Original Article

Studies of Oestrogen and Progesterone Receptors in Reproductive Organs of Prepubertal Gilts with Reproductive Disturbance

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Abstract

The present study aims to study the localization of steroid receptor, oestrogen receptor alpha and progesterone receptor by immunohistochemical technique in the reproductive organs of prepubertal culling gilts with reproductive disturbance. The genital organs from 20 culled gilts before puberty were collected. Historical data, reasons of culling and the macroscopic lesions of the reproductive tracts were recorded. The results of immunohistochemistry revealed that the expression of ER and PR was lower in prepubertal gilts with reproductive lesion compared to normal gilts. However, a significant difference was found only in some compartments of the cervix and uterus. In the cervix, a significant lower expression of PR was found in the surface epithelium and muscular layer of culling gilts with reproductive disturbance while the expression of ERα was similar between these groups of gilts. In the uterus, significant differences were observed for ERα score in the stroma and the myometrium, while PR immunostaining was significantly lower only in the myometrium of prepubertal gilts with reproductive lesion. Comparing between different compartments of the reproductive tissues, it was found that the muscular layers both in the cervix and the uterus were the most dynamic tissue for the changes of steroid receptors. The results from the present study showed the difference in the expression of ERα and PR in different reproductive organs of culled gilts. The changes in the expression of these steroid receptors in some compartments of the cervix and uterus may involve with pathological status found in these culled gilts. As the regulation of steroid hormones through the expression of their receptors differed according to specific cell types, therefore their expression between normal and reproductive failure gilts should also vary. Moreover, the expression of ERα and PR in the reproductive organs of culled gilts indicated that there are responses to the influence of these steroid hormones and therefore, may have significant roles in reproductive physiology as well as pathology.

Keywords: oestrogen receptor, prepubertal gilts, progesterone receptors, reproductive disturbance

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บทคัดย่อ

การศึกษาตัวรับฮอร์โมนเอสโตรเจนและโปรเจสเตอโรนในอวัยวะสืบพันธุ์สุกรสาวก่อนวัยเจริญพันธุ์ที่มีปัญหาระบบสืบพันธุ์

