Original Article

Morphological Changes and Infiltration of Immune Cells in the Endometrium of Anoestrus Gilt in Relation to the Ovarian Appearance and Serum Progesterone

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Abstract

The present study investigates morphological changes and distribution of the leukocyte subpopulation in the endometrium of anoestrus gilts in relation to reproductive cycles and serum progesterone (P_4). Selected genital organs from 30 gilts culled due to anoestrus were examined. The genital organs were classified according to the ovarian appearance into 3 groups, i.e. inactive (n = 10); follicular (n = 10); and luteal phase (n = 10). Blood samples were collected prior to slaughter to determine serum P_4. Seven tissue samples were randomly collected from the uteri of the gilts and were examined for histological structures, i.e. epithelial types and height, number of blood vessel, secretory vesicle and endometrial glands. Number of leukocyte subsets, i.e. lymphocytes, neutrophils, eosinophils, macrophages and plasma cells were counted. On average, age and body weight at culling of the gilts were 306.4±39.9 d (range 233-407 d) and 150.4±24.8 kg (range 104.0-205.5 kg). Lymphocyte was the most common immune cell in all tissue layers and in all stages of the reproductive cycle. Lymphocytes in glandular layer in the inactive phase was higher than in the follicular (p=0.02) and luteal phases (p=0.05). Neutrophils in both epithelial and subepithelial layers in follicular phases was higher than luteal and inactive phases (p<0.001). Eosinophil in subepithelium in the luteal phase was higher than inactive (p=0.004) and follicular phases (p<0.001). An increase in the serum P_4 resulted in an increase number of uterine glands (p<0.001), a decrease number of lymphocytes in all tissue layers (p<0.05), a decrease number of neutrophils in subepithelial layers (p=0.03) and an increase in the number of eosinophils in subepithelial layers (p<0.001). In conclusion, the infiltration of the leukocyte subpopulation in the endometrium of anoestrus gilts is largely dependent on the ovarian function. Neutrophils and eosinophils were common immune cells in follicular and luteal phases, respectively.

Keywords: anoestrus, endometrium, gilt, immune cell, progesterone

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บทคัดย่อ

การเปลี่ยนแปลงทางสัณฐานวิทยา และการสะสมของเซลล์ระบบภูมิคุ้มกันในเยื่อบุโพรงมดลูกสุกรสาวที่ไม่เป็นสัดสัมพันธ์กับลักษณะของรังไข่ และฮอร์โมนโปรเจสเตอโรนในซีรั่ม)

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การศึกษาครั้งนี้ ทำการตรวจการเปลี่ยนแปลงทางสัณฐานวิทยาและการกระจายตัวของเซลล์เม็ดเลือดขาวในเยื่อบุโพรงมดลูกสุกรสาวที่ไม่แสดงอาการเป็นสัดสัมพันธ์กับวงรอบระบบสืบพันธุ์ และปริมาณฮอร์โมนโปรเจสเตอโรนในซีรั่ม (P_4) อวัยวะสืบพันธุ์ถูกคัดเลือกจากสุกรสาวจำนวน 30 ตัว ซึ่งถูกคัดทิ้งเนื่องจากสาเหตุไม่แสดงอาการเป็นสัด ทำการแบ่งอวัยวะสืบพันธุ์ออกเป็น 3 กลุ่ม ตามลักษณะที่ปรากฏบนรังไข่ ได้แก่ ระยะรังไข่ไม่ทำงาน (10 ตัว) ระยะฟอลลิเคิล (10 ตัว) และระยะลูเทียล (10 ตัว) เก็บตัวอย่างเลือดจากสุกรสาวทุกตัวก่อนส่งโรงฆ่าสัตว์เพื่อตรวจฮอร์โมน P4 สุ่มเก็บตัวอย่างชิ้นเนื้อจากมดลูกสุกรสาวตัวละ 7 ตำแหน่งเพื่อตรวจวินิจฉัยลักษณะทางจุลกายวิภาค ได้แก่ ชนิดและความสูงของเยื่อบุโพรงมดลูก จำนวนหลอดเลือด ปริมาณเซลล์ที่มีการสร้างสารคัดหลั่ง และต่อมมดลูก นอกจากนี้ยังทำการตรวจนับจำนวนของเซลล์เม็ดเลือดขาวกลุ่มต่างๆ ได้แก่ ลิมโฟไซต์ นิวโทรฟิล อิโอซิโนฟิล แมคโครฟาจ และ พลาสม่าเซลล์ โดยเฉลี่ยสุกรสาวที่ทำการศึกษามีอายุ 306.4±39.9 วัน (พิสัย 233-407 วัน) และน้ำหนักตัว 150.4±24.8 กิโลกรัม (พิสัย 104.0-205.5 กิโลกรัม) ลิมโฟไซต์เป็นเซลล์ของระบบภูมิคุ้มกันที่พบมากที่สุดในเยื่อบุโพรงมดลูกของสุกรสาวทุกระยะ ลิมโฟไซต์ในชั้นแกรนด์ของเยื่อบุโพรงมดลูกในระยะที่รังไข่ไม่ทำงานมีปริมาณสูงกว่าระยะฟอลลิเคิล (p=0.02) และระยะลูเทียล (p=0.05) นิวโทรฟิลในชั้นเยื่อบุโพรงมดลูกและชั้นใต้เยื่อบุโพรงมดลูกในระยะฟอลลิเคิลสูงกว่าในระยะลูเทียล และระยะที่รังไข่ไม่ทำงาน (p<0.001) อิโอซิโนฟิลในชั้นใต้เยื่อบุผิวโพรงมดลูกในระยะลูเทียลสูงกว่าระยะรังไข่ไม่ทำงาน (p=0.004) และระยะฟอลลิเคิล (p<0.001) การสูงขึ้นของฮอร์โมน P_4 ทำให้มีปริมาณต่อมมดลูกมากขึ้น (p<0.01) จำนวนลิมโฟไซต์ในทุกชั้นของเยื่อบุโพรงมดลูกลดลง (p<0.05) จำนวนนิวโทรฟิลในชั้นใต้เยื่อบุโพรงมดลูกลดลง (p=0.03) และจำนวนอิโอซิโนฟิลในชั้นใต้เยื่อบุโพรงมดลูกเพิ่มขึ้น (p<0.001) โดยสรุป การสะสมของเซลล์เม็ดเลือดขาวชนิดต่างๆ ในเยื่อบุโพรงมดลูกของสุกรสาวที่ไม่แสดงการเป็นสัดขึ้นกับการทำหน้าที่ของรังไข่ นิวโทรฟิลและอิโอซิโนฟิลเป็นเซลล์ระบบภูมิคุ้มกันที่พบได้มากในระยะฟอลลิเคิลและระยะลูเทียลตามลำดับ

