throughout this phase, numerous antral follicles are subjected to atresia and some antral follicles grow continuously until ovulation (Guthrie, 2005).

Luteal phase begins after ovulation and is characterized by the presence of dominant corpora lutea (CL) and small follicles (Tummaruk et al., 2009a). The ovaries in this phase are lacking of large antral follicles due to high concentration of oestrogen and inhibin levels (Soede et al., 2011). In addition, there is very low

number of large follicles probably capable to ovulate (Schwarz et al., 2008). The ruptured follicles and the corpus luteum produce progesterone for 14 - 16 days (Guthrie, 2005). Therefore, this period is under the influence of progesterone. When ovulation occurs, oestrogen and inhibin decrease, leading to the removal of FSH suppression. Thereafter, the numbers of small and medium sized follicles increase during this phase (Soede et al., 2011).

The follicles competency is associated with oestrogen during follicular phase (Guthrie et al., 1995a; Knox, 2005). The development of large follicles and minimized numbers of small and medium size follicles is coincided with a decrease of circulating FSH level (Knox et al., 2003; Guthrie, 2005). Moreover, the selection of ovulatory follicle is correlated with the change of follicle dependence from FSH to LH (Knox, 2005).

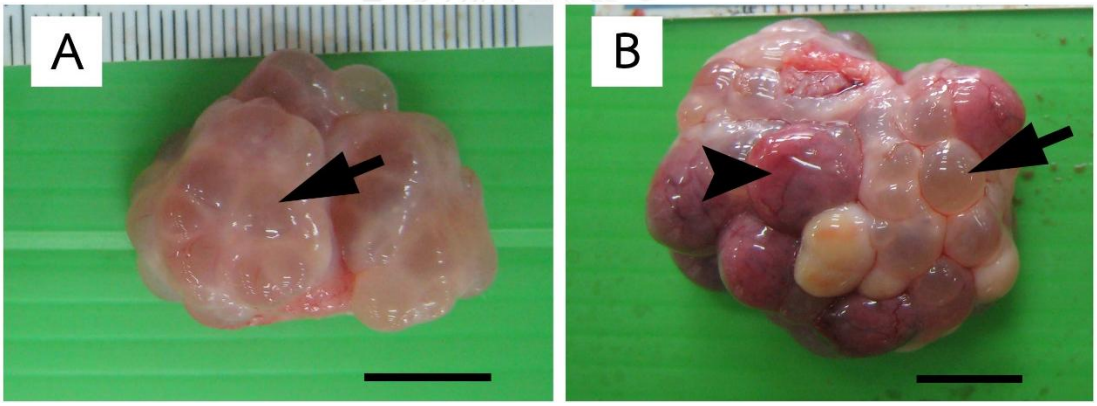

Figure 1. Macroscopic of the pig ovary (A) non-cyclic ovary (B) cyclic ovary (bar = 1 cm). Follicle characterized by clear fluid-filled structure (arrow). Corpora lutea (CL) are structures on the ovaries with pink or tan or yellow color (arrowhead).

1.4.4 Follicle development

The folliculogenesis can be determined by both follicle proliferation and apoptosis (Chun and Hsueh, 1998; Robker and Richards, 1998). The follicular atresia is rising during folliculogenesis and progress slowly during the beginning of the follicular phase (Young and McNeilly, 2010). However, ovarian follicle development includes proliferation and differentiation of the somatic cells lead to antral formation. A cavity of antral follicle contains fluid, which is produced from granulosa cells (Pineda, 2003). The maturation of follicles is initiated by an increase of granulosa cells

proliferation. In general, the ovarian follicles are categorized into four types (Figure 2), i.e., primordial follicles, primary follicles, secondary (preantral) follicles and tertiary (antral) follicles (Moniruzzaman and Miyano, 2010).

The transformation of preantral to antral follicles is controlled by intraovarian signals including gonadal steroids, growth factors and cytokines (Sirotkin, 2011). At this stage, follicles are most sensitive to the process of follicular atresia (Fortune, 2003; Orisaka et al., 2009). The large antral follicles become gonadotropin dependent and most of them become atresia, while some are developed to be pre-ovulatory follicle and subsequently ovulated (Edson et al., 2009). At the end of the maturation process, granulosa cells and theca cells synthesize and secrete steroid hormones (i.e., androgen and oestrogen), peptide hormones, prostaglandins and other substances in adequate amounts to cooperate in follicular maturation and transmit signals to coordinate hypothalamic-pituitary-ovarian axis functions (Caárdenas and Pope, 2002).

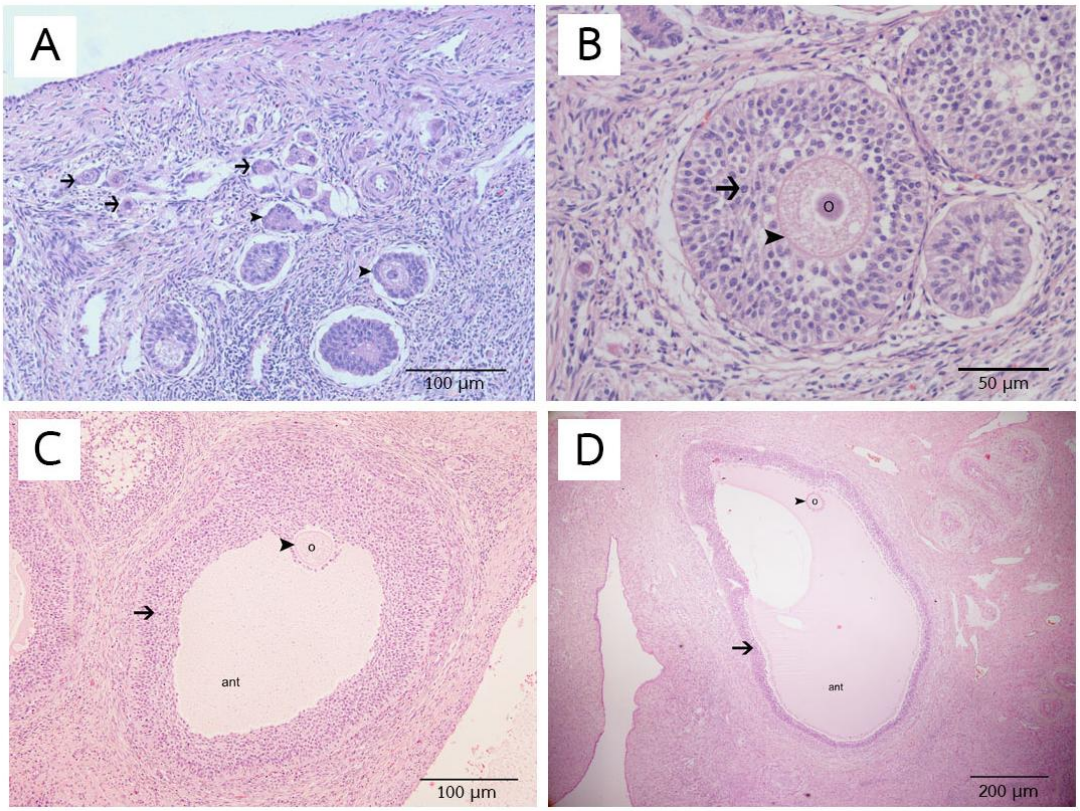

Figure 2. Follicle development initiates from primordial follicle to preovulatory follicle. (A) Primordial follicles (arrow) and primary follicles (arrowhead). (B) Preantral follicle

surrounded by multiple granulosa cells layers (arrow), zona pellucida (arrowhead) encircles an oocyte (O). (C) Antral follicle, antrum is formed and (D) preovulatory follicle.

1.4.5 Quantifying number of ovarian follicles and determination of proliferation score

The number and type of follicles in the ovary are indicators of folliculogenesis. This is associated with a number of hormones and growth factors (Myers et al., 2004). To quantify the number of ovarian follicles, ovarian tissue sections are conventionally performed on hematoxylin and eosin (H&E) tissue section. However, an immunohistochemical labelling of oocytes within the ovaries using an antibody against PCNA has been reported in rats (Muskhelishvili et al., 2005; Picut et al., 2008). Compared with H&E stained, the counting of ovarian follicles in PCNA-stained follicles have dramatically reduced variability from 11% to 0.2% and reduced the counting time by 46% (Muskhelishvili et al., 2005). An earlier study of the PCNA method has also been done in pig ovaries (Tomanek and Chronowska, 2006). However, the appearance of small follicles has not yet been clearly identified. The immuno-staining of PCNA is depending on several factors, e.g., the number of proliferating cells in the tissue sample, the concentration of primary antibodies, the pretreatment of tissue with heat-induced epitope retrieval (HIER) technique and the type of fixative (Muskhelishvili et al., 2005). It has been demonstrated that the primordial follicle of bovine pre-culture ovarian tissue is not stained by PCNA if the tissue sample is fixed in Bouin's solution (Wandji et al., 1996). In early report in rat formalin-fixed, paraffin-embedded ovaries, PCNA is strongly visualized when combination of HIER technique and a high concentration of primary antibody are used (Muskhelishvili et al., 2005). However, to our knowledge, HIER technique for PCNA has not been done in pig ovarian tissue.

1.4.6 Follicular atresia

In the ovaries of mammal, more than 99% of the ovarian follicles undergo atresia or the degenerative change during the different stage of follicle development (Hirshfield, 1991). The degeneration of atretic follicles in the mammalian ovaries can

be described by apoptotic cell death of granulosa and theca interna cells (Nakayama et al., 2000). Fortune (2003) found that most of the follicles become atresia during the transitional process from small to large size follicles. Nowadays, the mechanisms and the organization in granulosa cell apoptosis of porcine ovaries are still unclear. Apoptosis is associated with tissue regression, tissue remodeling and also participates in ovarian physiology in the process of follicular atresia (Berardinelli et al., 2004; Janowski et al., 2012; Yang et al., 2013). The detection of apoptotic cells based on morphology that described as pyknotic and chromatolytic properties (Majno and Joris, 1995; Clarke and Clarke, 1996). The identification of nuclear fragmentation using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is one of the most frequently methods accepted for apoptotic cell marker in histological sections and has been established since 1992 (Gavrieli et al., 1992; Negoescu et al., 1996; Sanders, 1997). To identify follicular atresia by TUNEL method in porcine follicles, the presence of apoptotic granulosa cells in the different areas within a follicle was used to categorize the stage of atretic follicle (Manabe et al., 2002). Nakayama et al. (2000) found that apoptotic cells of porcine granulosa cells were outstanding in inner surface. They found abundance of apoptotic cells in both granulosa cells and theca interna.

1.4.7 Luteinizing hormone receptor

The reproductive function is dependent on the pulsatile release of gonadotropin hormones. LH is known to regulate ovarian development and therefore, allow gilts attained puberty (Evans and O'Doherty, 2001). LH stimulates the biosynthesis of androgens from cholesterol by the theca cells. The granulosa cells uptake and aromatize androgen to oestrogen beneath the impact of FSH (Orisaka et al., 2009). LH promotes the maturation of granulosa and theca cells in the ovary and generate follicular development as well as steroidogenesis (Burns and Matzuk, 2002). During prepubertal period, the decline of corticosteroid and increasing LH secretion are possibly affected in stimulation of follicle maturation, which induce the first ovulation (Prunier et al., 1993). Many studies have concentrated the function of LH during preovulatory stage as LH takes a several roles throughout follicle

maturation (Meduri et al., 1996; Gebarowska et al., 1997; Bukovsky et al., 2003). However, LH action during preantral follicle development has less concern (Wu et al., 2007).

The same as other hormones, LH elicits its function through LHR, the G protein-coupled receptor superfamily (Wu et al., 2007). LHR is developed in the granulosa cell and reached its highest concentration in the mature follicle before ovulation in domestic animals (Pineda, 2003). In human, the localisation of LHR revealed that LHR is expressed in the theca interna of antral follicles and granulosa cells of preovulatory follicles and also observed in granulosa cells during the CL formation but not during CL regression (Takao et al., 1997). In rats, LHR is located in granulosa cells of preovulatory follicles, ovarian theca interna and luteal cells (Bukovsky et al., 1993). However, the expression of LHR in the porcine ovaries was observed in theca cells of preantral and antral follicles, granulosa cells of large antral follicles and also in external luteal cells (Meduri et al., 1992; Wu et al., 2007).

1.4.8 Oestrogen receptors

In ovarian follicles, oestrogen activates proliferation and inhibits apoptosis of the granulosa cells (Drummond and Fuller, 2010a). Moreover, oestrogen regulates the differentiation of granulosa cells by the way of inducing FSH capability to improve LHR expression (Quirk et al., 2004). The theca interna and the granulosa cells, cooperate in the synthesis of oestrogen under positive control of FSH and LH but under negative influence of inhibin (Pineda, 2003). In addition, corpus luteum can also originate oestradiol-17β in many species such as human (Saunders et al., 2000), rat (Sar and Welsch, 1999) and pig (Schams and Berisha, 2002) that perform as a paracrine/autocrine regulator.

Oestrogen regulates their functions through interaction with specific protein receptors, the oestrogen receptors (ER) (Pavao and Traish, 2001), which have been identified into two subtypes ERα and ERβ. These two receptors perform the individual physiological roles in female reproduction (Pelletier and El-Alfy, 2000). In ERβ knockout mice, the incidence of ovulation failure and decreased total number of

offspring was observed (Krege et al., 1998) while the folliculogenesis in ERα knockout mice is arrested at the stage of antrum together with large follicles developing into haemorrhagic and cystic follicles (Drummond et al., 2002). It was found that ERβ is more abundant compared to ERα therefore ERβ plays an essential role in follicular development (Rosenfeld et al., 2001; Emmen et al., 2005; Knapczyk et al., 2008). Sar and Welsch (1999) indicated that the expression of ERβ in rat ovary was particularly appeared in granulosa cells of both primary and growing follicles. In the porcine ovary, the immunohistochemical expression of ERβ was found in all types of the follicles with a higher amount than ERα (Slomczynska and Wozniak, 2001). The ERα positive staining was observed in granulosa cells of antral follicles but not in theca interna and luteal cells, whereas ERβ positive cell was demonstrated mostly in theca interna as well as luteal cells (Knapczyk et al., 2008).

1.5 Research Objectives

1) To investigate the association between the number of follicles, gilt age, body weight and average daily gain using the PCNA immuno-histochemical labelling technique

2) To investigate the localisation and distribution of apoptosis in the ovaries of gilts associated with the type of follicles, granulosa cells apoptosis and the ovarian appearance

3) To examine the immuno-staining of ERβ in the gilt ovarian tissue associated with antral follicles and ovarian appearance

4) To evaluate the immuno-staining of LHR in the cyclic gilt ovarian tissue associated with the type of follicles

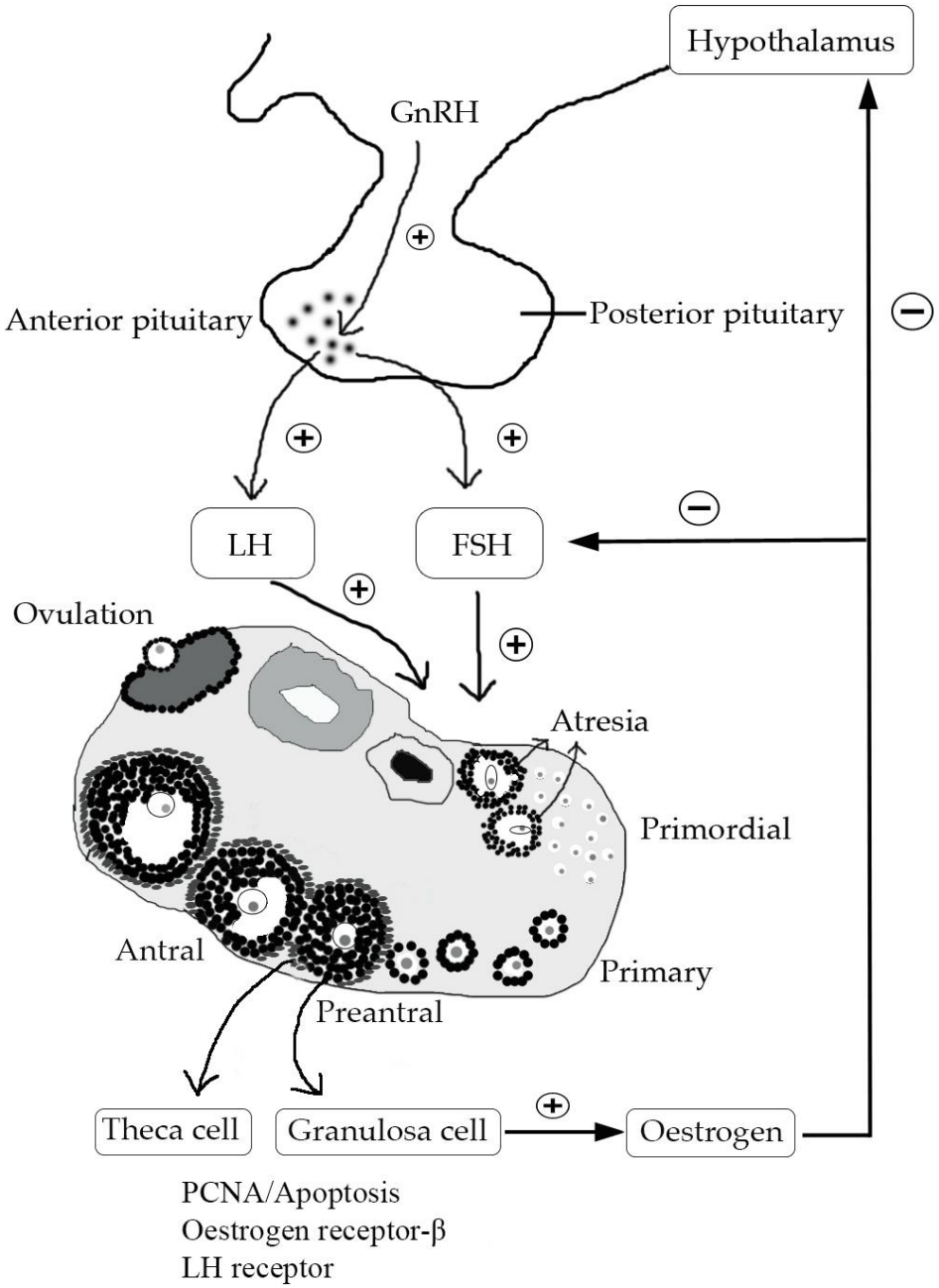

Figure 3. Diagram of this study including; PCNA, apoptosis, ERβ and LHR; Gonadotropin releasing hormone (GnRH) stimulates FSH and LH production from anterior pituitary. These two gonadotropins stimulate the folliculogenesis from primordial, primary, preantral and antral follicles. Granulosa and theca cells of follicles response by proliferate and differentiate, steroid hormone (i.e. oestrogen and progesterone) and growth factors production as well as presenting LH and oestrogen receptors to growing further until ovulation.