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การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่าและโปรเจสเตอโรนในอวัยวะสืบพันธุ์โดยวิธีอิมมูนโนฮิสโตเคมีของสุกรสาวทดแทนก่อนวัยเจริญพันธุ์ที่ถูกคัดทิ้งเนื่องจากปัญหาความล้มเหลวทางระบบสืบพันธุ์ โดยทำการเก็บตัวอย่างอวัยวะสืบพันธุ์จากสุกรสาวที่ถูกคัดทิ้งจำนวนทั้งหมด 20 ตัว ทำการบันทึกข้อมูลทางระบบสืบพันธุ์ สาเหตุของการคัดทิ้งและพยาธิสภาพที่ตรวจพบ ผลจากขบวนการอิมมูนโนฮิสโตเคพบการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่า และตัวรับฮอร์โมนโปรเจสเตอโรนในปริมาณน้อยกว่าในสุกรสาวที่มีปัญหาทางระบบสืบพันธุ์เปรียบเทียบกับสุกรสาวปกติที่ถูกคัดทิ้งโดยมีนัยสำคัญทางสถิติ ($p < 0.05$) ในบางส่วนของเนื้อเยื่อคอมดลูกและมดลูก ในคอมดลูกพบว่าการแสดงออกของตัวรับฮอร์โมนโปรเจสเตอโรนน้อยกว่าอย่างมีนัยสำคัญในชั้นเยื่อบุและชั้นกล้ามเนื้อคอมดลูกของสุกรสาวที่พบรอยโรคทางระบบสืบพันธุ์ ในขณะที่ไม่พบความแตกต่างของการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่าในส่วนต่าง ๆ ของคอมดลูก ในส่วนของมดลูกสุกรพบว่ามีความแตกต่างของการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่าในชั้นเนื้อเยื่อเกี่ยวพันและกล้ามเนื้อมดลูกระหว่างสุกรสาวปกติที่ถูกคัดทิ้งก่อนวัยเจริญพันธุ์และสุกรที่พบรอยโรค สำหรับการแสดงของตัวรับฮอร์โมนโปรเจสเตอโรนพบว่ามีความแตกต่างเฉพาะในชั้นกล้ามเนื้อของมดลูกเท่านั้น เมื่อพิจารณาความแตกต่างระหว่างส่วนต่าง ๆ ของอวัยวะสืบพันธุ์ที่ศึกษาในครั้งนี้พบว่าส่วนกล้ามเนื้อของคอมดลูกและกล้ามเนื้อมดลูกเป็นเนื้อเยื่อที่มีการเปลี่ยนแปลงของการแสดงออกของตัวรับฮอร์โมนมากที่สุด จากผลการทดลองในครั้งแสดงให้เห็นถึงความแตกต่างของการแสดงออกของตัวรับฮอร์โมนในส่วนต่าง ๆ ของอวัยวะในระบบสืบพันธุ์ของสุกรสาวที่ถูกคัดทิ้งซึ่งอาจจะมีความเกี่ยวข้องกับพยาธิสภาพที่พบในอวัยวะสืบพันธุ์เหล่านี้ เนื่องจากการทำงานของฮอร์โมนผ่านตัวรับฮอร์โมนนั้นมีความแตกต่างและจำเพาะในเซลล์สืบพันธุ์แต่ละชนิดดังนั้นการแสดงออกของตัวรับฮอร์โมนเหล่านี้ย่อมมีความแตกต่างกันทั้งในสุกรสาวที่ปกติและสุกรสาวที่มีปัญหาทางระบบสืบพันธุ์ ทั้งนี้การที่พบการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่าและตัวรับฮอร์โมนโปรเจสเตอโรนในครั้งนี้ แสดงให้เห็นว่ามีการตอบสนองต่ออิทธิพลของฮอร์โมนที่มีส่วนเกี่ยวข้องกับสรีรวิทยาและพยาธิวิทยาของอวัยวะในระบบสืบพันธุ์ของสุกรสาวที่ถูกคัดทิ้ง

คำสำคัญ: ตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่า สุกรสาวก่อนวัยเจริญพันธุ์ ตัวรับฮอร์โมนโปรเจสเตอโรน ความล้มเหลวของระบบสืบพันธุ์

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Introduction

In general, culling and replacement rate of sows with gilts was approximately 35-55% each year (D'Allaire and Drolet, 1999) and therefore the numbers of replacement gilts in herd has a great effect on the overall output of the farm. The replacement gilts were introduced to herd at about 20-22 week of age when they have reached puberty. The factors which influence age at puberty varied such as breed, season, feeding including the influence from internal hormones which have the important roles in controlling the function of female reproductive organs.

The reproductive problems which lead to culling were varied such as anoestrus, repeat mating, not pregnant, vaginal discharge, abortion and dystocia. There are several earlier studies on pathological investigation of gilt reproductive organs from slaughter house (Dalin et al., 1997; Ehnvall et al., 1981; Heinonen et al., 1998) and the results showed the high number of culling pigs with no pathological lesions of reproductive organs. Kunavongkrit et al. (1988) studied the morphological of ovaries and uteri from the slaughter house in Thailand and revealed that 16.4% of culling gilts had reproductive lesions

while 83.6% showed no pathological lesions of these reproductive organs. Moreover, 59.7% of normal culling gilts had ovulated. This is confirmed by the recent study in Thailand that about half of the gilts culled had shown normal reproductive organ (Tummaruk et al., 2009). Therefore, in addition to the macroscopic examination, there should be further studies of steroid hormones and their influences through specific steroid receptors in the reproductive organ of culling gilts. These may lead to better understanding of the mechanism of reproductive problems involved with the mechanisms under the influences of steroid hormones and their receptors.