คำสำคัญ: ไม่เป็นสัด เยื่อบุโพรงมดลูก สุกรสาว เซลล์ระบบภูมิคุ้มกัน ฮอร์โมนโปรเจสเตอโรน

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Introduction

In general, the annual removal rate of sows in swine commercial herds varies between 30 and 60% (Engblom et al., 2007). Common removal reasons include reproductive disorders, old age, low productivity, locomotor problems, milking problems, death and health problems (Stein et al., 1990; Lucia et al., 2000; Tummaruk et al., 2006). Reproductive disturbances are the most common unplanned removal reason in both sows and gilts (Lucia et al., 2000; Stein et al., 1990; Tummaruk et al., 2006). Reproductive failure in young sows and gilts has been recognized as an economic loss in the pig industry. Therefore, the culling decision is important for ensuring optimal productivity and economy. Investigations on the reproductive problems among gilts are necessary. On average, Landrace x Yorkshire (LY) crossbred gilts in Thailand attain puberty at about 200 days of age (Tummaruk et al., 2009). In general, the gilts that exhibit first estrus at later than 9 months of age (270 days) are considered delayed puberty (Einarsson et al., 1974). It has been demonstrated that 51% of the culling gilts in commercial swine herds in Thailand were classified as anoestrus (Tummaruk et al., 2006). The reason for the loss of replacement gilts due to anoestrus problems might be, at least in part, due to hot and humid climate. It has also been found that the proportion of gilts culled due to anoestrus in the summer was higher than in winter (56% versus 49%) (Tummaruk et al., 2006).

The reproductive function of the female pigs is difficult to examine under field conditions. Post-mortem examination of the reproductive organs is therefore a useful tool to obtain a potential source of