1.6 Research Outline

This recent thesis was operated in the Department of Obstetrics, Gynaecology and Reproduction, the Department of Anatomy, Faculty of Veterinary Science, Chulalongkorn University from June 2010 to 2014. The study was concentrated in the follicle development and hormone receptors, especially ERβ and LHR in gilt follicles associated with reproductive performances. This dissertation contains 6 chapters (Chapter 1 - 6).

Chapter 1 consisted of the introduction of this thesis, importance and rational, keywords, research coherence, literature review, research objectives, research outline and research benefits.

Chapter 2 was the application of PCNA immuno-staining technique in the ovaries of 19 Crossbred LY gilts to determine the number and type of follicles. Ovarian tissue sections were incubated with monoclonal anti-PCNA. The number of each follicle categorization was counted and calculated as follicles/100 μm^2. The association between the number of follicles, gilt age, body weight, average daily gain, age at first oestrus, ovulation rate and weight of the ovaries were investigated in this chapter.

Chapter 3 was the study of apoptotic cell localisation between 20 of non-cyclic and 27 of cyclic crossbred LY gilts. The TUNEL assay was used in this study to locate apoptotic cells in gilt's follicles. The evaluation and comparison were perform between preantral and antral follicles, granulosa and theca cells layers. The percentage of apoptotic cells in each follicular type between non-cyclic and cyclic gilts was evaluated and compared.

Chapter 4 was the study of the ERβ immunohistochemical distribution in the follicles from 25 non-cyclic and 30 cyclic crossbred LY gilts using anti-ERβ immunohistochemical procedure. The immunolocalisation of ERβ in the ovarian follicles and corpus luteum was demonstrated. Moreover, the ERβ immuno-staining in the follicular layers between non-cyclic and cyclic gilts was evaluated and compared in this chapter.

Chapter 5 was the study of the LHR immuno-staining in the follicles from 5 follicular phase and 16 luteal phase of crossbred LY gilts using the anti-LHR immunohistochemical procedure. The immunolocalisation of LHR in the ovarian follicles and corpus luteum was presented in this chapter. In addition, the immuno-staining of LHR in the follicular layers in relation to the follicle stage and cyclic gilt ovaries were also examined.

Chapter 6 was the general discussion and conclusions of this thesis. In addition, research limitations and suggestion for further investigations were included in this chapter.

1.7 Research Benefits

Anoestrus is the majority cause leading to exclude gilts out of the pool in the swine herd of Thailand. This problem influences the economics losses due to unexpected and extensive culled gilts. The follicle development and hormone receptors involved in follicular growth and ovulation is important mechanism related the folliculogenesis and oestrus cycle. Therefore, the use of immuno-staining may give a better understanding on the developmental process of follicle maturation, follicular atresia in relation with the hormonal receptors in the replacement gilts culled due to reproductive failure, especially anoestrus associated with several reproductive performances. These challenge to find the reasons why gilts do not attain puberty even their performances (i.e. age, body weight and ADG) are accessible. This dissertation investigated the number of proliferation and apoptosis of ovarian follicles as well as oestrogen β and LH receptors. The report of this dissertation i.e., follicle proliferation and apoptosis, ERβ and LHR immunoexpressions in gilt ovarian follicles might explain factors effecting anoestrus and delayed puberty.

CHAPTER II

The use of proliferating cell nuclear antigen (PCNA) immuno-staining technique to determine number and type of follicles in gilt ovary

(Published in Livestock Science (2012) 150: 425-431)

2.1 Abstract

The present study determined the number and type of follicles in gilts ovarian tissue using the proliferating cell nuclear antigen (PCNA) immuno-histochemical labelling technique and investigated the association between the number of follicles, gilt age, body weight, average daily gain, age at first oestrus, ovulation rate and weight of the ovaries. Ovarian tissues were obtained from 19 gilts aged 267.8 ± 19.2 days, weighting 145.7 ± 11.8 kg. The tissues were incubated with mouse monoclonal anti-PCNA. The follicles were categorized as primordial, primary and growing follicles. PCNA immuno-staining enhanced the visualization of small follicles and the efficacy to distinguish primordial and primary follicles. The gilt ovarian tissue contained 19.8 ± 8.5 follicles per 100 μm^2 (range 6.0 to 42.0). The number (and proportion) of primordial, primary and growing follicles were 13.1 ± 6.9 (64.2%), 6.2 ± 3.3 (32.7%) and 0.5 ± 0.2 (3.1%) follicles/100 μm^2, respectively. The number of primary follicles in the gilt ovary positively correlated to body weight (r = 0.50, *P* = 0.032) but negatively correlated to age at first oestrus (r = -0.54, *P* = 0.015). In conclusion, PCNA technique can be applied to quantify the precise number and distinguish the type of follicles in the ovarian tissue of porcine species. Gilts with a higher body weight and earlier age at first observed oestrus have a higher density of primary follicles in the ovarian tissue than those with lower body weight and later age at first observed oestrus.

2.2 Introduction

The number and type of follicles in the ovary are indicators of folliculogenesis, which is associated with several hormones and growth factors (Myers et al., 2004). To quantify the number of ovarian follicles, ovarian tissue sections are conventionally performed on hematoxylin and eosin (H&E) tissue section. This method is time consuming and it is difficult to differentiate the type of small follicles (Bolon et al., 1997; Bucci et al., 1997). However, an immunohistochemical labelling of oocytes within the ovaries using an antibody against proliferating cell nuclear antigen (PCNA) has been reported in rats (Oktay et al., 1995; Muskhelishvili et al., 2002; Picut et al., 2008). The PCNA method enhances the visibility of primordial and primary follicles of the rat's ovary. Compared with H&E stained, the counting of ovarian follicles in PCNA-stained follicles had dramatically reduced variability from 11% to 0.2% and reduced the counting time by 46% (Mushkelishvili et al., 2005). An earlier study of the PCNA method has also been done in pig ovaries (Tomanek and Chronowska, 2006). However, the appearance of small follicles has not yet been clearly identified.

The immuno-staining of PCNA is depending on several factors, e.g., the number of proliferating cells in the tissue sample, the concentration of primary antibodies, the pretreatment of tissue with heat-induced epitope retrieval (HIER) technique and type of fixative (Muskhelishvili et al., 2005). It has been demonstrated that the primordial follicle of bovine pre-culture ovarian tissue is not stained by PCNA if the tissue sample is fixed in Bouin's solution (Wandji et al., 1996). In rat formalin-fixed, paraffin-embedded ovaries, PCNA is strongly visualized when combination of HIER technique and a high concentration of primary antibody are used (Muskhelishvili et al., 2005). However, to our knowledge, the HIER technique for PCNA has not been done in pig ovarian tissue. The aims of the present study were to develop the optimal protocol for PCNA immunohistochemical labelling in pig ovarian tissue and to determine the number and type of the follicles in the gilt ovaries. In addition, the association between the number of follicles and some reproductive

data (i.e., age and body weight of the gilt, average daily gain (ADG), age at first observed oestrus, ovulation rate and weight of the ovary) was investigated.

2.3 Materials and methods

2.3.1 Data, sample collection and tissue processing

The ovaries of 19 Landrace x Yorkshire crossbred gilts were selected from our previous study (Tummaruk et al., 2009a). The genital organs collected from slaughterhouses were placed on ice and transported to the laboratory within 24 h of culling. Historical data for all gilts was collected, including the gilt identity, date of birth, date of first oestrus, date of culling and body weight at culling. Age at culling, age at first oestrus and ADG from birth to culling ADG (g/d) = (BW at culling-1.5/age at culling) x 1000 was calculated. The ovaries were weighed using an electronic balance (BJ 210C, Precisa, Instruments Ltd., Switzerland). The appearance of the ovaries was used to assess the stage of the oestrous cycle. Corpora lutea (CL) were defined as colored structures appearing on the ovaries which showed pink, tan or yellow, at a size of 7 to 12 mm in diameter. Corpora albicantia (CA) were defined as regressed and shrunken CL. Follicles were defined as transparent fluid-contained structures in the ovaries (Tummaruk et al., 2009a). Only the ovaries that were classified as luteal phases were included in the present study (19 gilts). The luteal phase was characterized by ovaries containing CL with or without small follicles and/or CA. The ovulation rate was defined as the total number of CL from both ovaries. 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 paraffin block (Tissue-Tek TEC, Sakura, Tokyo, Japan). The paraffin embeddings were cut into 5 µm thickness using microtome (Shandon, Anglia scientific instrument ltd., Cambridge, UK). For each ovarian tissue, two sections were cut serially and each section was placed on a separate slide, resulting in two sets with nearly identical ovarian sections. One set of the section was stained with H&E, while the other was stained by PCNA immuno-histochemistry.

2.3.2 Immunohistochemistry

PCNA immuno-staining technique has been modified after previous studies in the rat's ovarian tissue (Muskhelishvili et al., 2005; Picut et al., 2008) and in mammary tumors of cats (Taweechart et al., 2004). Briefly, the pig ovarian tissues were deparaffinized and placed in an antigen retrieval solution (0.01 M citrate buffer, pH 6.0) for 10 min in a microwave oven at 600 watt. Endogenous peroxidase was blocked using freshly prepared 3% hydrogen peroxide for 10 min at room temperature (RT). Nonspecific staining was blocked with 1% bovine serum albumin for 30 min at 37°C. 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. Staining was developed with 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma, Germany) for 3 min at RT. The sections were counterstained with Mayer's hematoxylin for 1 min, dehydrated through graded series of ethanol, placed into xylene and mounted with mounting media. During the steps of the staining procedure, slides were washed 3 times in phosphate-buffered saline (PBS) for 5 min each time. For negative control, PBS was used instead of the primary antibody.

2.3.3 Histological examination

Histological examination for PCNA immuno-staining was carried out in one ovarian tissue section per gilt. The ovarian follicles were quantified under light microscope with magnification of 40X for determining growing follicles and 100X for determining primordial and primary follicles (BX40, Olympus, Japan). For each section, 10 microscopic fields were arbitrarily selected for investigation and were counted in 25 squares of ocular micrometer that corresponded to 156.25 μm^2 (40X) and 25.0 μm^2 (100X) of real tissue area. Therefore, the total tissue area used to determine the number of primordial follicles, primary follicles and growing follicles was 250 μm^2, 250 μm^2 and 1562.5 μm^2, respectively. The quantity of follicles in the selected area was determined. Changing the location of the ocular micrometer was done across the entire area in a non-overlapping manner. Histological examination of

the samples was accomplished by only one person (D. Phoophitphong) who was unaware of the identity of the gilts. In the present study, only the numbers of follicles with an intact oocyte was counted to minimize the possibility of including degenerated oocytes. The number of follicles was expressed as the total number of follicles per 100 μm^2 of the tissue section.

2.3.4 Follicle categorisation

The follicles were classified into 3 categories as described earlier (Oktay et al., 1995; Picut et al., 2008) i.e., (I) "primordial follicles" were follicles having an oocyte surrounded by one or more flattened pregranulosa cells at the periphery of the follicle and no cuboidal cells (Figure 4A) (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 4B) 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 4C-G). For each category of follicles, both the number and the proportion of follicles were calculated. The number of follicles counted per 100 μm^2 of the real tissue section was calculated by: number of follicles = (number of counted follicles/ tissue area) x 100. The proportion of follicles was calculated by: proportion of each category of follicles = (number of each category of follicles/ number of all follicles) x 100.

2.3.5 Gilt categorisation

The gilts were classified on the criteria of body weight, ADG and age at first oestrus. According to body weight, gilts were grouped into 'light' (<150 kg, n = 12, mean ± SD = 138.9 ± 8.7) and 'heavy' (≥150 kg, n = 7, mean ± SD = 157.6 ± 5.2) weight. Due to ADG, gilts were divided into 'slow' (<600 gram/day, n = 14, mean ± SD = 512 ± 31) and 'fast' (≥600 gram/day, n = 5, mean ± SD = 623 ± 25) growth. As for age at first oestrus, gilts were classified as 'early' (<210 day, n = 9, mean ± SD = 202.8 ± 3.3) and 'delayed' (≥210 day, n = 10, mean ± SD = 229.8 ± 16.8) age at first oestrus groups.

2.3.6 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 the ovarian follicles were presented as mean ± SD. Proportional data was presented as a percentage. Proportions of each category of the ovarian follicles were analyzed by frequency analysis using PROC FREQ of SAS. Pearson's correlation was conducted to determine the association among the number of each category of follicles, the proportion of each category of follicles, age of the gilt, body weight, ADG, age at first oestrus, ovulation rate and weight of the ovary. One-way analysis of variance was used to analyze the effect of body weight classes (<150 vs ≥150 kg), ADG classes (<600 vs ≥600 g/day) and age at first oestrus classes (<210 vs ≥210 day) on number of follicles. Each of the factors was included in the statistical model one at a time. Least-squares means were calculated and were compared using the least significant different test. $P < 0.05$ was regarded to be statistically significant.

2.4 Results

The reproductive data of the gilts are presented in Table 1. On average, the gilts were 267.8 ± 19.2 day old and weighed 145.7 ± 11.8 kg. The ovaries weighed 6.5 ± 2.1 grams and the ovulation rate was 15.8 ± 4.3 (Table 1).

PCNA immuno-staining method could be used to determine the number of primordial, primary and growing follicles in the gilt ovarian tissue (Figure 4-5). Figure 4 demonstrates the appearance of different types of follicles in the gilts ovarian tissue in H&E tissue section compared with PCNA immuno-staining tissue sections. PCNA immuno-staining enhanced the visualization of small follicles and increased the ability to distinguish primordial and primary follicles (Figure 4B). Oocytes of all follicles types were also heavily stained by PCNA. The pre-granulosa cells surrounding the oocyte of the primordial and primary follicles were stained by PCNA (Figure 5A-B) but with a weaker staining compared to the granulosa cells surrounding the growing follicles (Figure 5C-G).

Table 1 Descriptive statistics of reproductive data and number of ovarian follicles in 19 gilts

Parameters	Mean ± SD	Range
Body weight at culling (kg)	145.7 ± 11.8	124 - 165
Age at culling (days)	267.8 ± 19.2	240 - 296
Age at first oestrus (day)	217.0 ± 18.4	196 - 272
Average daily gain (g/day)	541.0 ± 58.1	450 - 665
Weight of the ovary (gram)	6.5 ± 2.1	3.5 - 10.1
Ovulation rate	15.8 ± 4.3	5 - 23
Total number of follicles per 100 μm^2	19.8 ± 8.5	6.0 - 42.0
Number of primordial follicles (follicles/100 μm^2)	13.1 ± 6.9	3.2 - 27.6
Number of primary follicles (follicles/100 μm^2)	6.2 ± 3.3	2.4 - 14.0
Number of growing follicles (follicles/100 μm^2)	0.5 ± 0.2	0.3 - 0.9

Descriptive statistics on the number of follicles are presented in Table 1. On average, each of the 100 μm^2 of the ovarian tissue contained 19.8 ± 8.5 follicles. The number of follicles in the ovarian tissue per 100 μm^2 varied among gilts from 6.0 to 42.0 follicles. The numbers of primordial, primary and growing follicles were 13.1 ± 6.9 (range 3.2 - 27.6), 6.2 ± 3.3 (range 2.4 - 14.0) and 0.5 ± 0.2 (range 0.3 - 0.9) follicles/100 μm^2, respectively. The proportion of primordial, primary and growing follicles in each ovarian tissue section were 64.2% (range 33.3 - 83.0), 32.7% (range 13.8 - 64.7) and 3.1% (range 1.0 - 7.0), respectively.

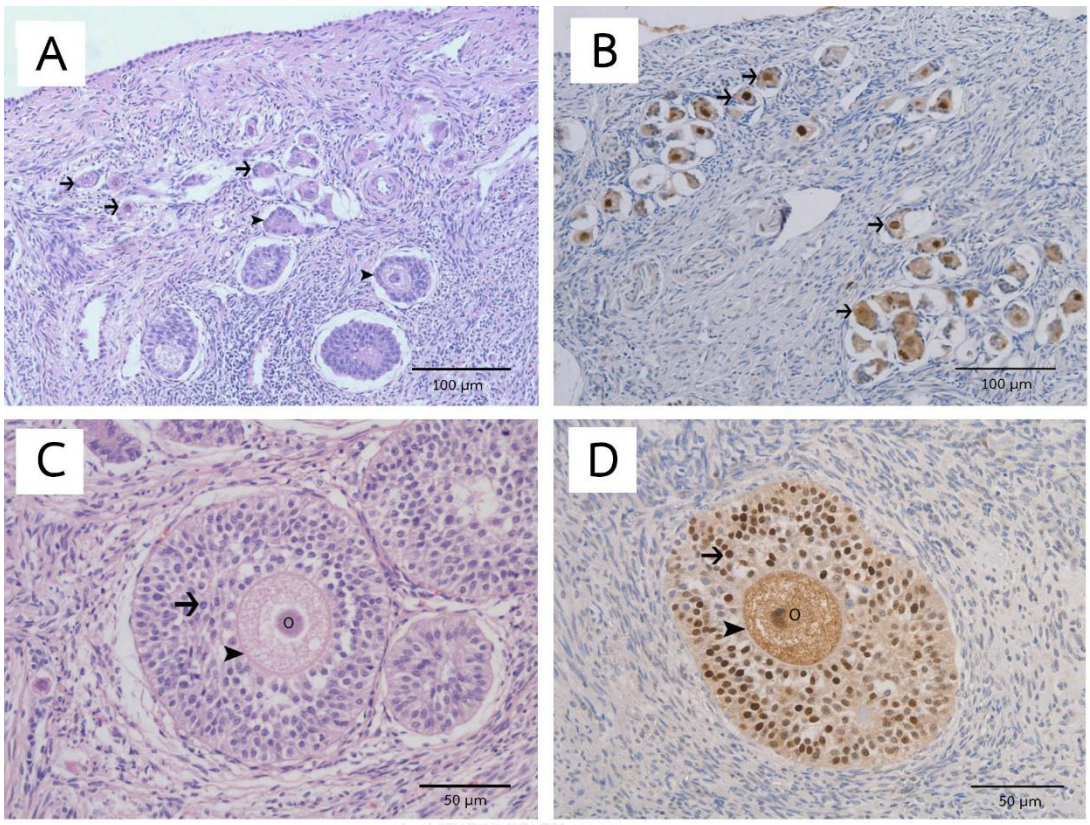

Figure 4. Gilt ovary stained with H&E and PCNA immunohistochemistry (A, B) and growing follicle (C, D). (A) Primordial follicle (arrow) and primary follicle (arrowhead) in H&E tissue section are difficult to detect. (B) PCNA immunohistochemistry can enhance the visualization of primordial (arrow) and primary follicles. (C) Growing follicle which intact oocyte (O) and surrounded by zona pellucida (ZP) (arrowhead) and granulosa cells (arrow) compared with growing follicle that both and oocyte and granulose cell were stained by PCNA immunohistochemistry (D). Original magnification: 40X (A-B), 200X (C-D).