information on infertility problems (Dalin et al., 1997; Heinonen et al., 1998; Karveliene et al., 2007; Tummaruk et al., 2009). Earlier studies have found that factors that cause reproductive failure alter the physiological status of the sow's endometrium in different pathways (reviewed by Dalin et al., 2004). However, the infiltration of immune cells in cyclic gilts and sows is also influenced by the oestrous cycle and hormones (Bischof et al., 1994; Jiwakanon et al., 2006; Kaeoket et al., 2001). Progesterone (P_4) increases tissue proliferation, gland development and protein secretion in the porcine endometrium (Mahaboob Basha et al., 1979). In addition, it has been shown that P_4 increases the susceptibility of the endometrium to bacterial infection and may subsequently cause endometritis (De Winter et al., 1995; De Winter et al., 1996; Wulster-Radcliffe et al., 2003). The infiltration and distribution of leukocytes in the porcine endometrium during the oestrous cycle has been comprehensively evaluated (Bischof et al., 1994; Jiwakanon et al., 2006; Kaeoket et al., 2001). Lymphocytes are the predominant leukocyte in the endometrium of the cyclic gilts. Surprisingly, numerous neutrophils were found in the endometrium of pre-pubertal gilts in some studies (Bischof et al., 1994; Jiwakanon et al., 2006). It has also been shown that the infiltration of immune cells in the sow endometrium, e.g., lymphocytes, neutrophils, macrophages, eosinophils, mast cells and plasma cells, is influenced by stage of the oestrous cycle, pregnancy period and endometritis (Bischof et al., 1995; De Winter et al., 1995; Engelhardt et al., 2002; Kaeoket et al., 2001; Kaeoket et al., 2003). In practice, the replacement gilts that have delayed puberty and/or delayed age at first mating might have been exposed to foreign antigens during oestrous cycle and, hence, have increased risk of uterine malfunction. Earlier studies have demonstrated that gilts that have delayed age at first mating tend to have poor reproductive performance and short longevity (Koketsu et al., 1999; Schukken et al., 1994). To our knowledge, infiltration of the immune cells in the endometrium of anoestrus gilts has not been elucidated. The objective of the present study was to investigate the infiltration of immune cells in the endometrium of gilts culled due to anoestrus in relation to the ovarian appearance and the serum progesterone (P_4).

Materials and Methods

Post-mortem examination and tissue collection: Genital organs from 30 Landrace x Yorkshire crossbred gilts from five commercial swine herds in Thailand were used in the present study. All of the gilts were culled due to anoestrus and none had been mated. After slaughter, the genital organs including ovary, oviduct, uterus, cervix, vagina, vestibule, vulva and urinary bladder were collected, placed on ice and transported to the laboratory within 24 h of culling. The genital organs were examined to assess the stage of the oestrous cycle and gross pathology. Ovarian appearance and component structures, i.e. corpora lutea (CL), corpora albicantia (CA) and follicles, on the ovaries were carefully examined. The ovarian appearance and component structures were defined according to previous study (Tummaruk et al., 2009). The organs were selected according to the stages of the reproductive cycle: inactive (n=10); follicular (n=10); and luteal phases (n=10). 'Inactive phase' was defined as the case where ovaries had only small follicles (<7 mm) lacked CL or CA. 'Luteal phase' was characterized by ovaries that contained CL with or without small follicles and/or CA. 'Follicular phase' was characterized by ovaries that had large follicles (7-12 mm in diameter) together with CA. Numbers of follicles, CL and CA were counted. The oviducts and uterine horns were dissected from the mesosalpinx and mesometrium and were measured for length. The uterus from utero-tubal junction to uterine body was dissected and weighed using an electronic balance (KD300, max=5 kg, d=1 g, TANITA corporation, Bangkok, Thailand). The uterine horns were opened longitudinally and the endometrium was investigated. Seven tissue samples were collected from the uterus of the gilts including the proximal, middle and distal part of each uterine horn and uterine body. The samples were fixed in 10% neutral buffered formalin for at least 24 h, embedded in paraffin and processed by use of an automatic tissue processor (Histokinette 2000 automatic tissue processor, Reichert-Jung, London, UK). Each sample was embedded in a paraffin block using an embedding instrument (Tissue-Tek TEC, Sakura, Tokyo, Japan). The paraffin embedding was cut with 5 μm thickness using microtome (Shandon, Anglia scientific instrument ltd., Cambridge, UK). The slides were left overnight at 37°C. The tissues were deparafinized using xylene, passed through different concentrations of alcohol and were stained using haematoxylin for 5 min and eosin for 30 sec (H&E).

Serum progesterone assays: Blood samples were collected from the jugular vein prior to slaughter. The blood samples were centrifuged at 3,000 rpm (1,160 xg) for 10 min. The serum was collected and stored at -20°C until assay. The serum progesterone (P_4) level was determined by a solid-phase radioimmunoassay (Coat-A-Count®, CA, USA). The method has earlier been evaluated (Tummaruk et al., 2004). The assay was performed according to the manufacturer's instructions. The kit provides a reagent and a tube, coated with antibodies to P_4. The calibrators represented 0, 0.3, 1.6, 31.8, 63.6 and 127.2 nmol/l. A 0.1-ml aliquot of calibrators, the undiluted samples and 1.0 ml of iodinated P_4 (approximately 75,000 cpm), were pipetted into the appropriate tube, in duplicate. After 3 h incubation at room temperature, the incubation was removed by simple decantation and each tube was counted for 1 min in a gamma counter. The P_4 antiserum is highly specific for P_4 with low cross-reactivity to other naturally-occurring steroids. The sensitivity of the assay was 0.06 nmol/l. The assay procedure followed that shown in the manufacture's manual. Briefly, 100 μl of the serum sample was put in tubes coated with P_4 antibody, in duplicate. 1.0 ml of ^{125}I-Progesterone was added to every tube and incubated for 3 hours at room temperature (25°C). The liquid was removed from all

tubes, and the tubes were counted for 1 minute in gamma counter. A known amount of P_4 was added to every assay in order to calculate the intra-assay coefficients of variation, which were 6.86% and 3.25% for low and high P_4 concentrations, respectively.