Correlations between the number of ovarian follicles and all reproductive data are presented in Table 2. The number of primary follicles in ovaries were positively correlated with body weight (r = 0.50, *P* = 0.032) but negatively correlated with age at first oestrus (r = -0.54, *P* = 0.015). The proportion of primary follicles was also increased when either the body weight (r = 0.45, *P* = 0.052) or the ADG of the gilt rose (r = 0.46, *P* = 0.049). Neither the weight of the ovary nor the ovulation rate correlated with the number of ovarian follicles (Table 2).

Table 2 Pearson's correlation between number and type of each category of the gilt's ovarian follicles (I: primordial, II: primary and III: growing follicles) and reproductive data

Parameters	Number			Proportion		
	I	II	III	I	II	III
Body weight at culling	NS	r = 0.50 (*P*=0.032)	NS	r = -0.42 (*P*=0.070)	r = 0.45 (*P*=0.052)	NS
Age at culling	NS	NS	NS	NS	NS	NS
Age at first oestrus	NS	r = -0.54 (*P*=0.015)	NS	NS	NS	r = 0.53 (*P*=0.020)
Average daily gain	NS	NS	NS	r = -0.47 (*P*=0.043)	r = 0.46 (*P*=0.049)	NS
Weight of the ovary	NS	NS	NS	NS	NS	NS
Ovulation rate	NS	NS	NS	NS	NS	NS

NS = not significant

The density and proportion of follicles in the ovarian tissues of gilts by body weight, ADG and age at first oestrus are presented in Table 3. As can be seen from the table, gilts with a body weight of ≥150 kg had a greater number of primary follicles per 100 μm^2 (8.7 vs 4.7, P = 0.006) than gilts with a body weight of <150 kg. Furthermore, the proportion of primary follicles tended to be higher in gilts with a body weight of ≥150 kg compared to gilts with a body weight of <150 kg (40.1% vs 28.3%, P = 0.070). Likewise, gilts with a high ADG (623 ± 25 gram/day) had a higher proportion of primary follicles (45.1% vs 28.2%, P = 0.012) than gilts with a low ADG (512 ± 31 gram/day). Gilts with normal age at first oestrus (202.8 ± 3.3 days) had a higher number of primary follicles (8.0 vs 4.5, P = 0.020) than gilts with a delayed age at first oestrus (229.8 ± 16.8 days) (Table 3). The total number of follicles per 100 μm^2 of the ovarian tissue tended to be higher in gilts with a younger age at first oestrus than gilts with an old age at first oestrus (22.5 vs 17.3, P = 0.18) (Table 3).

Table 3 Density and proportion of follicles in the ovarian tissues of gilts by body weight and average daily gain (least squares mean ± SEM).

Ovarian follicles	Body weight (kg)		Average daily gain (gram/day)		Age at first oestrus (day)	
	≥150	<150	≥ 600	< 600	≥210	<210
Number of primordial follicles	12.8±2.7[a]	13.2±2.0[a]	7.9±2.8[c]	14.9±1.7[d]	12.2±2.2[e]	14.0±2.3[e]
Number of primary follicles	8.7±1.0[a]	4.7±0.8[b]	7.4±1.5[c]	5.7±0.9[c]	4.5±0.9[e]	8.0±0.9[f]
Number of growing follicles	0.48±0.08[a]	0.54±0.06[a]	0.53±0.09[c]	0.51±0.06[c]	0.50±0.07[e]	0.53±0.07[e]
Proportion of primordial follicles	57.3±4.8[a]	68.3±3.6[a]	51.2±5.0[c]	68.9±3.0[d]	67.7±4.2[e]	60.4±4.4[e]
Proportion of primary follicles	40.1±4.8[a]	28.3±3.6[a]	45.1±5.1[c]	28.2±3.0[d]	28.6±4.2[e]	37.2±4.4[e]
Proportion of growing follicles	2.6±0.7[a]	3.4±0.5[a]	3.7±0.8[c]	2.9±0.5[c]	3.7±0.6[e]	2.4±0.6[e]
Total number of follicles/100 μm^2	22.0±3.2[a]	18.4±2.5[a]	15.9±3.7[c]	21.1±2.2[c]	17.3±2.6[e]	22.5±2.7[e]

[a,b/c,d/e,f] Different superscripts within a row differ significantly ($P < 0.05$)

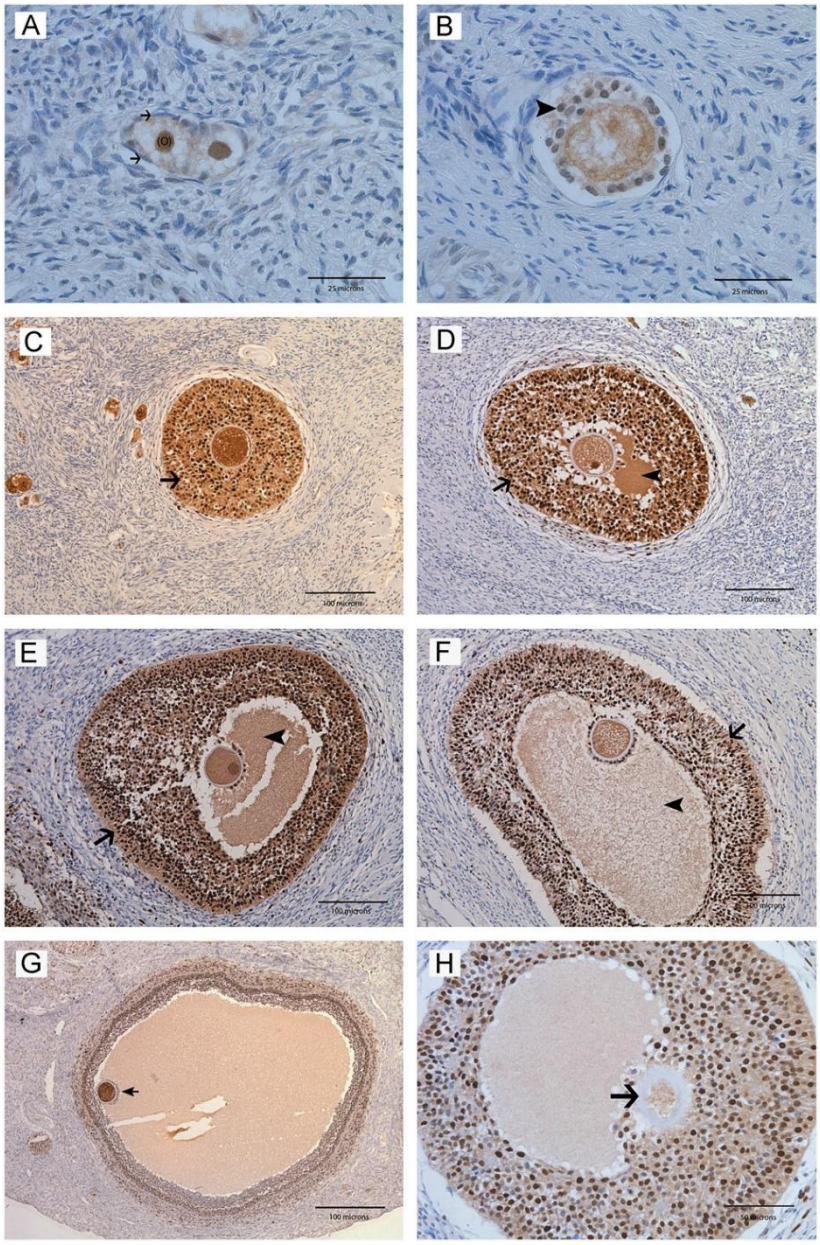

Figure 5. Different category of follicles with proliferating cell nuclear antigen (PCNA) immunohistochemical labeling (A) Primordial follicles with an oocyte (O) surrounded by one or more flattened pre-granulosa cell (arrow) at the periphery of the follicle without cuboidal cells. (B) Primary follicles with an oocyte surrounded by either a mixture of flattened pre-granulosa cells and plumb cuboidal granulosa cells (arrowhead) or single layer of cuboidal granulosa cell (C-F). Growing follicles: pre-antral follicle (C) with small area of fluid-filled (arrowhead) and antral follicles (D) with a visible zona pellucida and /or multiple layers of granulosa cells (arrow). (G) Graffian follicles with an oocyte surrounded by cumulus cells (arrow). (H) Atretic follicles with a remnant of oocyte and zona pellucida (ZP) (arrow). Magnification 40x (G), 100x(C-F), 200x (H) and 400x (A and B).

2.5 Discussion

PCNA is a standard marker for proliferating cells because it contains an auxiliary protein of DNA polymerase-δ enzymes which is important for DNA synthesis (Wood and Shivji, 1997; Muskhelishvili et al., 2005). PCNA is a useful marker for ovarian follicle count in rats because of the distinct ability of marking oocyte nuclei with PCNA antibody which significantly increases in speed and accuracy of counting (Muskhelishvili et al., 2005). Furthermore, when using H&E-stained, the primordial follicles are easily overlooked (Muskhelishvili et al., 2005). In the present study, a modified PCNA immuno-staining protocol in the pig ovarian tissue was demonstrated. PCNA immuno-histochemical labelling of oocytes was demonstrated for the first time in the rat's ovarian tissue since 1995 (Oktay et al., 1995). At the beginning, PCNA immuno-reactivity appeared only in the pre-granulosa cells of early primary follicles and all following stages, while primordial follicles were not stained by PCNA. However, the combination use of heat-induced epitope retrieval technique and a high concentration of primary antibody has been demonstrated to increase the visualization of primordial follicles in rat ovaries (Muskhelishvili et al., 2005). In pig ovaries, immuno-histochemical localization of PCNA had also been investigated (Tomanek and Chronowska, 2006). However, no remarkable PCNA staining of primodial follicles can be demonstrated. The reason might arise from the heat-induced epitope retrieval technique that was not been implemented in that study (Tomanek and Chronowska, 2006). In our study, PCNA immuno-histochemical labelling technique was modified by treating the pig ovarian tissue with antigen retrieval solution (0.01 M citrate buffer, pH 6.0) for 10 min in a microwave oven at 600 watt and using a dilution of 1:200 of mouse monoclonal anti-PCNA. The results revealed that the oocyte nuclei and either the pre-granulosa or the granulosa cells surrounding the oocyte could be clearly distinguished from the ovarian tissue. Thus, the PCNA immuno-histochemical labelling technique developed in our study could be used as a tool for ovarian follicle count in the pig ovary.

The present study demonstrated that it is difficult to distinguish the primordial and primary follicles from the other remaining ovarian tissue by H&E

staining only. The reason was due to that the H&E staining is a proper method for demonstrating the histological appearance of the cells but cannot be used to study the cells function. In general, hematoxylin stains blue in the nucleus of cells, while eosin stains red in other part of the cells, red blood cells and connective tissues. The H&E staining is commonly used for evaluating overall histological morphology in many types of tissues that were fixed in formalin and embedded in paraffin. Ovarian tissues of pubertal gilts differed from other tissues in several aspects, such as it contains several stages of the follicles and oocytes. Either the oocytes or the granulosa cells surrounding the follicles in the gilt ovarian tissue are highly proliferated and a rather high density of these follicles was commonly observed in the porcine species (i.e., 19.8 ± 8.5 follicles/100 μm^2). H&E staining cannot distinguish proliferating cells and the other remaining tissues, hence the small follicles are hardly seen and cannot be precisely determined (Figure 4). The present study revealed that the PCNA immunohistological method enhanced the visibility of small follicles. This finding is in agreement with a number of earlier studies in other species (Muskhelishvili et al., 2005; Picut et al., 2008).

The present results provide fundamental on data follicle density in pig ovarian tissue and differentiate the type of follicles in the gilt ovary. It should be noted that all of the gilt ovaries included in the present study were obtained from pubertal gilts (approx. 9 months of age) and all of them had ovulated. To our knowledge, no quantitative data on the number and type of follicles in the gilt ovary has been reported. It was found that majority of the follicles in the pubertal gilts ovarian tissue were primordial follicles (64.2%), while 32.7% were primary growing follicles and 3.1% of follicles that had reached a large size (pre-antral and antral follicles). At this age, the gilt's ovarian tissue contained 19.8 follicles/100^2 μm. Of these follicles, 13.1, 6.2 and 0.5 were primordial, primary and growing follicles, respectively.

In the present study, the density of follicles in the gilt ovaries is associated with their body weight, ADG and age at first observed oestrus, but not associated with age of the gilts, weight of the ovary and ovulation rate. It was found that gilts

with a body weight of >150 kg, an ADG of >600 g/day or those exhibiting first standing oestrus before 210 days of age had a higher density of total follicles in the ovarian tissue, particularly the primary follicles, than those with poorer performance. The association among these factors and number of primary follicles indicates that gilts with a heavy body weight or high ADG and those exhibiting first standing oestrus normally are supposed to have a higher number of proliferating cells than those with a poor reproductive performance. Recently, it has been demonstrated that some hormones/protein related to energy homoeostasis and reproduction, e.g., obestatin (Meszarosova et al., 2008), leptin (Sirotkin and Meszarosova, 2010), and ghrelin (Sirotkin, 2011), are able to increase porcine granulosa cells proliferation and the expression of PCNA in granulosa cells cultured in vitro. This implies that the gilts with a high body weight, high ADG and attaining puberty early might have a higher level of these hormones as well as other growth factors than those with a poor performance. Under field conditions, breeding replacement gilts with a heavy body weight and high ADG as well as those with an early age at puberty resulted in a better subsequent reproductive performances and herd productivity (Tummaruk et al., 2001; Tummaruk et al., 2007; Patterson et al., 2010).

In conclusion, PCNA technique can be applied to quantify precise numbers and distinguish the type of follicles in the ovarian tissue of porcine species. Gilts with a higher body weight and earlier age at first observed oestrus have a higher density of primary follicles in the ovarian tissue than those with a lower body weight and later age at first observed oestrus.

CHAPTER III

Apoptotic cell localisation of preantral and antral follicles in relation to non-cyclic and cyclic gilts

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3.1 Abstract

The objective of this study was to determine apoptotic cell localisation in preantral and antral follicles of porcine ovaries. Additionally, the proportion of cells undergoing apoptosis was also compared between delayed puberty gilts and normal cyclic gilts. Ovarian tissues were obtained from 34 culled gilts with an age and weight of 270.1 ± 3.9 days and 143.8 ± 2.4 kg, respectively. The gilts were classified according to their ovarian appearance as 'non-cyclic' (n = 7) and 'cyclic' (n = 27) gilts. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to determine apoptotic cell expression in different compartments of the ovarian tissue sections. All apparent preantral (n = 110) and antral (n = 262) follicles were evaluated by using image analysis software. It was found that apoptotic cells were expressed in both granulosa (22.2%) and theca cell layers (21.3%) of the follicles in the porcine ovaries. The proportion of apoptotic cells in the granulosa layer in the follicles was positively correlated with that in the theca layer (r = 0.90, $P < 0.001$). Apoptosis did not differ significantly between preantral and antral follicles in either granulosa (27.8% and 26.4%, $P > 0.05$) or theca cell layers (28.6% and 26.5%, $P > 0.05$). The proportion of apoptotic cells in non-cyclic gilts was higher than cyclic gilts in both granulosa (31.7% and 22.6%, $P < 0.001$) and theca cell layers (34.8% and 20.2%, $P < 0.001$). The present study indicated that apoptosis of the granulosa and theca cell layers in the follicles was more pronounced in the ovarian tissue of delayed puberty gilts than cyclic gilts. This implied that apoptosis could be used as a biologic marker for follicular

development/function and also that apoptosis was significantly associated with anoestrus or delayed puberty in gilts, commonly observed in tropical climates.

3.2 Introduction

Apoptosis, or programmed cell death, occurs during folliculogenesis of ovarian tissue. Matsuda-Minehata et al. (2006) stated that more than 99% of mammalian follicles, including in pigs, undergo degeneration or atresia during follicular growth and development due to granulosa cell apoptosis. The period of follicle development during the preantral to early antral stage is more susceptible to atresia than the later stage of follicles (Orisaka et al., 2009). In general, granulosa cells are closely associated with oocyte development through their endocrine and paracrine signaling (Goto et al., 2013; Yang et al., 2013). Therefore, granulosa cell dysfunction might lead to poor oocyte quality and infertility (Gilchrist et al., 2004; Su et al., 2009). Moreover, granulosa cell apoptosis has been reported to be a cause of follicular atresia in mammalian ovaries (Yang et al., 2013), which leads to infertile animals. To date, various techniques have been used to assess apoptotic cells in many types of tissues. Over the past decades, the detection of DNA fragmentation has been widely used to determine the presence of apoptotic cells in histologic sections (Stadelmann and Lassmann, 2000). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay is a method frequently applied to detect DNA fragmentation in tissue sections, including in pigs (Durlej et al., 2012). In pigs, the number of ovulations (i.e., litter size at birth) is one of the most important criteria determining reproductive performance (Tummaruk and Kesdangsakonwut, 2015). Due to the female puberty gradation and physiologic condition, folliculogenesis and its regulation are important for female pigs to regulate many reproductive functions including puberty attainment, oestrus behaviour and ovulation (Brussow et al., 2002; Ratky et al., 2005; Schwarz et al., 2008). Tummaruk et al. (2009a) reported that a high proportion of gilts in the tropical country of Thailand were culled due to anoestrus, and greater than 50% of those culled had non-cyclic ovaries. Of the gilts culled due to reproductive problems, ovarian abnormalities, such

as ovarian cysts, ovarian adhesions, unilateral small ovary and ovo-testis, were reported as causes (Tummaruk et al., 2009a). These findings indicate that ovarian dysfunction in gilts is one of the most common reasons leading to culling of replacement gilts in tropical climates. Therefore, additional knowledge of porcine ovarian function and pathology, as well as its associated factors, is needed. Follicular development in cyclic gilts is different from non-cyclic gilts. In cyclic gilts, follicle development occurs under the influence of corpus luteum (CL) development, while this is not the case in non-cyclic gilts (Murphy et al., 2001). Furthermore, the number of ovulations in pigs is also associated with the number of large follicles present during follicle development (Driancourt et al., 1992). Nevertheless, follicles selected to develop until ovulation may not always provide healthy oocytes. Therefore, a biologic marker indicating the competency of follicles in porcine ovaries should be carefully determined in order to be used as an indirect measure of oocyte quality. In cyclic gilts, some follicles may be able to avoid atresia by exposure to follicle stimulating hormone (FSH) (Knox, 2005). Therefore, it is hypothesised that follicular cell apoptosis can be used as a biologic marker to indicate the difference in follicle development between non-cyclic and cyclic gilts. The objective of the present study was to determine apoptotic cell localisation in preantral and antral follicles of the porcine ovary. Additionally, the proportion of cells undergoing apoptosis was also compared between delayed puberty (i.e., non-cyclic) and normal cyclic gilts.

3.3 Materials and methods

3.3.1 Animals, data and sample collection

Ovarian tissue samples were collected from 34 Landrace x Yorkshire crossbred culled gilts. The organs and historical data of the gilts were obtained from two commercial swine herds in Thailand. The historical data of the gilts included the herd and gilt identity, breed, date of birth, date of entry into the herd, date of first observed oestrus, date of culling, and body weight at culling. The age at culling and average daily gain (ADG) from birth to culling was calculated. The ovaries were collected immediately after slaughter and were placed on ice and transported to the

laboratory at Chulalongkorn University within 24 h. 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 assess reproductive status (i.e., non-cyclic versus cyclic gilts). The ovarian structures including the CL, corpora albicantia (CA) and follicles were defined as previously described by Tummaruk et al. (2009a), and the number of CL was counted to determine the number of ovulations. The ovary was defined as 'non-cyclic' when it contained only small follicles and had no CL or CA, and it was defined as 'cyclic' when it contained CL or CA and follicles. After that, 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 blocks were cut into a 4 µm thick sections using a microtome (Shandon, Anglia scientific instrument ltd., Cambridge, UK) which were placed on gelatin-coated slides.

3.3.2 TUNEL assay

All ovarian tissue sections were stained by TUNEL method using a commercial kit (ApopTag® Peroxidase kit; Millipore, CA, USA). The immunohistochemical procedure for the TUNEL assay was performed following the manufacturer's protocol. Briefly, the ovarian tissue sections were deparaffinised in xylene and rehydrated through graded ethanol dilutions. The tissue sections were placed in 0.01 M citrate buffer (pH 6.0) in a microwave oven at 750 watts for 15 min (three cycles of 5 min each) to retrieve antigenicity. Endogenous peroxidase was blocked using freshly prepared 3.0% hydrogen peroxide (H_2O_2) at room temperature (RT) for 10 min. Equilibration buffer was applied to the slides for 10 sec at RT. Thereafter, the sections were incubated with TUNEL reaction mixture: terminal deoxynucleotidyl transterase (TdT enzyme) and reaction buffer in a humidified chamber for 1 h at 37 °C. After incubation with the reaction mixture, stop/wash buffer was applied to the sections for 10 min at RT. Anti-digoxigenin conjugate was dropped onto the slides and incubated for 30 min at RT. The sections were stained with 3, 3'-diaminobenzidine tetrahydrochloride hydrate for 2 min at RT (ImmPACT DAB™,

Vector Laboratories, Burlingame, CA, USA). The sections were then counterstained with Mayer's hematoxylin for 1 min, washed with running water and mounted with mounting media. During the steps in the staining procedure, the slides were washed three times in phosphate-buffered saline (PBS) for 1 min each time. A negative control section was prepared by replacing TdT solution with PBS.

3.3.3 Follicle categorisation

The follicles were classified according to the reproductive status of the gilts into two groups: non-cyclic (n = 7) and cyclic (n = 27) ovaries. The types of the follicles that were evaluated included preantral (included only secondary follicles, n = 110) and antral follicles (n = 262) as described by Phoophitphong et al. (2012). A preantral follicle was defined as a follicle that presents an oocyte with visible zona pellucida that is surrounded by multiple layers of granulosa cells without antral formation, while the antral follicle presents an oocyte with zona pellucida that is surrounded by multiple layers of granulosa cells with antral formation (Figure 6). Follicles were also classified into two stages consisting of normal and atretic follicles, as was described in detail by Nakayama et al. (2000). A normal follicle was defined as a follicle that contained cumulus cells encircling oocytes, well-retained granulosa and theca cells and a granulosa layer attached to the basement membrane. An atretic follicle was defined as a follicle containing an oocyte without cumulus cells or with only a small amount of cumulus cells (Figure 6). In atretic follicles, granulosa and theca cells showed nuclear condensation, the granulosa layer was detached from the basement membrane, and phagocytic cells were observed in the follicular antrum.

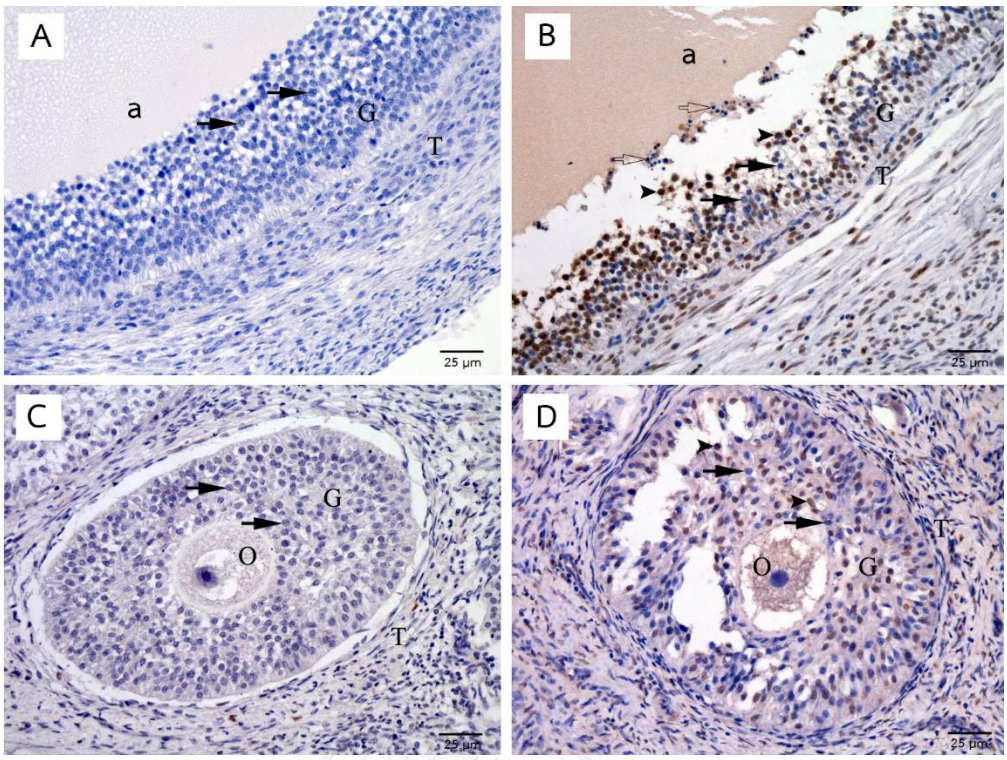

Figure 6. Follicle categorisation. Normal follicle of antral and preantral follicles (A, C) characterised by well-retained granulosa (G) and theca cell layers (T) that were attached to the basement membrane. Atretic follicle of antral and preantral follicles (B, D) characterised by shrunken oocyte cytoplasm, loose granulosa layer and/or granulosa layer detached from the basement membrane, and phagocytic cells within the follicular antrum (a).

3.3.4 Determination of apoptotic cells

The ovarian sections were captured using a digital camera (MicroPublisher 5.0 RTV QImaging®, Surry, BC, Canada) under a light microscope (BX-50, Olympus, Tokyo, Japan) with corresponding software. Preantral and antral follicles from all ovaries were evaluated for TUNEL staining. All of the visible preantral and antral follicles in the ovarian tissue section of the gilts were evaluated. Measurement of TUNEL positive stained cells in both granulosa and theca cell layers was evaluated using image analysis software (Image-Pro® PLUS 6.0 Programming software, Media Cybernetics, Inc., MD, USA). The percentage of TUNEL positive cells was calculated as: TUNEL positive cells (%) = (number of TUNEL positive cells/total numbers of cells counted in each field) x 100.

3.3.5 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 error of means (SEM) and the range of all the continuous data were calculated. The percentage of apoptotic cells was presented as mean ± SEM. Multiple analysis of variance (ANOVA) was used to analyse the apoptotic cells using the general linear mixed model procedure of SAS. The statistical model included the fixed effect of follicle types (preantral, antral, normal and atretic follicles), reproductive status (non-cyclic and cycling gilts) and the interaction between follicle type and reproductive status. The gilt's identity was included in the statistical model as a random effect. Least-squares means were calculated and compared using the least significant difference test with the Tukey-Kramer adjustment for multiple comparisons. Correlation coefficients were calculated by a Pearson correlation procedure. Statistical significance was set at $P < 0.05$.

3.4 Results

The average, age at culling, body weight at culling and ADG of the gilts are shown in Table 4.

Table 4 Descriptive statistics on age, body weight and average daily gain of gilts and the percentage of apoptotic cells in granulosa and theca cell layers in porcine ovaries.

Variables	Number of observation	Mean ± SEM	Range
Age (day)	34	270.1 ± 3.9	229 - 345
Body weight (kg)	34	143.8 ± 2.4	117 - 179
Average daily gain (g/day)	34	528.6 ± 9.0	438 - 665
Apoptotic cells (%)			
Granulosa cells	372	22.2 ± 1.4	0 - 100
Theca cells	372	21.3 ± 1.4	0 - 98

3.4.1 Localisation of apoptotic cells in the porcine ovary

Apoptotic cells were detected in the granulosa and theca layers in both preantral and antral follicles as shown in Figure 6. The apoptotic cells were detected notably in nucleus of granulosa and theca cells (Figure 6B, D). For normal follicles, some apoptotic-stained cells were detected in the granulosa and theca layers (Figure 6A, C). Likewise, a number of apoptotic cells were also detected in atretic follicles. The proportion of apoptotic cells in the granulosa layer was positively correlated with that in the theca layer (r = 0.90, $P < 0.01$, n = 372). In addition, body weight of the gilts was significantly correlated with the proportion of apoptotic cells in both the granulosa (r = -0.10, $P < 0.05$) and theca layers (r = -0.17, $P < 0.05$). However, age and ADG of the gilts did not show a correlation with the proportion of apoptotic cells in the follicles ($P > 0.05$).

3.4.2 Apoptotic cell expression in the follicles of non-cyclic and cyclic gilts

The average percentage of apoptotic cells in the granulosa (31.7% vs 22.6%, $P < 0.01$) and theca cell layers (34.8% vs 20.3%, $P < 0.01$) of the ovary in non-cyclic gilts was higher than in cyclic gilts. Additionally, apoptotic cells by reproductive status and follicle type in granulosa and theca cell layers are presented in Table 5. The average proportion of apoptotic cells in the granulosa layer of antral follicles in non-cyclic gilts was higher than that in cyclic gilts ($P < 0.05$). However, for preantral follicles, no difference in the proportion of apoptotic cells in the granulosa layer was observed between non-cyclic or cyclic gilts ($P > 0.05$). The average proportion of apoptotic cells in the theca cell layer in non-cyclic gilts was higher than that in cyclic gilts in both preantral ($P < 0.05$) and antral follicles ($P < 0.05$).

Table 5 The percentage of apoptotic cells (least squares means ± SEM) in granulosa and theca cell layers in preantral and antral follicles of non-cyclic and cyclic gilts.

Reproductive status	Follicular type	Number of follicle	Apoptotic cells (%)	
			Granulosa layer	Theca layer
Non-cyclic	Preantral	21	32.1 ± 3.8[a]	35.0 ± 4.3[a]
	Antral	65	31.2 ± 2.1[a]	34.6 ± 2.4[a]
Cyclic	Preantral	89	23.5 ± 1.8[ab]	22.1 ± 2.1[b]
	Antral	197	21.6 ± 1.2[b]	18.4 ± 1.4[b]

[a,b] Different superscripts within a column indicate significant difference ($P < 0.05$).

3.4.3 Apoptotic cell expression in normal and atretic follicles

The average percentage of apoptotic cells in the granulosa layer of atretic follicles was higher than that in normal follicles (49.0% vs 5.2%, $P < 0.01$). Likewise in the theca layer, the average percentage of apoptotic cells in atretic follicles was greater than in normal follicles (46.7% vs 8.4%, $P < 0.01$). In the theca layer, the proportion of apoptotic cells in normal follicles of cyclic gilts was lower than that of non-cyclic gilts (1.7% vs 15.2%, $P < 0.01$), as shown in Figure 7.

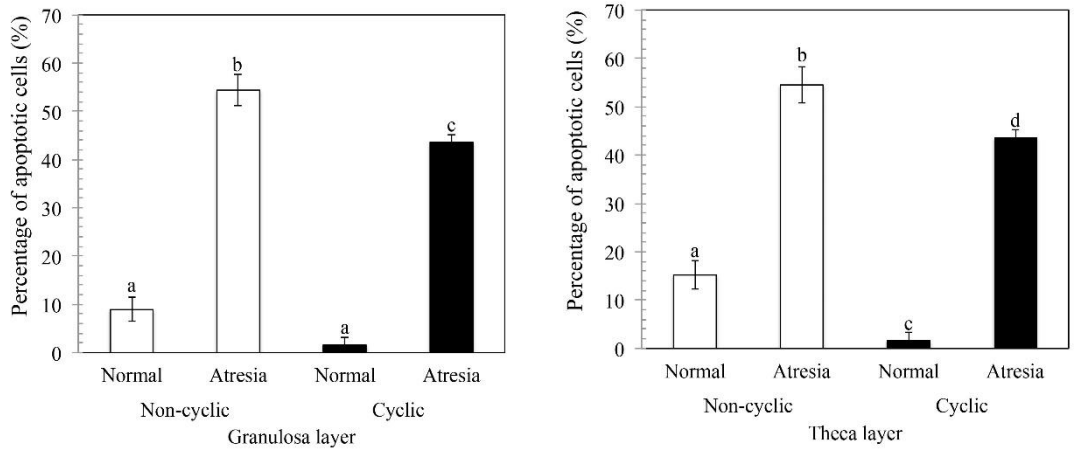

Figure 7. Percentage of apoptotic cells in granulosa and theca cell layers of normal and atretic follicles in non-cyclic and cyclic gilts. a,b,c,d Different characters indicate a significant difference ($P < 0.05$).

3.4.4 Apoptotic cell expression in preantral and antral follicles

The average proportion of apoptotic cells in the granulosa (27.8% vs 26.4%, $P > 0.05$) and theca cells layers (28.6% vs 26.5%, $P > 0.05$) in preantral follicles did not differ significantly compared to that in antral follicles. The proportion of apoptotic cells in the granulosa and theca cell layers of normal and atretic preantral and antral follicles are presented in Figure 8. For atretic follicles, the average proportion of apoptotic cells in the granulosa (51.9% vs 46.1%, $P > 0.05$) and theca cell layers (50.0% vs 43.3%, $P > 0.05$) of preantral follicles did not differ significantly compared to antral follicles. Likewise, in normal preantral and antral follicles, there was no difference in apoptotic cells between the granulosa (3.8% vs 6.6%, $P > 0.05$) and theca cell layers (7.1% vs 9.7%, $P > 0.05$).

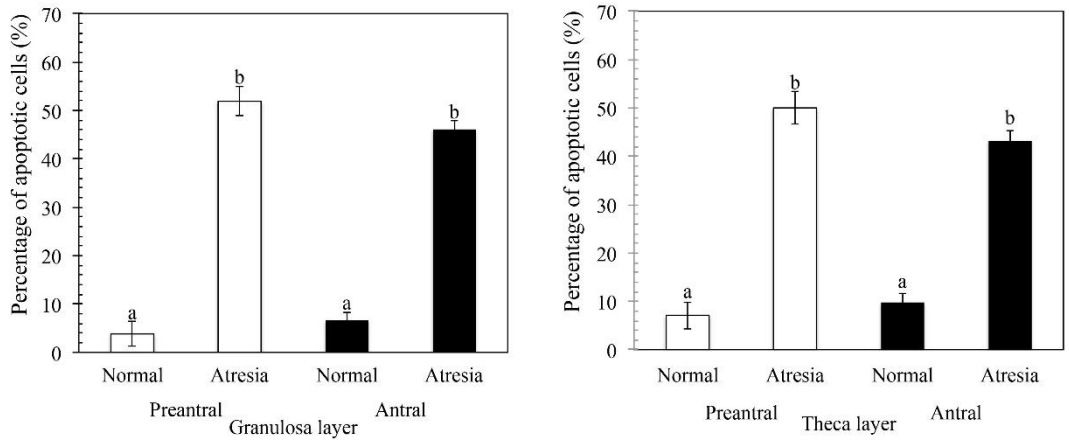

Figure 8. Percentage of apoptotic cells in granulosa and theca cell layers of normal and atretic follicles in preantral and antral follicles. a,b Different characters indicate a significant difference ($P < 0.05$).