Histological examination: The sections were divided into three layers for histological examination: epithelium, subepithelium, and glandular. Immune cells, e.g. lymphocytes, neutrophils, eosinophils, macrophages and plasma cells in each layer were quantified under light microscope (400x) (Figures 1a-c). For each section and each layer, 20 microscopic fields were arbitrarily selected for investigation. Ocular micrometer with 25 squares corresponded to 15,625 μm² (400x) of real tissue area, and 125 μm of real tissue length was used for counting the number of immune cells in each area by movement of the ocular micrometer across the entire area in a non-overlapping manner (Kaeoket et al., 2001). Histological examination of the samples was done by the same person who was unaware of the identity of the gilts (Y. Teamsuwan). Uterine samples of seven parts in H&E stain were evaluated. The number of immune cell counts was expressed as the total number of cells per uterine section (20 microscopic fields). The height (μm) of the surface epithelium was measured by the Image-Pro Plus version 6.0 using 400x magnification (Media Cybernatics, Inc. MD USA, 2006) and the morphology of the surface epithelium was reported using the descriptive categories of simple cuboidal, simple columnar, pseudostratified cuboidal and pseudostratified columnar. In the subepithelial connective tissue layer, the number of vessels in cross sections including capillaries, venules, veins, arterioles and arteries were counted at 400x. The levels of subepithelium edema were estimated by a scoring system as 0: none, 1: slight, 2: moderate and 3: marked with 100x magnification. With magnification 100x, counting was made for the number of transversely sectioned glands in the glandular connective tissue layer including of upper and lower parts of the tubular endometrial glands. Furthermore, the qualitative estimation of the secretory vesicles of the glandular epithelium were graded as 0: none, 1: slight, 2: moderate and 3: marked under the light microscope with 400x magnification.

Statistical analyses: Data were analyzed using SAS version 9.0 (SAS Inst., Cary, NC, USA). Numbers of cells were presented as the mean number of cells per 20 ocular fields (312,500 μm²) in seven tissue sections. Number of vessels and number of sectioned glands were presented as the total number of vessels/glands per 20 ocular fields. The height of the surface epithelium was measured in 20 positions in each section and was presented as mean. The data were analyzed using a general linear model procedure (PROC GLM). Normal distribution of the data was tested using the UNIVARIATE procedure option NORMAL. A natural logarithmic transformation was applied to the number of immune cells to achieve the assumption required for analysis of variance. Least squares means were obtained and were compared using the least significant different test. The score of subepithelial edema and secretory vesicles were analyzed using the Wilcoxon's rank-sum test (PROC NPAR1WAY). Spearman's correlation was used to analyze the association between P_4 and the size of the leukocyte subpopulation. A value of $P \leq 0.05$ was regarded to be statistically significant.

Results

Reproductive data and gross morphology: On average, age and body weight at culling of the gilts were 306.4±39.9 d (range 233-407 d) and 150.4±24.8 kg (range 104.0-205.5 kg). The weight of the uteri was 504.8±335.9 g (range 58-1,120 g). The weight of the uterus differed between inactive and cyclic ovaries ($p<0.001$) (Table 1). Three gilts had cystic ovaries (single cyst in one gilt and multiple cysts in two gilts). The age at culling, body weight at culling, uterine weight and level of serum P_4 in each group of gilts are presented in Table 1.

Table 1 Age at culling, body weight at culling, uterine weight and levels of serum progesterone (P_4) in inactive (n=10), follicular (n=10) and luteal (n=10) phases

Group	Age at culling (d)	Body weight (kg)	Uterine weight (g)	P_4 (nmol/l)
Inactive	300.3±40.8[a]	156.2±17.3[a]	160.1±99.1[a]	1.8±1.9[a]
Luteal	300.1±31.0[a]	148.5±22.0[a]	662.7±169.9[b]	53.4±40.7[b]
Follicular	284.4±26.3[a]	141.0±16.4[a]	562.1±295.0[b]	3.5±3.0[a]
All (n=30)	294.9±33.0	148.6±19.1	461.6±296.2	19.6±33.3

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

Morphological changes: A number of endometrial morphologies, e.g., type of surface epithelium, mitotic figures, number of vessels, oedema score, number of endometrial glands and number of secretory vesicles, changed according to the reproductive cycle (Table 2). The types of surface epithelium of the endometrium were pseudostratified cuboidal and simple columnar during the inactive phase and were pseudostratified columnar during the follicular and luteal phases. The heights of the surface epithelium in the inactive, follicular and luteal phases were 26.3±8.9, 26.4±6.3 and 26.9±5.9 μm, respectively ($p>0.05$). Mitotic figures

were frequently observed in the surface epithelium during the follicular phase. Most of the vessels in the endometrium were located in the subepithelial connective tissue layer close to the surface epithelium. The types of vessels included capillaries, arterioles, venules, arteries and veins. The number of vessels in the follicular phases was higher than in the inactive phase (Table 2). Edema of the subepithelial layer in the follicular phase was higher than in the luteal and inactive phases (p<0.05) (Table 2). The number of endometrial glands in the luteal phase was higher than in the follicular and inactive phases (p<0.05). The secretory vesicles in the glandular epithelium of the luteal phase were approximately 4 times higher than in the inactive phase (p<0.001) (Table 2).

Table 2. Number of vessels, number of uterine glands, score of edema and score of secretory vesicle (means±SD) in inactive (n=10), follicular (n=10) and luteal (n=10) phases

Group	Vessels	Uterine glands	Edema	Secretory vesicle
Inactive	17.8±2.6^{c}	167.1±29.0^{c}	0.8±0.9 ^{b}	0.6±0.8^{c}
Luteal	27.7±4.1^{b}	799.7±120.7^{b}	0.9±1.1^{b}	2.6±0.7^{b}
Follicular	53.6±6.9^{a}	345.2±32.8^{a}	2.3±0.9^{a}	1.6±0.7^{a}
All (n=30)	33.1±16.1	437.3±280.2	1.3±1.0	1.6±1.0

$^{a, b, c}$ different superscripts within column differ significantly (p<0.05)

Infiltration of leukocyte subpopulation

The surface epithelium: Figures 1 and 2 show the infiltration of the leukocyte subpopulation in the surface epithelium of the endometrium in each group of gilts. Lymphocytes were predominantly immune cells in all groups. Neutrophils in the follicular phase were higher than in the luteal (p=0.003) and inactive phases (p=0.01).

The subepithelial connective tissue layer: Infiltration of the leukocyte subpopulation in the subepithelial connective tissue layer of the endometrium is presented in Figures 1 and 3. Neutrophils (p<0.001), macrophages (p<0.05) and plasma cells (p<0.05) in the follicular phase were higher than in the luteal and inactive phases. Eosinophils in the luteal phase were higher than in the follicular (p<0.001) and inactive phases (p=0.003).

The glandular connective tissue layer: Infiltration of the leukocyte subpopulation in the glandular layer is presented in Figures 1 and 4. Lymphocytes in the inactive phase were higher than in the follicular (p=0.02) and luteal phases (p=0.05). Neutrophils in the follicular phase were higher than in the luteal (p=0.01) and inactive phase (p=0.01).

Correlation between P_4 and morphological changes and leukocyte subpopulation: Serum P_4 was positively correlated with the weight of the uterus (p=0.003) and the number of uterine glands (p=0.003) (Table 3). The number of lymphocytes in the surface epithelium (p=0.02), subepithelial connective tissue layer (p=0.15) and glandular connective tissue layer (p=0.002) decreased when serum P_4 increased (Table 3). In the subepithelial connective tissue layers, the number of eosinophils increased when the serum P_4 increased (p=0.04). The number of neutrophils, macrophages and plasma cells were not correlated with serum P_4 (p>0.05).

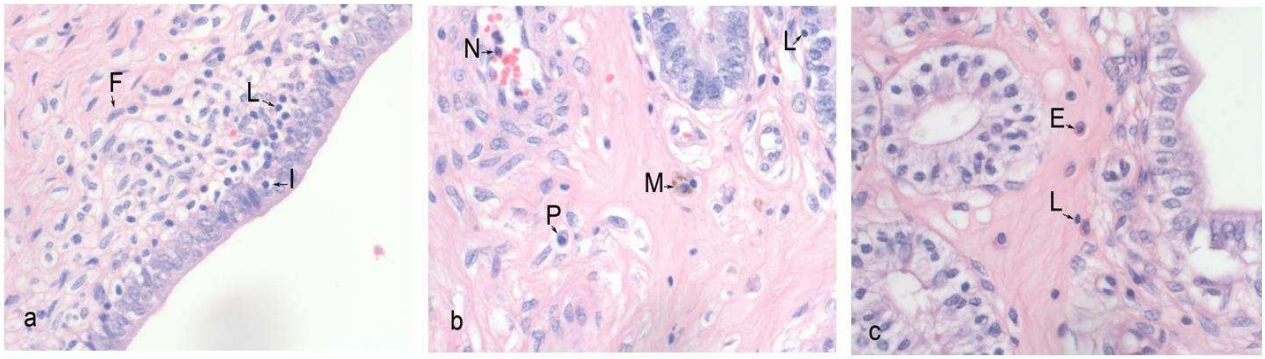

Figure 1 The gilt's endometrium by light microscopy (a) inactive phase (b) follicular phase (c) luteal phase. I: intraepithelial lymphocyte, L: lymphocyte, F: fibroblast, M: macrophage, P: plasma cell, N: neutrophil, E: eosinophil, 400x mag , H&E staining.