3.5. Discussion

In this present study, the proportion of apoptotic cells in non-cyclic gilts was higher than that in cyclic gilts. This indicated that the follicles of non-cyclic gilts were more susceptible to apoptosis than those of cyclic gilts. The average age of non-cyclic gilts (270 days) in the present study was higher than previously reported in Landrace x Yorkshire crossbred gilts in Thailand which attained puberty at 200 days (Tummaruk et al., 2009b) and were defined as having a delayed puberty problem. Therefore, delayed puberty of the gilts observed under field conditions might be

partly explained by a high proportion of apoptotic follicles. This might subsequently cause suboptimal growth of the follicles and lack of ovarian steroid production for initiating oestrus behaviour. The main difference in sex steroid production and intraovarian factors between prepubertal and pubertal gilts is low oestradiol concentration throughout the prepuberty period (Evans and O'Doherty, 2001). To our knowledge, oestrogen also affects follicular growth and follicular cell differentiation by increasing the proliferation of granulosa cells (Schams and Berisha, 2002). In the prepubertal period, follicular growth and ovarian morphologic changes are regulated by intraovarian factors. These factors serve as an autocrine and/or paracrine aspect to activate (e.g., insulin-like growth factor 1 (IGF-1), inhibin and transformation growth factor) or to inhibit (e.g., activin, epidermal growth factor and follicle regulatory protein) follicle development (Bolamba et al., 1994). Moreover, Yu et al. (2004) found that changes in oestradiol and progesterone levels are related to the regulation of follicle development and atresia. It was found that the oestradiol level in atretic follicles is significantly lower than in healthy follicles (Yu et al., 2004).

Moreover, apoptotic cell expression between granulosa and theca cell layers was positively correlated. This indicated that apoptosis of granulosa and theca cells is closely associated and may cause atresia of follicles. Studies have revealed that apoptosis of granulosa cell induces follicular atresia through intracellular signals and apoptotic stimuli (Liu et al., 2003; Yu et al., 2004; Matsuda-Minehata et al., 2006). More than 99% of follicles undergo degeneration or atresia during follicular growth and development because of granulosa cell apoptosis (Matsuda-Minehata et al., 2006). Ovarian granulosa and theca cells interact during preantral follicular development which is known as the two-cell, two gonadotropin theory (Hillier et al., 1994; Magoffin, 2005). Granulosa cells are responsible for apoptosis when the granulosa cell differentiation occurred and initially sustain apoptosis (Nakayama et al., 2000; Matsuda-Minehata et al., 2006). It has been suggested that theca cells raised under a culture system are unable to maintain their structure and function without the presence of granulosa factors such as IGF-1 and inhibin B (Kotsuji et al., 1990; Magoffin, 2005). In the preantral stage, theca cells are the source of androgen production, provide increased blood supply for the transport of ovarian factors to

growing follicles and enhance the susceptibility to gonadotropins (Orisaka et al., 2009; Young and McNeilly, 2010). Therefore, the granulosa and theca cells are essential ovarian stromal cells involved in folliculogenesis.

In the granulosa cell layer, the number of apoptotic cells in atretic follicles was higher than in normal follicles. Granulosa cells of atretic follicles undergo changes in cell morphology, including DNA fragmentation, and can be detected by TUNEL. Apoptotic cells were also detected in the normal follicles, although the number of apoptotic cells was lower. This finding is similar to previous reports in sheep (Jolly et al., 1997) and cows (Blondin and Sirard, 1995) in which granulosa cell apoptosis was also detected in the healthy follicles. Moreover, the results in the present study are consistent with Sun et al. (2012), in that apoptotic cells were found in both granulosa and theca cell layers of normal and atretic follicles. Apoptosis of granulosa cells can be described as atretic follicle degeneration occurring in mammalian animals (Hughes and Gorospe, 1991; Tilly and Hsueh, 1992). To our knowledge, the proper balance between apoptosis and proliferation is essential for follicle development. Inappropriate balance has led to follicular cysts (Sun et al., 2012). Therefore, apoptosis is considered an imperative mechanism of follicular atresia.

In the present study, apoptotic cells were observed in both the granulosa and theca cell layers of both preantral and antral follicles. Furthermore, the proportion of apoptotic cells was greater in preantral follicles than antral follicles. This might due to the fact that the preantral follicle is sensitive to gonadotropins (Matsuda-Minehata et al., 2006; Orisaka et al., 2009). Previous studies revealed that in the early antral stage of follicular development, the majority of follicles become atretic (Hirshfield, 1991; Depalo et al., 2003; Matsuda-Minehata et al., 2006). This occurs in the transition period from preantral to antral follicles. Granulosa cell apoptosis is visible in the preantral stage along with the smallest size of antral follicles (Gougeon, 1996). At this stage, the follicles are adaptive to the gonadotropin dependence and are mainly regulated by intraovarian regulators such as growth factors, cytokines and steroids (Orisaka et al., 2009). Follicles chosen for further development are assumed to get proper gonadotropin and intraovarian signals for

survival, while follicles undergoing atresia receive insufficient survival factors to complete maturation (Hu et al., 2004).

In conclusion, apoptosis is detected in both preantral and antral follicles of the porcine ovary. The expression of cellular apoptosis was observed in both granulosa and theca cell layers of the porcine ovarian tissue. Apoptosis of the granulosa and theca cells in the follicles was more pronounced in the ovarian tissue of delayed puberty gilts (i.e., non-cyclic) than cyclic gilts. This implies that apoptosis can be used as a biologic marker for follicular development/function and is significantly associated with anoestrus or delayed puberty in gilts, which is commonly observed in tropical climates.

CHAPTER IV

Immunoexpression of oestrogen receptor β in the ovarian follicle in relation to non-cyclic and cyclic gilts

4.1 Abstract

Oestrogen receptor β (ERβ), the main subtype of oestrogen receptor, involves in the follicle maturation which is need for antrum forming and preovulatory follicle development. The objective of the present study was to determine the immunoexpression of ERβ in non-cyclic and cyclic gilts using immunohistochemistry. Ovaries were obtained from 55 Landrace x Yorkshire crossbred non-cyclic (n = 25) and cyclic gilts (n = 30). The expression of ERβ was determined by using immunohistochemistry on paraffin sections using polyclonal antibodies against ERβ. The percentage of ERβ expressing in antral follicles was determined by using Image-Pro® Plus software. The ERβ immunoexpression was observed in the theca interna cells of follicles and in the luteal cells of corpus luteum (CL), while the immunoexpression was not obviously detected in granulosa cells. On the average, ERβ immunoexpression was detected in 22.3% of theca cells and 85.7% of the luteal cells. The ERβ immunoexpression in the theca cells of antral follicles in non-cyclic gilts was lower than that in cyclic gilts (17.5% and 26.4%, respectively, $P < 0.001$). Moreover, the average percentage of ERβ immunoexpression was positively correlated with body weight (r = 0.22, $P < 0.01$) and age (r = 0.27, $P < 0.01$) of the gilts. It can be concluded that ERβ immunoexpression in the porcine ovaries can be detected in both antral follicles and CL. Moreover, cyclic gilt exhibits higher ERβ immunoexpression than non-cyclic gilt. The results suggest that ERβ might play an essential role for follicular development and might be associated with puberty attainment and the reproductive status.

4.2 Introduction

Oestrogen, the sex steroid hormone, plays the important roles in regulation of mammal reproductive system as well as in gilts and sows (Drummond et al., 1999). The ovary is a major target organ for biological functions of, which signaling through the nuclear receptors within the ovary (Mangelsdorf et al., 1995; Knapczyk et al., 2008). The theca interna and granulosa cells are major component of the follicles that synthesize oestrogen (Gilling-Smith et al., 1997). The most active oestrogen produced and secreted by antral follicles is oestradiol-17β. Besides, corpus luteum (CL) can also secrete oestradiol-17β, which involves as an autocrine/paracrine regulator (Einspanier et al., 1991). In general, oestrogen regulates reproductive function and follicle maturation by binding to their nuclear protein, oestrogen receptors (ERs) (Conneely, 2001). Alteration in the expression of ERs may leads to ovulation failure and abnormal reproductive function (Drummond et al., 2002).

Under field condition, the alteration of ovarian steroids production which regulates reproductive organs might be occur due to several factors, such as, stressful management, exogenous hormone administration and reproductive status (Castagna et al., 2004; Szulanczyk-Mencel et al., 2010). These may leads to the culling of young animals from the commercial herd. In swine commercial herd, 40–60% of sows are culled and are replaced by gilts annually (Engblom et al., 2007; Tummaruk et al., 2010a). Our previous study has demonstrated that a certain number of replacement gilts were culled due to reproductive failure, especially anoestrus and/or abnormal oestrous behaviour (Tummaruk et al., 2009a). Delayed puberty and anoestrus cause an increased non-productive day (NPD) in commercial farm, which needed more production cost to solve these problems. The mechanism associated with anoestrus or poor oestrous behaviour in gilts is therefore needed to be explored.

In general, ERs consist of two isoforms, i.e., oestrogen receptor α (ERα) and β (ERβ) (Knapczyk et al., 2008). ERα is the classical ER that was identified since 1986, while ERβ was identified later (Saunders, 1998). Although ERα and ERβ are encoded on different chromosome, they had a considerable sequence homology in their

domain (Saunders, 1998). Therefore, it could be speculated that the presence of any of the ERs subtypes indicates the tissue action of oestrogen. The expression and distribution of both ERβ and ERα has been demonstrated in the ovaries of both cyclic sows and pregnant sows (Slomczynska et al., 2001; Knapczyk et al., 2008). Emmen et al. (2005) found that ERβ plays the important role in follicular maturation in mice. In ERβ knockout mice, a high incidence of ovulation failure and a decrease number of offspring was observed (Krege et al., 1998). Slomczynska et al. (2001) demonstrated that ERβ is highly expressed in granulosa cells of the follicles in pig. In porcine ovary, the ERβ is more pronounce over ERα that ERβ expressed differently between different stages of the reproductive cycles in all types of follicles, while ERα was found only in large pre-ovulatory follicles and early corpora lutea (Slomczynska and Wozniak, 2001). Nevertheless, the expression and distribution of ERβ in relation to reproductive status in non-pregnant gilts has not been examined. We hypothesized that the expression of ERβ in the ovarian tissue of gilts might be dissimilar between non-cyclic and cyclic gilts. Therefore, the objective of the present study was to demonstrate the presence of ERβ in the ovaries of non-cyclic and cyclic gilts using immunohistochemistry.

4.3 Materials and methods

4.3.1 Animals, data and sample collection

Ovarian tissues were collected from 55 Landrace x Yorkshire crossbred gilts. The organs and historical data of the gilts were obtained from two commercial swine herd in Thailand. The historical data of the gilts included the herd and gilt identity, breed, date of birth, date of entry into the herd, date of first observed oestrus, date of culling, and body weight at culling. The age at culling was calculated. The ovaries 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 210 C, max = 210 g, d = 0.01 g, Precisa Instruments Ltd., Switzerland). The appearance of the ovaries was used to define the reproductive status (i.e., non-cyclic and cyclic gilt). The ovarian structures including CL, corpora

albicantia (CA) and follicles were defined according to a previous study (Tummaruk et al., 2009a). The number of CL was counted and defined as the number of ovulations. The ovary was defined as 'non-cyclic' when it contained only small follicles and had no CL or CA, and as 'cyclic' when it contained CL or CA and follicles.

4.3.2 Tissue processing and immunohistochemistry

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 paraffin blocks (Tissue-Tek TEC, Sakura, Tokyo, Japan). The paraffin blocks were cut into a 5 µm thick section using a microtome (Shandon, Anglia scientific instrument ltd., Cambridge, UK). Thereafter, the ovarian tissue sections were deparaffinized in xylene and rehydrated through decreasing graded ethanol dilutions. The tissue sections were placed in 0.01 M citrate buffer (pH 6.0) in a microwave oven at 750 watts for 15 min (3 times x 5 min) to retrieve antigenicity. Endogenous peroxidase was blocked using 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 rabbit polyclonal anti-ERβ (Santa Cruz Biotechnology, Inc., Texas, USA) as a primary antibody at a dilution of 1:100 at 4 °C overnight. After incubation with the primary antibody, the sections were incubated with biotinylated secondary antibody-horse anti-mouse anti-rabbit IgG (1:200, 30 min at RT, Vector Laboratories, Burlingame, CA, USA) and avidin-biotin-peroxidase complex (30 minutes at RT, Elite, Vectastain®, ABC kit, Vector Laboratories, Burlingame, CA, USA). Staining was developed with 3, 3'-diaminobenzidine tetrahydrochloride hydrate (3 min at RT, Peroxidase DAB, Vector Laboratories, Burlingame, CA, USA). The sections were counterstained with Mayer's hematoxylin for 30 sec, and mounted with mounting media. During the steps in the staining procedure, the slides were washed 3 times in phosphate-buffered saline (PBS) for 5 min each time. For the negative control, PBS was used instead of the primary antibody.

4.3.3 Determination of ERβ staining

The follicle was classified as antral follicle according to Phoophitphong et al. (2012). Antral follicle is a follicle having an oocyte and zona pellucida surrounded by multiple layers of granulosa cells with antral formation as shown in Figure 9. Antral follicles and corpora lutea from the ovarian tissue section of each gilt were randomly captured by a digital camera (QImaging®, BC, Canada) attached to light microscope (Olympus BX51, Olympus, Japan) at 200X magnification. In total, the ERβ immuno-staining was determined in antral follicles (n = 268) and CL (n = 160). To evaluate the proportion of immunoreaction area, the ERβ positive stained area were evaluated using image analysis software (Image-Pro® PLUS 6.0 Programming software, Media Cybernetic, Inc.). The percentage of ERβ positive area was calculated as: ERβ positive area (%) = (total ERβ positive area/total area in each microscopic field) x 100.

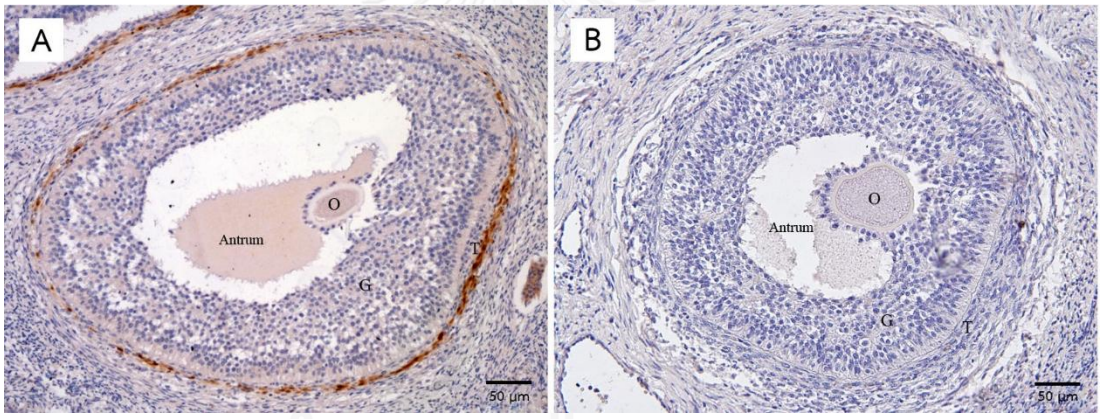

Figure 9. Localisation of ERβ immunoexpression in paraffin sections of gilt antral follicles. The antral follicle is the follicle with a central oocyte and zona pellucida surrounded by multiple layers of granulosa cells with antral formation. ERβ positive immunostained was found in theca cells of antral follicle (A). Negative control (B). Magnification 100X. G: granulosa cells layer; O: oocyte; T: theca cells layer.

4.3.4 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 error and the range of all the continuous data were calculated by the MEANS procedure. Data was presented as mean ± standard error of means (SEM). The correlation was analyzed by the CORR procedure (Pearson's Correlation Coefficients Procedure).

Normal distribution of dependent variable was tested by the UNIVARIATE procedure. The percentage of ERβ immunoexpression in antral follicles and CL was assigned as a dependent variable. The ovarian status (non-cyclic and cyclic) was included in the statistical model as an independent variable. The gilt's identity was included in the statistical model as a random effect. The percentage of ERβ immunostained area between ovarian status was compared by the NPAR1WAY procedure, using Wilcoxon's rank sum test. The level of statistically significant was set at *P* value less than 0.05.

4.4 Results

Descriptive data for the age, body weight and average daily gain of the gilts and ERβ immunoexpression are presented in Table 6. Of all the gilts, 30 gilts (52.5%) had cycling ovaries and 25 gilts (47.5%) had non-cyclic ovaries. All of the gilts with cycling ovaries had dominant CL and the ovulation rate was 16.7 ± 3.4 (ranged 9.0 - 23.0).

Table 6 Gilts' historical data and ERβ immunoexpression in the antral follicles of non-cyclic and cyclic gilts (least squares mean ± SEM)

Variables	Non-cyclic gilts (n = 25)	Cyclic gilts (n = 30)	*P* value
Age (day)	256.9 ± 0.8	280.0 ± 0.8	< 0.001
Body weight (kg)	142.0 ± 0.5	146.2 ± 0.4	< 0.001
Average daily gain (g/d)	547.9 ± 1.6	522.6 ± 1.5	< 0.001
ERβ immunoexpression (%)	17.5 ± 0.5	26.4 ± 0.5	< 0.001

The ERβ immunoexpression was clearly detected in the nucleus and cytoplasm of theca cells of antral follicles (Figures 9 and 10) and also in preantral follicles (Figure 10). Nevertheless, the immunolocalisation pattern for ERβ in the theca cell layer was dispersedly stained. In addition, the ERβ immunoexpression was also observed in the nucleus and cytoplasm of luteal cell of CL (Figure 11). The immunolocalisation pattern in the CL was tightly stained.

The average percentage of ERβ immunoexpression was detected at 22.3% (range 0 - 95.0%) of theca cells and 85.7% (range 2.8 - 100%) of luteal cells. The average percentage of ERβ immunoexpression in theca cell of antral follicles of non-cyclic gilts was lower than that in the cyclic gilts (17.5% and 26.4%, respectively, $P < 0.001$). Moreover, the average percentage of ERβ immunoexpression was positively correlated with body weight ($r = 0.22$, $P < 0.01$) and age ($r = 0.27$, $P < 0.01$) of the gilts.