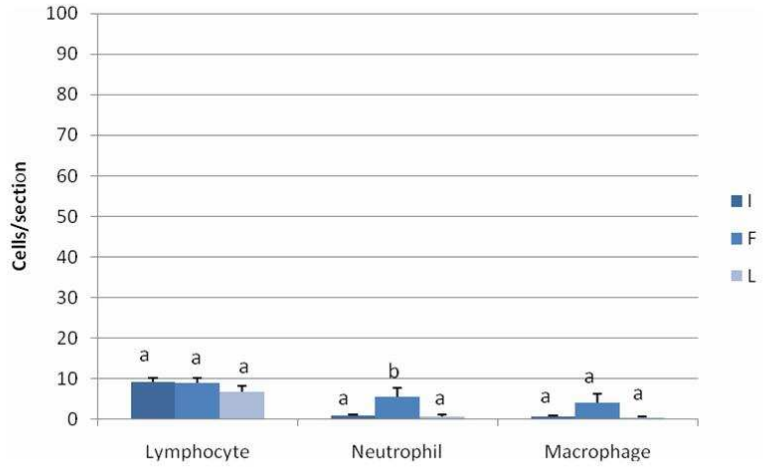

Figure 2 Number of leukocyte subsets in the surface epithelium of endometrium in inactive (I), follicular (F) and luteal (L) phases (mean±SEM) [a,b]different superscripts differed significantly (p<0.05)

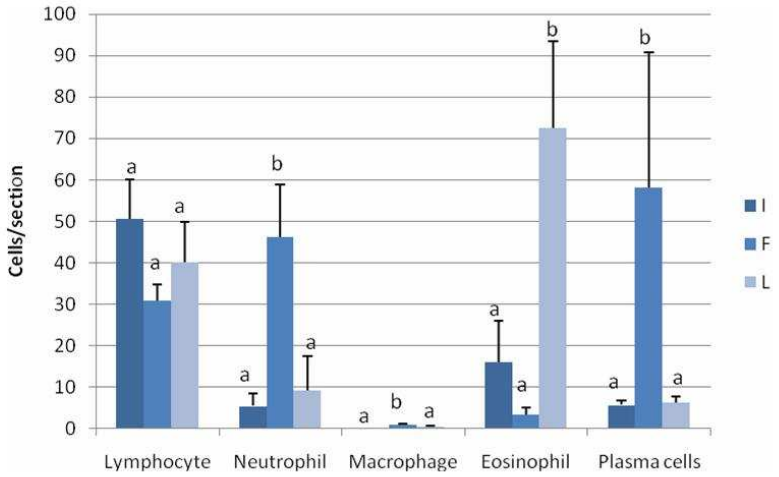

Figure 3 Number of leukocyte subsets in the subepithelial layer of endometrium in inactive (I), follicular (F) and luteal (L) phases (mean±SEM) [a,b]different superscripts differed significantly (p<0.05)

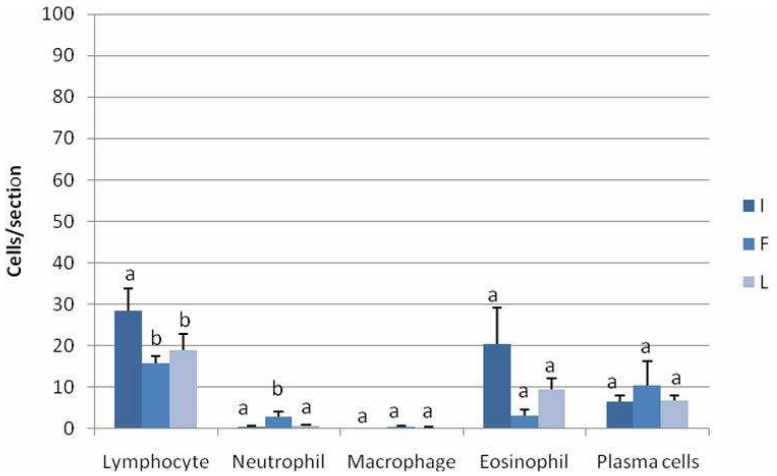

Figure 4 Number of leukocyte subsets in the glandular layer of endometrium in inactive (I), follicular (F) and luteal (L) phases (mean±SEM) [a,b]different superscripts differed significantly (p<0.05)

Table 3. Correlation (Spearman's correlation) between uterine weights, number of uterine glands, leukocyte subpopulation and serum progesterone in anoestrus gilts