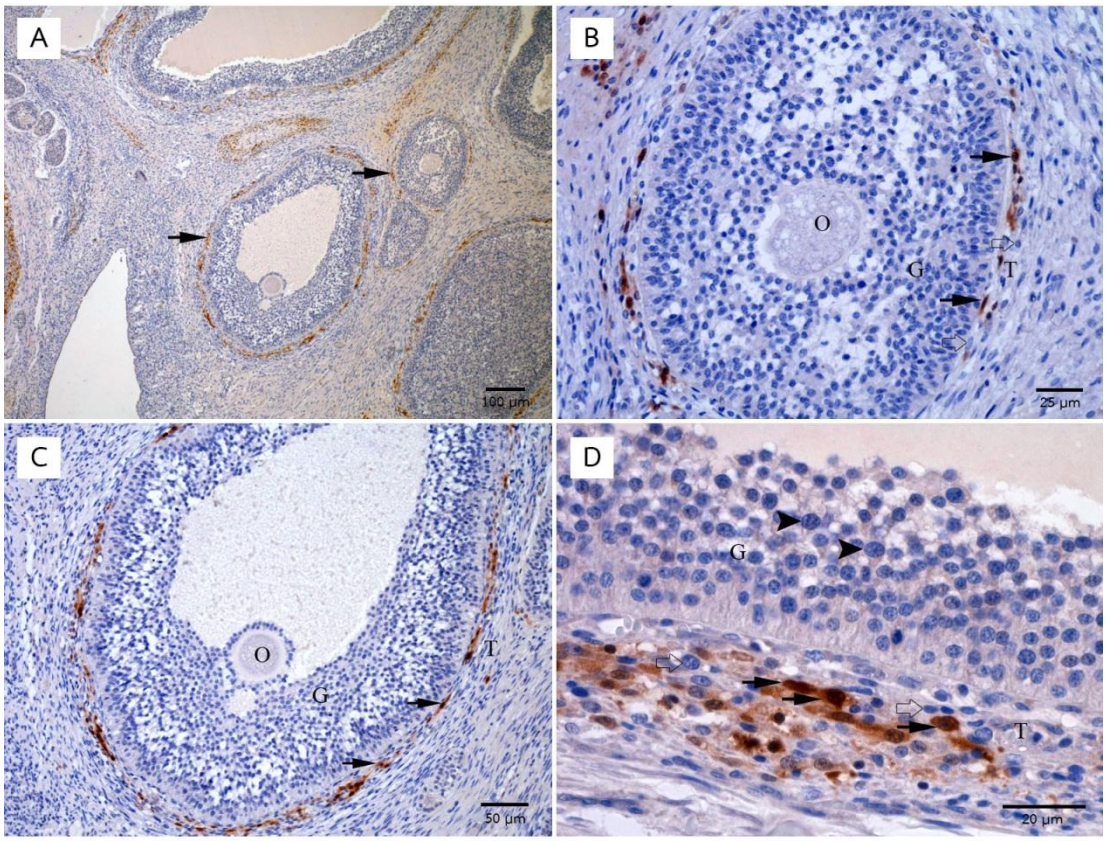

Figure 10. Localisation of ERβ immunoexpression in preantral (A and B) and antral (A and C) follicles. ERβ immunoexpression was found both in the nucleus and cytoplasm (arrow) of the theca cells (D). Negative immunoexpression in the theca cells (clear arrow) and granulosa cells (arrowhead) stained blue (D). Magnification 40X (A), 100X (B), 200X (C) and 400X (D). G: granulosa cells layer; O: oocyte; T: theca cells layer.

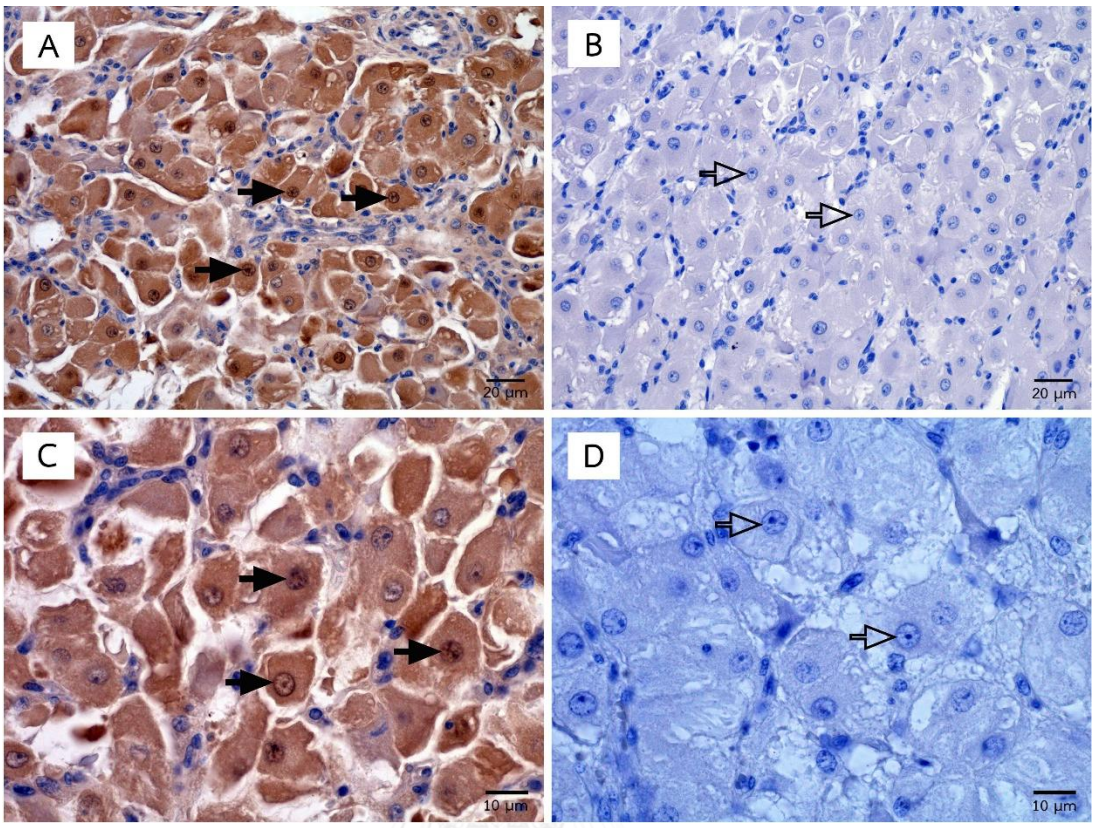

Figure 11. Localisation of ERβ immunoexpression in the corpora lutea of cyclic gilts. ERβ immunoexpression (black arrow) (A, C) and ERβ negative immunoexpression (clear arrow) (B, D) in luteal cells. ERβ positive immunoexpression was detected in the nucleus and cytoplasmic compartment of the luteal cell. Magnification 200X (A, B) and 400X (C, D).

4.5 Discussion

In the present study demonstrated the ERβ immunoexpression in the porcine ovaries. The immuno-staining for ERβ was shown mainly in the theca cell layer of ovarian follicles, while the staining in the granulosa layer was not notable. Moreover, immuno-staining of ERβ was also detected in luteal cells of CL. The difference of ERβ immunoexpression in ovarian follicles of non-cyclic and cyclic gilts was clearly observed. This indicated that antral follicles of non-cyclic gilts could response to oestradiol-17β in a lower degree than those of cyclic gilts. This may be the result from insufficient ERβ -mediated oestradiol reaction in the ovary to activate healthy preovulatory follicles. Comparing to the ERα immuno-staining, the ERβ is the dominant subtype of ER in the ovary, while ERα is more abundant in the uterus

(Pelletier et al., 2000; Slomczynska and Wozniak, 2001). Therefore, ERβ may regulate the ovarian activity during reproductive stage of gilts, and it should be the main subtype which involves in reproductive problem regarding the ovarian function such as inovulation or anoestrus.

The immunoexpression of ERβ detected in theca cell of gilts ovary is in agreement with the earlier studies in cyclic and pregnant porcine ovary using mouse monoclonal anti-human ERβ (Slomczynska and Wozniak, 2001; Knapczyk et al., 2008). In addition, ERβ mRNA was observed in both granulosa and theca layers of porcine antral follicles by *in situ* hybridization technique (Slomczynska et al., 2001). The difference in results may be due to the difference of laboratory technique. Even though the ERβ is observed in the ovaries, the differences of ERβ localisation among mammalian species are documented (Rosenfeld et al., 2001; Drummond and Fuller, 2010b). In bovine ovaries, ERβ immunoexpression was detected in granulosa and theca cells layers (Salvetti et al., 2007). However, the expression of this receptor in theca cells of antral follicles is low in human and absent in rat (Rosenfeld et al., 2001). To our knowledge, theca interna cells (Lautincik et al., 1994) and granulosa cells of fully grown follicles (Richards, 1994) are the major source of oestrogens that affect developing follicles by enhancing proliferation of granulosa cells and inducing the amount of gonadotropins receptors (Schams and Berisha, 2002). Ovarian theca cells are known as endocrine cells involved in steroidogenesis and closely related with developing follicles. Theca cells produce androgens and diffuse into granulosa cells where cytochrome P450 aromatase metabolized androgens to oestradiol (Magoffin, 2005; Tajima et al., 2007; Young and McNeilly, 2010).

The present study also observed the immuno-staining for ERβ in porcine luteal cells. This finding has been documented in the previous articles that porcine CL not only produce progesterone but also the oestradiol-17β (Slomczynska and Wozniak, 2001; Knapczyk et al., 2008). The CL is known as a temporary endocrine gland produces progesterone and also oestradiol-17β in domestic animals, which serves as an autocrine and/or paracrine regulator (Schams and Berisha, 2002). It is accepted that maternal recognition of pregnancy in swine signaling through oestrogen and other substances (Spencer and Bazer, 2004). In the CL of pregnant

swine, the intensity of ERβ immunoexpression continuously increased since the pregnancy occurred (Knapczyk et al., 2008). Thus, porcine CL might response via ERβ to control their function during gestation period. In addition, in *in vitro* study of young porcine CL, progesterone secretion is stimulated by oestradiol-17β (Pitzel et al., 1993). Therefore, porcine CL is served as a binding site of oestradiol-17β in order to regulate the physiological events during the reproductive cycle.

The data obtained from this study indicated the difference of ERβ immunoexpression between non-cyclic and cyclic gilts. The lower ERβ immunoexpression in non-cyclic gilts compared with cyclic gilts was clearly observed in antral follicles. This may suggest that non-cyclic ovary has inadequate ERβ to mediate oestradiol within antral follicles. Thus, ERβ is possibly involved in the ovarian follicular growth through oestrogen action. The function of steroids within the ovary has been described including oestrogen and its receptors (Rosenfeld et al., 2001; Drummond et al., 2002; Schams and Berisha, 2002; Couse et al., 2005). The ERβ acts within the follicles as a regulator to mediate oestrogen to granulosa cells, which were induced by Follicles stimulating hormone (FSH) to differentiate and develop to a preovulatory follicle and response to the gonadotropin stimulation (Couse et al., 2005). Moreover, there is an evidence in ERβ knockout mice that the follicle development was interrupted in which small ovaries, low ovulation rate and decreased offspring were also observed (Krege et al., 1998). For this reason, ERβ in porcine antral follicle is essential for follicular development and should be correlated with puberty attainment and the reproductive cycle.

The ERβ immunoexpression in the theca cell layer and luteal cell, as shown in the current study, suggesting the physiological reaction of oestradiol-17β with its receptor in these ovarian cells and corpus lutea among reproductive status of gilts. Furthermore, ERβ is therefore one of the major steroid receptor for intraovarian activity and physiological reproductive maturation.

CHAPTER V

Immunohistochemical localisation of luteinizing hormone receptor in the cyclic gilt ovary

5.1 Abstract

Luteinizing hormone receptor (LHR) is a specific membrane receptor that binds to luteinizing hormone (LH), resulting in androgen and progesterone production, and hence the regulation of LHR expression is necessary for follicle maturation, ovulation and corpus luteum formation. We examined the immunolocalisation of LHR in cyclic gilt ovaries. Ovaries were obtained from 21 gilts aged 326.0 ± 38.7 days and weighing 154.6 ± 15.7 kg. The tissues were incubated with rabbit anti-LHR polyclonal antibody. The follicles were categorized as primordial, primary, preantral and antral follicles. Ovarian phase was categorized as either follicular or luteal phases. The immunolocalisation of LHR was clearly expressed in primary, preantral and antral follicles. LHR immuno-staining was detected in the cytoplasm of granulosa, theca interna and luteal cells. LHR immuno-staining was evaluated using imaging software. LHR immuno-staining in the theca interna cells in antral follicles was almost twice as intense as that in preantral follicles (65.4% vs 38.3%, $P < 0.01$). LHR immuno-staining was higher in follicular phase than the luteal phase (58.6% vs 45.2%, $P < 0.05$). In conclusion, the expression of LHR in the theca interna cells of antral follicles in the follicular phase was higher than in the luteal phase. This indicates that LHR may impact follicular development from the primary follicle stage onwards.

5.2 Introduction

Development of the ovarian follicle during antral formation depends on the secretion of pituitary gonadotropins. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) play an essential role in this process by regulating the

responsiveness of granulosa and theca cells. Folliculogenesis and the regulation of this mechanism vary depending on the female pubertal status and physiological stage (Brussow et al., 2002; Ratky et al., 2005). During folliculogenesis, granulosa cells increase their responsiveness to FSH and LH, and thereafter, oestradiol production begins. As the ovarian follicle grows, granulosa cells continue to proliferate and differentiate until the preovulatory stage is reached (Robker and Richards, 1998). Selection of the ovulatory follicle is related to changes in gonadotropin dependence in the follicle from FSH to LH. Conversion to LH dependence is suggested by increasing expression of LH receptor (LHR) mRNA in both granulosa and theca interna cells during the first and third days of the follicular phase, becoming highest in large follicles, particularly on day 5 (Guthrie, 2005). LHR is a specific membrane receptor that binds to LH, resulting in androgen and progesterone production. Hence the regulation of LHR expression is necessary for follicle maturation, ovulation and corpus luteum formation (Takao et al., 1997; Liang et al., 2012). Studies have demonstrated the expression of LHR on granulosa cells of large antral follicles and theca cells of preantral and antral follicles in sheep (Eckery et al., 1997), bovine (Bao et al., 1997), porcine (Meduri et al., 1996), mouse (Wu et al., 2000) and human ovaries (Yung et al., 2014). Nevertheless, to our knowledge, no study has investigated LHR immunoexpression at different stages of the follicles (i.e., from primordial to preovulatory follicles) in porcine ovaries.

In general, the ovarian follicle develops in response to regulation by FSH and LH. However, most studies have concentrated on the role of FSH in the follicular development and final differentiation of granulosa cells at the antral and preovulatory stage. LH has also been suggested to act as a survival and differentiation factor throughout follicular maturation (Cortvrindt et al., 1998; Wu et al., 2000). Therefore, the distribution of LHR during follicular development should be investigated. The use of immunohistochemical staining will provide an insight into the regulation of ovarian follicular growth by LHR in gilts. The objective of the present study was to illustrate and evaluate the immuno-staining of LHR in different types of ovarian follicles during the follicular and luteal phases in cyclic gilts.

5.3 Materials and methods

5.3.1 Animals, data and sample collection

Ovarian tissues from 21 Landrace x Yorkshire crossbred gilts were obtained from slaughterhouses. Historical data about the gilts were collected from two commercial swine herds in Thailand, and included the herd and gilt identity, breed, age and body weight at culling. The reproductive organs were collected immediately after slaughter, placed on ice and transported to the laboratory within 24 h. The appearance of the ovaries was used to define ovarian status (i.e., follicular and luteal phases). The ovarian structures including corpora lutea (CL), corpora albicantia (CA) and follicles were defined according to a previous study (Tummaruk et al., 2009a). The ovary was defined as being in the 'follicular phase' when it contained follicles and in the 'luteal phase' when it contained CL or CA and small follicles.

5.3.2 Tissue processing and immunohistochemistry

The ovaries were fixed in 4% paraformaldehyde for 48 - 72 h, processed using 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). Thereafter, the ovarian tissue sections were deparaffinized in xylene and rehydrated through graded ethanol dilutions. The tissue sections were placed in 0.01 M citrate buffer (pH 6.0) in a microwave oven at 800 watts for 35 min (7 times x 5 min) to retrieve antigenicity. Endogenous peroxidase was blocked using freshly prepared 3% hydrogen peroxide (H_2O_2) at room temperature (RT) for 15 min. Nonspecific staining was blocked with normal goat serum (Vector Laboratories, Burlingame, CA, USA) at RT for 30 min. The slides were then incubated with rabbit polyclonal anti-LHR (sc 25828, Santa Cruz Biotechnology, Inc., Texas, USA) as a primary antibody at a dilution of 1:100 at 4°C overnight. After incubation with the primary antibody, the sections were incubated with biotinylated secondary antibody-goat anti-rabbit IgG at a dilution of 1:200 at RT for 30 min (Vector Laboratories, Burlingame, CA, USA) followed with avidin-biotin-peroxidase complex

for 30 min at RT (Elite®, Vectastain, ABC kit, Vector Laboratories, Burlingame, CA, USA). Staining was developed with 3, 3'-diaminobenzidine tetrahydrochloride hydrate for 3 min at RT (ImmPACT® DAB, Vector Laboratories, Burlingame, CA, USA). During staining, the slides were washed three times in phosphate-buffered saline (PBS) for 5 min each time. The sections were counterstained with Mayer's haematoxylin for 1 min, and mounted with mounting media. For the negative control, PBS was used instead of the primary antibody.

5.3.3 Evaluation LHR immunoreactivity

The follicles were divided into four categories: primordial, primary, preantral and antral follicles (Phoophitphong et al., 2012). To evaluate LHR immuno-staining, preantral and antral follicles were examined. A preantral follicle is a follicle that has an oocyte and visible zona pellucida surrounded by multiple layers of granulosa cells with no antral formation (Figure 12A). An antral follicle is a follicle that has an oocyte and zona pellucida surrounded by multiple layers of granulosa cells with antral formation (Figure 12B).

To determine the extent of LHR immuno-staining in the selected follicles, images of the stained tissue sections were captured by digital camera (MicroPublisher 5.0 RTV QImaging®, Surry, BC, Canada) attached to a light microscope (BX-50, Olympus, Tokyo, Japan). The immuno-staining of LHR in the granulosa and theca interna layers was evaluated using imaging software (Image-Pro® PLUS 6.0 Programming software, Media Cybernetics, Inc., USA). The LHR immuno-staining in each layer in a follicle was calculated as follows: LHR immuno-staining (%) = (the area of LHR-positive staining/the total area of the selected microscopic field) x 100. All preantral (n = 82) and antral follicles (n = 91) in all tissue sections were evaluated.

5.3.4 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 continuous data were calculated using the MEANS

procedure. The percentage area of LHR immuno-staining was presented as mean ± SD. Multiple analysis of variance (ANOVA) was used to analyse the percentage of LHR immuno-staining (%) using the general linear mixed model procedure of SAS. The statistical model included the fixed effect of follicle type (preantral and antral follicles), ovarian phase (follicular and luteal phases), and the interaction between follicle type and ovarian phase. The gilt's identity was included in the statistical model as a random effect. Least-square means were calculated and compared using the least significant difference test with Tukey-Kramer adjustment for multiple comparisons. $P < 0.05$ was regarded as statistically significant.

5.4 Results

Descriptive data on the age, body weight and average daily gain of the gilts and the LHR immuno-staining in the granulosa and theca interna layers are displayed in Table 7.

Table 7 Descriptive statistics for age, body weight, average daily gain of the gilts and luteinizing hormone receptor (LHR) immuno-staining in granulosa and theca interna layers.

Reproductive parameter	Number of observation	Means ± SD	Range
Age (days)	21	326.0 ± 38.7	284 - 481
Body weight (kg)	21	154.6 ± 15.7	131 - 203
Average dairy gain (g/day)	21	477.9 ± 54.7	358 - 628
LHR Immuno-staining (%)			
Granulosa layer	173	92.0 ± 16.0	17 - 100
Theca interna layer	173	48.1 ± 29.4	0.9 - 97.6

5.4.1 Immunohistochemical localisation of LHR

LHR immunoexpression was found in primordial, primary, preantral and antral follicles and in the luteal cells of the porcine ovary (Figure 13C-F), showing as a brown-pigmented precipitate located at the site of the antigen (Figure 13C-F). LHR immuno-staining was detected in the oocyte of primordial, primary, preantral and

antral follicles (Figure 13C-F). In the preantral and antral follicles, LHR immuno-staining was observed in the cytoplasmic compartment of the granulosa and theca interna cells (Figure 13E-F). LHR immuno-staining was also present in the cytoplasmic compartment of the luteal cells in the corpus luteum (Figure 13G-H).

5.4.2 Evaluation of LHR immunoreactivity

LHR immuno-staining differed between regions of the preantral and antral follicles (Figure 12). The amount of LHR immuno-staining in the granulosa layer was similar in antral and preantral (92.7% vs 92.0%, $P > 0.05$). However, in the theca interna layer, the amount of immuno-staining was higher in antral follicles than in preantral follicles (65.4% vs 38.3%, $P < 0.01$).

With regards the follicular and luteal phases (Figure 14), the amount of LHR immuno-staining in the granulosa layer of the follicular phase was similar to that in the luteal phase (92.8% vs 91.9%, $P > 0.05$). However, immuno-staining in the theca interna layer was higher in the follicular phase than in the luteal phase (58.6% vs 45.2%, $P < 0.05$).

Multiple comparisons of LHR immuno-staining in the granulosa and theca interna layers according to the follicular and luteal phases are presented in Table 7. As can be seen from the table, LHR immuno-staining in the granulosa layer did not differ significantly between phases and types of follicle. However, variations in the immuno-staining were detected in the theca interna layer. The highest LHR immuno-staining in the theca interna layer was observed in the antral follicles during the follicular phase (Table 7), with higher LHR immuno-staining in the antral follicles during the follicular phase than during the luteal phase (79.7% vs 51.2%, $P < 0.05$). However, there was no difference in LHR immuno-staining between the follicular phase and luteal phase in preantral follicles (Table 8).

Table 8 Luteinizing hormone receptor (LHR) immuno-staining in the granulosa and theca interna layers by follicle type and ovarian phase.

Ovarian phase	Follicle type	Number of follicle	LHR immuno-staining (%)	
			Granulosa layer	Theca interna layer
Follicular	Preantral	15	90.5±4.1[a]	37.4±7.0[bc]
	Antral	17	95.2±3.9[a]	79.7±6.6[a]
Luteal	Preantral	67	93.2±1.9[a]	39.2±3.3[c]
	Antral	74	90.2±1.8[a]	51.2±3.1[b]

[a,b,c] Different superscripts within column indicate significant difference ($P < 0.05$)

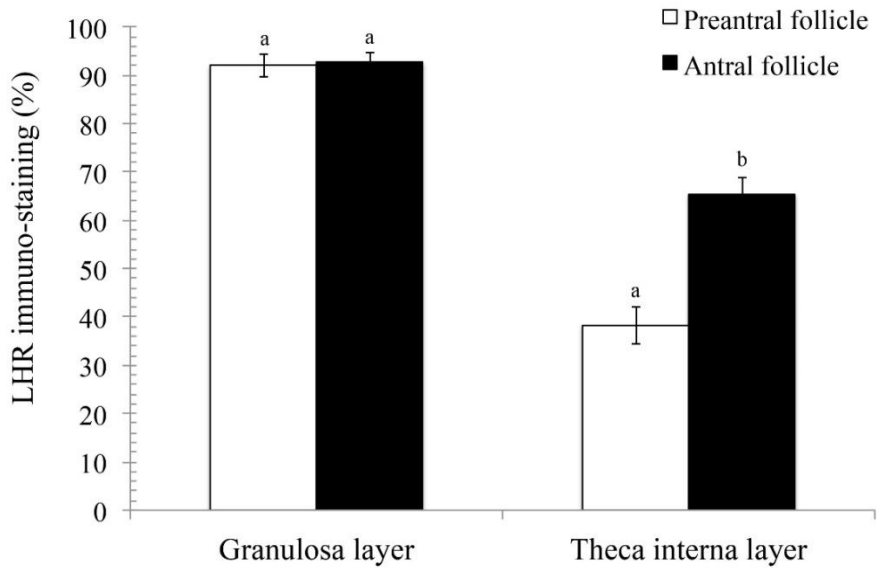

Figure 12. LHR immuno-staining in granulosa and theca interna layers of preantral (white bar) and antral (black bar) follicles. Different letters indicate significant difference ($P < 0.05$).

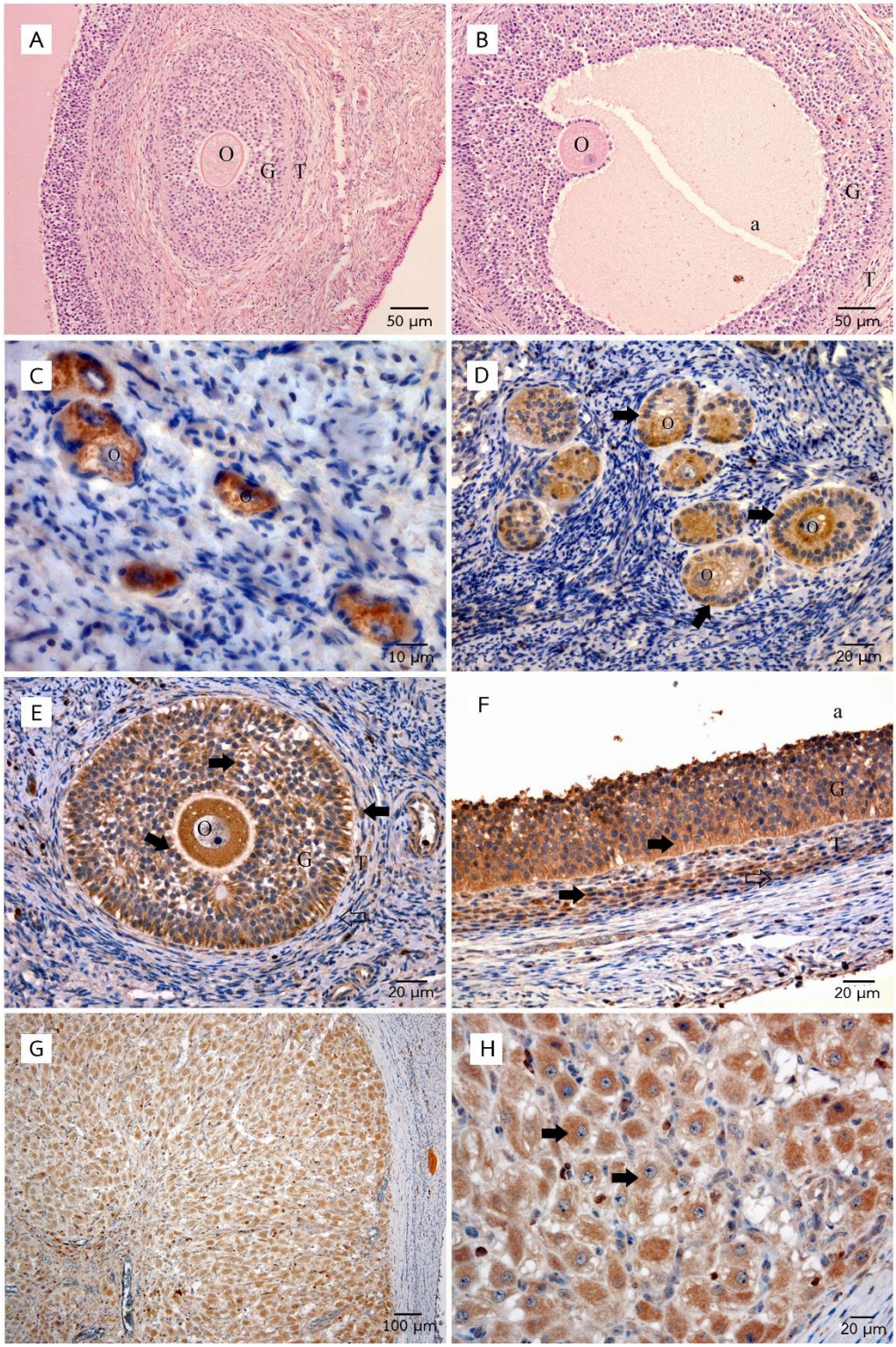

Figure 13. (A) Preantral follicle (i.e., an oocyte surrounded by multiple layers of granulosa cells without antrum formation); (B) Antral follicle (i.e., an oocyte encircled with multiple layers of granulosa cells and antral formation); (C) Luteinizing hormone receptor (LHR) immuno-staining during follicle development; primordial follicles; (D) LHR immuno-

staining in primary follicles; (E) LHR immuno-staining in preantral follicles; (F) LHR immuno-staining in antral follicles; LHR immuno-staining in cytoplasm of granulosa and theca interna cells of preantral and antral follicles (positive; black arrow, negative; clear arrow); (G-H) LHR immuno-staining in the cytoplasm of luteal cells in the corpus luteum (a: antrum, G: granulosa layer, O: oocyte, T: theca interna layer). Magnification (A, B) 100X; (D, E, F) 200X; (C, H) 400X.

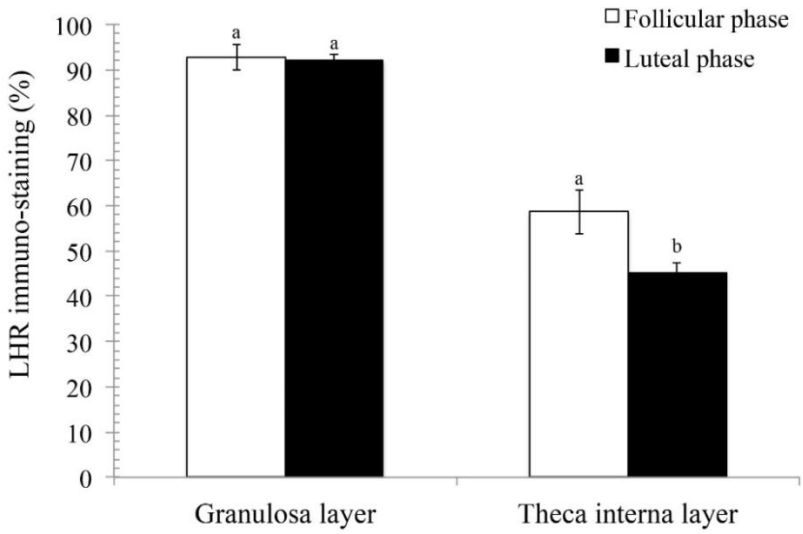

Figure 14. LHR immuno-staining in granulosa and theca interna layers in follicular (white bar) and luteal (black bar) phases. Different letters indicate significant difference ($P < 0.05$).

5.5 Discussion

LHR is a membrane receptor located on the surface of theca and granulosa cells and is involved in follicle development, ovulation and corpus luteum formation (Edson et al., 2009; Yung et al., 2014). LHR localisation was previously demonstrated in the porcine ovary using monoclonal antibodies (Meduri et al., 1992) and autoradiography (Gebarowska et al., 1997). However, the results of these studies are incompatible with our results. Meduri et al. (1992) found that LHR was not labeled on the primordial and primary follicle. In the present study, LHR immuno-staining was detected in the oocyte of all follicle types and was found in the granulosa cells of primary, preantral and antral follicles. However, LHR immuno-staining in the theca interna cells was detected in only preantral and antral follicles. The granulosa cells

of large antral and preovulatory follicles also exhibited LHR immuno-staining (Gebarowska et al., 1997). This was in accordance with our finding that the LHR was clearly stained in the granulosa and theca interna layers of antral follicles. The research materials and laboratory methods used in the present study may explain the differences in results. The present study demonstrated that the heat-induced epitope retrieval technique is an effective protocol for enhancing LHR immuno-staining in formalin-fixed and paraffin-embedded porcine ovaries. A similar technique has also been used to detect proliferating cell nuclear antigen (PCNA) (Phoophitphong et al., 2012). Thus, the LHR immunohistochemical labeling technique was further developed in this study and could be used for other types of antigen in porcine ovarian tissue.

In this study, LHR immunoreactivity was located in the cytoplasm of granulosa and theca interna cells of preantral and antral follicles. During follicle maturation, antral follicles need LHR in order to respond to the LH surge (Schwarz et al., 2008). An earlier study demonstrated that LHR immuno-staining was found in granulosa and theca cell layers in human preantral follicles (Yung et al., 2014). This indicates that LHR synthesis is initiated in preantral follilcles and is required for further growth of antral follicles.

Moreover, our results revealed high levels of LHR immuno-staining in the granulosa and theca interna layers of antral follicles during the follicular phase. This might be because the developing antral follicles need LHR to enhance their response to the LH surge during the follicular phase (Yung et al., 2014). In mouse follicles, functional LHRs are present in the theca layer even though the preantral follicle may not need LH for this follicular stage (Magoffin et al., 1994). This immunohistochemical study thus confirmed that the LHR is important for follicle development from preantral stage and that it increases in number during the antral stage. The presence of LHR and FSHR in the oviduct and uterus of prepubertal gilts has also been reported (Bukovsky et al., 2003; Liang et al., 2012).

In the present study, the high levels of LHR immuno-staining during the follicular phase suggest that the follicles may respond to the circulating level of LH secretion. During the follicular phase, gonadotropin dependence was switches from

FSH to LH dependence. This switch is associated with increasing LHR and decreasing FSH mRNA expression (Guthrie, 2005). In addition, preovulatory maturation is correlated with the expression of steroidogenic enzymes that are associated with an increase in LHR mRNA in both granulosa and theca interna cells (Guthrie, 2005). A study in LHR knock-out mice revealed that LHR is associated with steroidogenic enzymes and ovulatory mechanisms (Burns and Matzuk, 2002).

In the present study, LHR immunoreactivity was found in the theca interna cells of preantral follicles and was highest in antral follicles. Theca cells are first recognized when a follicle has more than two layers of granulosa cell and are the site of androgen production under the regulation of LH (Magoffin and Weitsman, 1994). Moreover, LHR mRNA is detectable when theca interna cells form over the granulosa cells (Bao and Garverick, 1998). Thus, we suggested that theca cells of preantral follicle raise and initiate to differentiate, produce LHR, steroidogenic enzymes and some androgens. As the follicle develops to the antral stage, theca cells mature and turn into steroidogenic cells by increasing androgen production (Young and McNeilly, 2010).

LHR immuno-staining was also expressed in the luteal cells of the CL, as in previous studies in pig (Gebarowska et al., 1997), human (Yung et al., 2014) and rat ovaries (Bukovsky et al., 2003). This indicates the association between LHR and CL formation in porcine ovaries. In the CL, the LHR immunoreaction was detected in the cytoplasm of luteal cells. LH is thought to activate progesterone production by granulosa cells and is also a luteotrophic factor for CL formation after ovulation and CL promotion during pregnancy (Takao et al., 1997). In rat and bovine CL (using monoclonal antibody and ^{125}I-hCG binding, respectively), LHR immuno-staining was also detected in the luteal cells (Chengini et al., 1991; Bukovsky et al., 1993). These findings indicate that luteal cells are one of the targets of LH signaling.

In conclusion, LHR is expressed in the granulosa cells of primary follicles and both granulosa and theca interna cells of preantral and antral follicles. LHR immuno-staining was detected in both the follicular and luteal phases of the porcine ovary. The presence of LHR on granulosa and theca interna cells of the follicles indicated that LH is closely associated with follicle development in porcine ovaries.

CHAPTER VI

GENERAL DISCUSSION

This thesis demonstrated the quantification and density of ovarian follicles using proliferative marker and evaluating follicular apoptosis in cyclic and non-cyclic gilts. Moreover, the immunolocalisation of ERβ receptor and LHR in the ovarian follicles associated with reproductive status was demonstrated.