Parameters	Correlation coefficient (*r*)	*P*-value
Uterine weight	0.61	0.003
Uterine glands	0.62	0.003
Surface epithelium		
-Lymphocyte	-0.42	0.02
Subepithelial layer		
-Lymphocyte	-0.27	0.15
-Neutrophil	-0.31	0.09
-Eosinophil	0.38	0.04
-Plasma cell	0.27	0.14
Glandular layer		
-Lymphocyte	-0.52	0.002
-Neutrophil	-0.30	0.10

Discussion

In the present study, lymphocytes were the predominant immune cells observed in all tissue layers of the endometrium in all groups of the anoestrus gilts. This finding is in accordance with previous findings in normal gilts (Bischof et al., 1994), normal sows (Kaeoket et al., 2001) and pre-pubertal gilts (Jiwakanon et al., 2006). These findings indicate that lymphocytes are a dominant immune cell and play a major role in the function of the gilts' endometrium. In the present study, the number of lymphocytes is not significantly different between the gilts that have inactive ovaries (true anoestrus gilts) and those that have cyclic ovaries (functional anoestrus gilts). This indicates that both true and functional anoestrus gilts had a similar number of lymphocytes in all tissues layers of the endometrium. Interestingly, the number of lymphocytes in the intra-epithelium and subepithelium of the endometrium was not significantly different among reproductive stages, but the number of lymphocytes in the glandular connective tissues layer in the gilts that had inactive ovaries was higher than those that were in either luteal or follicular phases. This indicates that the infiltration of lymphocytes in the endometrium occurs before the ovarian function. Jiwakanon et al. (2006) have demonstrated that the number of lymphocytes in the endometrium of pre-pubertal gilts was about 2 times higher than non-pregnant cyclic sows and most of them were T lymphocytes suggesting that lymphocytes may be important in the gilts. It is well known that the T lymphocytes are composed of T helper cells which modulate the local immune response, whereas, the functions of T cytotoxic cells are to recognize and destroy the infected cells (Piccinni, 2006). With these functions, it is possible that the regulation in immunologic surveillance has to be prepared in the gilts before reaching puberty and being ready for the invading of agents (or foreign antigen) by insemination.

In cyclic gilts, the number of lymphocytes in both intra-epithelium and sub-epithelium was negatively correlated with P4. In general, P4 is low in pre-pubertal gilts and increases after puberty is attained (Tummaruk et al., 2004). Therefore, the decrease in the number of lymphocytes might be due to the high serum P4 after puberty. Kaeoket et al. (2001) found that in post-weaning cyclic sows, the number of lymphocytes is also negatively correlated with P4. It has been demonstrated that high P4 increases the susceptibility of the endometrium to bacterial infection (Wulster-Radeliffe et al., 2003). Compared to earlier studies, the number of lymphocytes in different tissue layers of the endometrium was similar to that of weaned sows (Kaeoket et al., 2001). An earlier study has demonstrated that changes in steroid concentrations, particularly the increase in serum P4, regulate leukocytes functions, especially the lymphocytes (Jiwakanon et al., 2005; Kaeoket et al., 2001) and decrease the ability of the uterus in gilts to resist infection (Wulster-Radeliffe et al., 2003). In the present study, it was found that gilts that had anoestrus problems still had a normal number of lymphocytes in the endometrium. Similar to the experience with normal sows, the number of lymphocytes presented in all layers of the endometrium in cyclic gilts was negatively correlated with level of serum P4.

Neutrophils and macrophages are observed in all tissue layers of the gilts' endometrium, especially during the follicular phase in this study. This finding is in accordance with a previous study in normal sows (Kaeoket et al., 2001). In the present study, neutrophils in all tissue layers in the follicular phase were higher than in the luteal and inactive phases. In general, the number of neutrophils was positively correlated with oestrogen and negatively correlated with P4 (Kaeoket et al., 2001). It is known that neutrophils are the earliest phagocytic cells responding to acute inflammation. Neutrophils engulf and destroy foreign antigens and thereafter die. Both neutrophils and macrophages act as non-specific phagocytic cells, which is part of the innate immune defense mechanism of the female genital tract. In the sows' endometrium, a high number of neutrophils have been found after insemination (Kaeoket et al., 2003). It is known that uterine infections reduce

reproductive efficiency in pigs. It has been demonstrated that impaired function of neutrophils increase the susceptibility to uterine infection (Wulster-Radeliffe et al., 2003). In pigs, resistance to uterine infections is highest during oestrus and lowest during the luteal phase (Wulster-Radeliffe et al., 2003; Jana et al., 2004). Wulster-Radeliffe et al. (2003) demonstrated that inoculation of *Actinomyces pyogenes* and *Escherichia coli* on day 8 of oestrous (luteal phase) induced uterine infection in all gilts, while none of the gilts inoculated with bacteria on day 0 developed infection. The resistant to infection of the uterus is closely related to P4 and oestrogen concentration. It has been demonstrated that a high level of P4 down-regulates immune cell function (Jiwankanon et al., 2006; Kaeoket et al., 2001; Wulster-Radeliffe et al., 2003). In the uterine lumen of the pig, bacteria may be able to survive and proliferate during the follicular phase, leading to infection during the luteal phase. In the present study, pubertal anoestrus gilts that have a delayed first mating might have had exposure to a foreign antigen during the oestrous cycle and hence had increased risk of endometritis.