6.1 Proliferation and apoptosis of ovarian follicle

During the folliculogenesis, ovarian follicular cells, i.e., granulosa and theca cells proliferate and differentiate under the influence of the steroid hormones and intraovarian growth factors (Young and McNeilly, 2010). These factors are known as a regulator of follicle maturation and survival. The number of each type of the ovarian follicles reflects the ovarian functions. In Chapter II, we demonstrated that the density of primary follicles was associated with body weight and age at first observed oestrus in gilts. We also established the immunohistochemical procedure to evaluate the number of each type of follicles in the ovarian tissue sections. We demonstrated that the PCNA can be applied to quantify the number of follicles and to identify follicle types in the porcine ovary. PCNA is a useful marker for precisely quantifies the number of ovarian follicle because of the ability to distinguish the oocyte nuclei as well as proliferating cells (e.g., granulosa cells) from the ovarian stroma via PCNA antibody. This enhances the speed and accuracy of follicle determination. This finding is in agreement with the previous studies in rat (Muskhelishvili et al., 2005) and pig (Tomanek and Chronowska, 2006) indicating that PCNA is a helpful marker for ovarian follicle determination. Nevertheless, in the previous study in the porcine ovaries (Tomanek and Chronowska, 2006), no remarkable PCNA staining of primordial follicles has been demonstrated yet. Therefore, the heat-induced epitope retrieval (HIER) technique is implemented (Chapter II). In our protocol, the pig ovarian tissue is pretreated with HIER technique using 0.01 M citrate buffer, pH 6.0 in a microwave

oven at 600 watt. The oocyte nuclei and either the pre-granulosa and the granulosa cells are clearly distinguished from the ovarian stroma. Previously, hematoxylin and eosin (H&E) staining is a conventionally method used to quantify the number of ovarian follicles in the tissue sections. However, H&E staining cannot distinguish proliferating cells and the other remaining tissues, hence the small follicles are hardly seen and cannot be precisely determined (Bolon et al., 1997; Bucci et al., 1997). Both the oocyte and the granulosa cell are highly proliferated and a rather high density of follicles is found in the porcine ovarian tissue. The PCNA immunohistological labelling is therefore enhanced the visibility of small follicles (Chapter II). This novel technique is therefore recommended for further investigation on the porcine ovaries.

In general, more than 99% of follicles undergo degeneration or atresia during follicular growth and development because of granulosa cell apoptosis (Matsuda-Minehata et al., 2006). In Chapter III, we demonstrated that granulosa and theca cells apoptosis occurred in both preantral and antral follicles. In addition, the higher proportion of apoptotic cells in preantral follicles compared to antral follicles maybe due to the fact that preantral follicles get improper gonadotropins and receive inadequate survival factors to regulate maturation processes (Hu et al., 2004). Granulosa cells are responsible for apoptosis when the granulosa cells differentiation occurred and initially sustain apoptosis (Nakayama et al., 2000; Matsuda-Minehata et al., 2006). Granulosa cells morphological changes occurred during apoptosis, including DNA fragmentation, can be detected by TUNEL (Durlej et al., 2012). Moreover, the positive correlation of apoptotic cell expression between granulosa and theca cell layers is also observed, indicating intercellular communication among these cells. In preantral follicle, granulosa and theca cells are interacting which is known as the two-cell, two gonadotropin theory (Hillier et al., 1994; Magoffin, 2005). Moreover, the large proportion of follicular apoptotic cells in non-cyclic gilts compared to cyclic gilts is observed. This may be related to the different of ovarian steroid hormone, especially oestradiol, which are affects granulosa and theca cell differentiation (Evans and O'Doherty, 2001).

6.2 Oestrogen receptor β and LHR in ovarian follicles

Both ERβ and LHR are detected in preantral and antral follicles in different compartments of the porcine ovarian tissues. Oestrogen receptor β is a predominant subtype of oestrogen receptor in ovarian tissues (Slomczynska et al., 2001). ERβ plays an essential role in the follicle maturation and oestrogen responsiveness. Therefore, ERβ may regulate the ovarian activity during reproductive stage in gilts, and it is the main subtype which involves in reproductive problem regarding the ovarian function, such as ovulation failure and anoestrus. LHR is a necessary receptor for follicle development, ovulation and corpus luteum formation (Liang et al., 2012). LHR immunoexpression has been demonstrated in the porcine ovary using monoclonal antibodies (Meduri et al., 1992) and autoradiography (Gebarowska et al., 1997). However, the results of these studies are incompatible with our results (Chapter V). Meduri et al. (1992) revealed that LHR was not detected in primordial and primary follicle. However, we could demonstrate the LHR immuno-staining in the oocyte and the follicles including the primordial and primary follicles (Chapter V).

In Chapter IV, the immuno-staining of ERβ is detected in the theca interna cell layer of antral follicles. This indicated that theca cell is the source of oestrogen, which promote folliculogenesis and enhances granulosa cell proliferation (Schams and Berisha, 2002; Magoffin, 2005). However, ERβ mRNA is observed in both granulosa and theca layers of the porcine antral follicles by *in situ* hybridization technique (Slomczynska et al., 2001). Theca interna cells and granulosa cells of fully grown follicles are the main source of oestrogen that affect developing follicles by enhancing proliferation of granulosa cells and enhancing the expression of gonadotropins receptors (Schams and Berisha, 2002). Ovarian theca cells are known as endocrine cells involved in steroidogenesis by producing androgens and diffuse into granulosa cells where cytochrome P450 aromatase metabolized androgens to oestradiol (Magoffin, 2005; Tajima et al., 2007; Young and McNeilly, 2010). Thus, theca cells are closely related with developing follicles. We could clearly demonstrate that the expression of ERβ is most prominent in the theca cells rather than the granulosa cells in the porcine follicles.

The LHR immuno-staining is detected in the oocyte in all follicle types and is found in the granulosa cells of primary, preantral and antral follicles (Chapter V). The LHR immuno-staining is detected in the granulosa cells of large antral and preovulatory follicles (Gebarowska et al., 1997). In accordance with Chapter V, the immuno-staining of LHR is found in both granulosa and theca interna cells layers of preantral and antral follicles. Moreover, the greater immuno-staining of LHR in antral follicle than preantral follicle may be due to the fact that the developing antral follicle requires LHR to enhance the LH surge (Yung et al., 2014). Infertility and delayed puberty have been reported in human who lacks of LHR function (Misrahi et al., 1997). These findings indicated that these two hormone receptors involved in ovarian follicular growth, especially in antral follicles.

Corpus luteum (CL) is known as a temporary endocrine gland produces progesterone and also oestradiol-17β in domestic animals, which serves as an autocrine and/or paracrine regulator (Schams and Berisha, 2002). In the CL, we observe the immunoreactivity of ERβ (Chapter IV) and LHR (Chapter V) in luteal cells. Thus, the porcine CL may regulate their functions via ERβ and LHR. LH is also a luteotrophic factor for CL formation after ovulation and during pregnancy (Takao et al., 1997) and oestrogen stimulates progesterone production in the luteal cells of young porcine CL (Pitzel et al., 1993). Moreover in the ERβ knockout mice, low number of pups and litters is detected (Krege et al., 1998). Therefore, it has been accepted that the CL serves as an autocrine and/or paracrine regulators mediated through ERβ and LHR as suggested previously by Schams and Berisha (2002) and Gebarowska et al. (1997), respectively.

6.3 Difference between non-cyclic and cyclic gilts

Reproductive problems are commonly found in replacement gilts raised under tropical climates. Major problems led to culling of replacement gilts under field condition are “anestrus” and “delayed puberty”. In most cases, the ovaries of those gilts do not cyclic (Tummaruk et al., 2009a) even though their age should attain puberty and express fertile estrus. In Chapter III, non-cyclic gilts had more

follicular apoptotic cells than those cyclic gilts in both granulosa (31.7% vs 22.6%) and theca cell layers (34.8% vs 20.3%). This indicated that ovarian follicle of non-cyclic gilts is more susceptible to apoptosis than cyclic gilts. Therefore, delayed puberty of the gilts might be partly explained by a high proportion of apoptotic follicles and subsequently cause suboptimal growth of the follicles and lack of ovarian steroid production for initiating oestrus behaviour. In fact, follicular development during prepubertal period is regulated by intraovarian factors and changes of steroid hormone, especially oestradiol (Yu et al., 2004). Moreover, the low oestradiol level throughout prepubertal period compared with pubertal gilts has been reported (Evans and O'Doherty, 2001).

In Chapter IV, the difference of ERβ immuno-staining in antral follicles between non-cyclic (17.5%) and cyclic gilts (26.4%) was observed. This may be the result of antral follicles of non-cyclic gilts response to oestradiol-17β lower than those of cyclic gilts. Additionally, there is an insufficient ERβ-mediated oestradiol reaction in the ovary of non-cyclic gilts. Thus, ERβ is possibly involved in the ovarian follicular growth through oestrogen action. The function of steroids within the ovary has been described including oestrogen and its receptors (Rosenfeld et al., 2001; Drummond et al., 2002; Schams and Berisha, 2002; Couse et al., 2005). According to the evidence in ERβ knockout mice, ERβ regulates follicle development and ovulation rate (Krege et al., 1998). Therefore, it is suggested that the low ERβ in non-cyclic gilts involve with the less capacity of follicle development via ERβ expression. These might subsequently cause suboptimal follicular growth and reduced ovarian steroid production consequently affectd oestrous behaviour and puberty attainment.

6.4 Conclusions

Non-cyclic gilt remains a major cause of reproductive dysfunction and affects the economic loss in commercial swine herds in Thailand. Maturation of ovarian follicle requires cell proliferation and differentiation of both granulosa and theca cells and needs hormone receptors in order to response this process as

demonstrated in this thesis, i.e., proliferation, apoptosis, ERβ and LHR. Base on the information in this thesis, it can be concluded that:

- The PCNA can be used to distinguish follicle types, quantify the follicular number and calculate follicle density in the gilt ovarian tissue which is associated with reproductive performance.
- Gilts with a heavy body weight and early age at first observed oestrus have a high density of primary follicles in the ovarian tissue.
- Apoptosis is observed in both granulosa and theca cell layers of the porcine ovarian tissue.
- Apoptosis is detected in the ovarian tissue of non-cyclic gilts more than cyclic gilts.
- The lower ERβ found in non-cyclic gilts compare with cyclic gilts indicated that ERβ is a factor affecting the follicle maturation.
- High apoptosis and inadequate ERβ may cause abnormal folliculogenesis, which result in anoestrus, poor oestrus symptoms and/or delated puberty in gilts.
- LHR express in both preantral and antral follicles particularly in the theca cell of antral follicles during follicular phase.

6.5 Research limitations

In Chapter II, even though the PCNA can be used to detect proliferating cells but DNA repair can also be detected by this antibody. Therefore, using PCNA alone to determine cells proliferation should be interpret with caution. Nevertheless, the present study aimed to use PCNA to distinguish different types of follicles in the ovarian tissue only. The proliferation score based PCNA immuno-staining has not been determined yet. In addition, in the present study, the precised days of oestrus cycle could not be determined. The reproductive phase is classified into three major groups: inactive, follicular and luteal phase. In Chapter III and V, the number of ovaries in non-cyclic gilts and during follicular phase was lower than cyclic gilts and those in luteal phase. With the less number of gilts included in the experiment, the

power of the test maybe reduced. Thus, significant results may not be obtained. In Chapter IV, no serum oestradiol-17β evaluation in non-cyclic and cyclic gilts could be demonstrated. Therefore, the interpretation was made base on only the immunolocalisation to the ERβ in the ovarian tissue of gilts.

6.6 Suggestions for further investigations

Another proliferative marker, such as Ki-67, should be considered as an alternative marker to evaluate the degree of cells proliferation. Parallel with the LHR, FSH receptor in the ovarian follicle is also taking an important part of follicular maturation. Therefore, the evaluation of FSH receptor immuno-staining may explain the consequence of gonadotropins dependence from the preantral to antral transformation. Furthermore, growth factors related with follicle maturation, e.g., growth differentiation factor-9 (GDF-9), IGF-1 and transforming growth factor-β (TGF-β) should be studied by others laboratory experiment, e.g. enzyme-like immuno-assay (ELISA), polymerase chain reaction (PCR) and western blot in order to provide more details about hormone levels and the presence of follicle growth factors between non-cyclic and cyclic gilts. These could increase knowledge on many mechanisms behind the follicular growth in both cyclic and non-cyclic gilts. Therefore, understanding insight of follicle development and hormone function within the porcine ovaries should be further investigated.

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APPENDIX

List of publications and conferences

1. **Phoophitphong, D.**, Wangnaitham, S., Srisuwatanasagul, S., Tummaruk, P. 2012. The use of proliferating cell nuclear antigen (PCNA) immuno-staining technique to determine number and type of follicles in the gilt ovary. **Livestock Science.** 150; 425-431.
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 detection. **Comparative Clinical Pathology.** 24; 1385-1394. DOI 10.1007/s00580-015-2087-y.
3. 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. DOI 10.1007/s00580-014-2006-7.
4. Tummaruk, P., Boonwong, N., Chumthong, W., Saengthong, W., Jiraphan, S., **Phoophitphong, D.**, Srisuwatanasagul, S. Expression of oestrogen receptor **α** in the endometrium of porcine reproductive and respiratory syndrome virus infected gilts. **Comparative Clinical Pathology**. DOI 10.1007/s00580-016-2226-0.
5. **Phoophitphong, D**., Srisuwatagasagul, S., Koonjaenak, S., Tummaruk, P. 2016. Apoptotic cell localisation of preantral and antral follicles in relation to non-cyclic and cyclic gilts. **Reproduction in Domestic Animals**. DOI 10.1111/rda.12693.
6. **Phoophitphong, D**., Wangnaitham, S., Srisuwatanasagul, S., Tummaruk, P. 2011. Quantifying number of follicles within the pig ovary by proliferating cell nuclear antigen (PCNA) immuno-staining method. Proceeding of the 1st **Symposium of the Thai Society for Animal Reproduction** (TSAR). Bangkok, Thailand.
7. **Phoophitphong, D**., Olanratmanee, E., Srisuwatanasagul, S, Wangnaitham, R., 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. Proceeding of the 22nd **International Pig Veterinary Society** (IPVS) Congress. Jeju, South Korea.
8. **Phoophitphong, D**., Srisuwatanasagul, S., Tummaruk, P. 2012. The asoociation between number of follicles and body weight of the gilts. Proceeding of the 11th **Chulalongkorn University Veterinary Annual Conference** (CUVC). Bangkok, Thailand.

9. **Phoophitphong, D.**, Srisuwatanasagul, S., Tummaruk, P. 2012. The asoociation between number of follicles and body weight of the gilts. Proceeding of the 87th **Royal Golden Jubilee** (RGJ) Seminar Series. Bangkok, Thailand.
10. **Phoophitphong, D.**, Srisuwatanasagul, S., Tummaruk, P. 2013. Estrogen receptor β in normal and cystic ovary in gilts. Proceeding of the 15th **RGJ-Ph.D. Congress**. Pattaya, Thailand.
11. **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. Proceeding of the 51st **Kasetsart University Annual Conference.** Bangkok, Thailand.
12. **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. Proceeding of the 38th **International Conference on Veterinary Science** (ICVS). Bangkok, Thailand.
13. **Phoophitphong, D.**, Olanratmanee, E., Tummaruk, P. 2013. Factors influencing weaning-to-first-service interval in tropical sows. Proceeding of the 17th **European Society for Domestic Animal Reproduction Conference** (ESDAR). Bologna, Italy.
14. Boonwong, N., Chumtong, W., Saengthong, W., Jiraphan, S., **Phoophitphong, D.,** Srisuwatanasagul, S., Tummaruk, P. 2014. Expression of estrogen receptor alpha in the endometrium of gilts infected with porcine reproductive and respiratory syndrome virus. Proceeding of the 2nd **Symposium of the Thai Society for Animal Reproduction** (TSAR). Bangkok, Thailand.
15. 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. Proceeding of the 2nd **Symposium of the Thai Society for Animal Reproduction** (TSAR). Bangkok, Thailand**.**
16. **Phoophitphong, D.**, Srisuwatanasagul, S., Tummaruk, P. 2014. Localization of estrogen receptor β in the porcine ovary. Proceeding of the 2nd **Symposium of the Thai Society for Animal Reproduction** (TSAR). Bangkok, Thailand. (Poster Award)
17. Tummaruk, P., Phuansuriya, K., Amornchaisuwan, T., Sungsin, P., Wijitchot, W., **Phoophitphong, D.**, Knox, R.V. 2016. Proceeding of the 3rd **Symposium of the Thai Society for Animal Reproduction** (TSAR). Bangkok, Thailand.
18. Tummaruk, P., **Phoophitphong, D.,** Knox, R.V. 2016. Serum IGF-1 concentration is significantly associated with the response of gilts to estrus induction using gonadotropin. Proceeding of the 3rd **Symposium of the Thai Society for Animal Reproduction** (TSAR).

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19. **Phoophitphong, D** and Tummaruk, P. 2016. Follicle development, ovulation and evidence of silent heat in gilts after estrus induction using gonadotropin under tropical climate. Proceeding of the 24th **International Pig Veterinary Society Congress** (IPVS). Dublin, Ireland.
20. **Phoophitphong, D** and Tummaruk, P. 2016. Serum estradiol-17β concentration responses after PG600® injection in gilts. Proceeding of the 24th **International Pig Veterinary Society Congress** (IPVS). Dublin, Ireland.
21. **Phoophitphong, D.**, Srisuwatanasagul, S., Tummaruk, P. 2016. Leptin immune-staining in the porcine ovarian follicles. Proceedings of the 15th **Chulalongkorn University Veterinary Annual Conference** (CUVC). Bangkok, Thailand.
22. Phunansuriya, K., Amornchaisuwan, T., Sungsin, P., Wijitchot, W., **Phoophitphong, D.**, Tummaruk, P. 2016. Post-mortem examination on the reproductive organs of gilts on Day 3 and 7 after estrus induction by using gonadotropin in a tropical climate. Proceeding of the 15th **Chulalongkorn University Veterinary Annual Conference** (CUVC). Bangkok, Thailand.
23. **Phoophitphong, D.**, Srisuwatanasagul, S., Tummaruk, P. 2016. The luteinizing hormone receptor immunohistochemical staining in the cyclic gilt ovary. The 18th **International Congress of Animal Reproduction** (ICAR). Tour, France.

VITA

Miss Duangkamol Phoophitphong was born on March 8th, 1982 in Songkhla, Thailand. She received the Bachelor's degree (Doctor of Veterinary Medicine, D.V.M., 2nd class Honors) from the Faculty of Veterinary Medicine, Kasetsart University, Thailand in 2007. She has worked as a lecturer at the Faculty of Veterinary Technology, Kasetsart University for 4 years. She was responsible to the anatomy and histology lessons. Since 2011, she was permitted from her original affiliation to enter further education. She enlisted a Ph.D program in Theriogenology at the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University. She earned a scholarship from the Royal Golden Jubilee (RGJ) Ph.D Program, Thailand Research Fund and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund). All through the Ph.D program, she had 5 international publications and 18 proceedings.