Additionally, plasma cells were found in the follicular phases. This is in contrast to the findings of Jiwakanon et al. (2006) who found that the number of plasma cells in the connective tissue of the endometrium was relatively low in both pre-pubertal gilts and anoestrus sows. In general, plasma cells produce immunoglobulin and secrete this into the lumen (Kutteh and Mestecky, 1994). This action may have a significant role in the early defense against the invasion of microorganisms at the mucosal surfaces of the endometrium as in the case for intestinal mucosa (Bailey et al., 2009; Golby and Spencer, 2002). In the porcine oviduct, a higher number of plasma cells were found in the infundibulum compared to other parts (Jiwakanon et al., 2006). This indicates that the anoestrous gilts in the present study might have been exposed to some foreign antigen that stimulated plasma cell infiltration (Bischof et al., 1994; Dalin et al., 2004). For inseminated sows, Kaeoket et al. (2003) found no increase in the number of plasma cells in the subepithelial connective tissue layer of the endometrium after insemination, indicating that semen may not be the antigen that can stimulate the number of plasma cells. Unlike neutrophils and macrophages, plasma cells are developed from lymphoid lineage and react to pathogens in a specific manner. It has been demonstrated that some types of bacteria, such as *Escherichia coli*, *Streptococcus sp.* and *Staphylococcus sp.* can be isolated from the vulva of normal sows (Bara et al., 1993; Carabin et al., 1996). Gilts suffering from immuno-suppression and/or exposed to various stressful factors (e.g. acclimatization and transportation) are at a high risk of being infected. During oestrus, bacteria may invade the uterine lumen through the cervical opening and the exposure of the gilt's endometrium to these bacteria might have been occurred.

In this study, the occurrence of anoestrus in cyclic gilts might possibly have been caused by uterine infection. In an experimental study, Jana et al. (2004) demonstrated that the intrauterine infusion of *Escherichia coli* in gilts on Day 4 of the oestrus cycle resulted in endometritis and vaginal discharge in all the gilts. The administration of *Escherichia coli* resulted in a reduction of the plasma level of luteinizing hormone (LH) and oestradiol-17β on Days 15-18, and lower P4 and a higher level of PGFM on Day 8 after treatment. These inflammatory processes after bacterial infusion resulted in anoestrus. Furthermore, infusion of *Escherichia coli* in the uterine lumen of gilts reduced PGF2α in the utero-ovarian vein and reduced plasma P4 from Days 10-14 after treatment. This indicates that the development of the inflammatory process after the infusion of *Escherichia coli* could interrupt ovarian function of the gilts (Jana et al., 2007).

Noticeably, the eosinophils are the dominant immune cell during the luteal phases. This finding is in agreement with a previous study in normal sows (Kaeoket et al., 2001). However, in the present study, the number of eosinophils was approximately 20 times higher than that reported for normal sows (Kaeoket et al., 2001). The function of eosinophils in the uterus of the anoestrous gilts is unknown. It has been demonstrated that the largest number of eosinophils was found in the connective tissue of the subepithelial layer on Day 11 of pregnant sows (Kaoeket et al., 2003), during Days 10–14 of pregnancy in gilts (Bischof et al., 1995), and on Day 11 of non-pregnant sows (Kaeoket et al., 2001). These findings indicate that eosinophil infiltration is dependent on a high P4 level at a certain stage and not pregnancy. Irrespective of embryos being present or absent, the eosinophils in the endometrium of sows under P4 dominance may be associated with the dynamic changes in structure and function of the endometrium in preparation for a potential attachment of embryos (Jeziorska et al., 1995). A number of studies on the function of eosinophils in the endometrium have been reported. For instance, it was found that eosinophils synthesize a number of cytokines including vascular endothelial growth factor (VEGF), a potent multifunctional cytokine that exerts several important actions on the vascular endothelium (Horiuchi and Weller, 1997). VEGF is involved in placental vascularization and in vascular permeability that stimulates the transfer of the nutrients' mother to the fetus (Vonnahme et al., 2001). Furthermore, the eosinophils are associated with the inflammatory mediators to against micro-organisms (Gleich et al., 1993).

In conclusion, the infiltration of the leukocyte subpopulation in the endometrium of anoestrus gilts is largely dependent on the ovarian function. P4 played an important role in the infiltration of the leukocyte subpopulation in anoestrus gilts. Lymphocytes are the predominant immune cells for all reproductive stages. Neutrophils and eosinophils were common immune cells in follicular and luteal phases, respectively.

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