The ovarian steroid hormones mainly oestrogen and progesterone, interplay the roles of controlling the morphological and functions of female reproductive organs of all mammals e.g. control of reproductive cycle, ovulation as well as pregnancy (Cooke et al., 1998; Drummond, 2006; Lessey, 2003; Spencer and Bazer, 2002). These steroid hormones elicit their functions by binding through specific receptor proteins in target tissues (Jensen, 1991; Jensen and DeSombre, 1973; Yamashita, 1998), therefore the presence of steroid receptors is as important as the levels of steroid hormones since they involve with the effective functions of reproductive control. There are several studies reported about the different localization of steroid receptor proteins in various reproductive organs and it was shown that steroid receptors such as oestrogen receptors and progesterone receptors could be found mainly in the uterus, cervix and ovary (Mowa and Iwanaga, 2000; Pelletier and El-Alfy, 2000; Pelletier et al., 2000; Wang et al., 2000). However, the study of steroid receptors in newly wean anoestrous sow demonstrated the high presence of steroid receptors in the uteri though the level of steroid hormones, oestradiol 17-β and progesterone were low (Sukjumlong et al., 2004[a]). Moreover, the presence of ER in the gene level was involved with the reproductive performance of the pigs (Isler et al., 2002; van Rens et al., 2000). Though the studies of steroid receptors in normal reproductive tracts are widely documented, the data of these receptors expression in reproductive disturbance gilts is still lacking. Therefore, the present study aims to investigate the presence of steroid receptor, oestrogen alpha and progesterone receptor in reproductive organs of infertile gilts.

Materials and Methods

Animal and sample collection: Twenty crossbred Landrace x Yorkshire (LY) gilts were used. Historical data of all gilts was recorded. The genital organs of the slaughter gilts including ovary, uterus, and cervix were collected from the slaughterhouse within the herd or near the herd within 24 h after culling. The samples were placed in an ice box and sent to the laboratory within 24 h after slaughter. Post-mortem examination was performed on each part of the reproductive organs within 48 h after culling. The investigation focused on the abnormality of the ovaries, uterine horns and cervix. The reproductive status of the gilt was classified according to the appearance of the ovaries and the uterus. The gilts were defined as pre-puberty when the ovaries had no CL and contained follicles.

After post-mortem examination, the samples were dehydrated, routine histological processed, embedded in paraffin and 4 µm thick sections were cut from each block and mounted on Polysine™ slides (Menzel-Glazer, Germany). These sections were used for immunohistochemistry.

Immunohistochemistry: Before immunohistochemistry, sections were deparaffinized in xylene and rehydrated in graded alcohol. The immunohistochemical protocol was described previously by Sukjumlong et al. (2003). Briefly, antigen unmasking technique by mean of heating in the microwave (in 0.01M citrate buffer, pH 6.0) was performed in order to increase the antigen-antibody reaction. A standard avidin-biotin immunoperoxidase technique (Vectastain® ABC kit, Vector Laboratories, Inc., USA) was applied to detect ERα and PR. The primary antibodies used were mouse monoclonal antibody to oestrogen receptor alpha, ERα, (C-311: sc-787, Santa Cruz Biotechnology Inc., USA, dilution of 1:25) and mouse monoclonal antibody to PR (Immunotech, clone 10A9, dilution of 1:200). The incubation time for both primary antibodies was 1 h at room temperature. The normal gilt uterus and cervix during follicular phase which were known to express ERα and PR were served as positive controls. In the final step, a chromogen which was 3,3'-diaminobenzidine (DAB, Dakopatts AB, Älvsjö, Sweden) was added to visualize the bound enzyme (brown color). All sections were counterstained with Mayer's hematoxylin followed by mounting in glycerine-gelatin before investigation.

Classification of positively stained cells: The classification of positively stained cells was done separately in each compartment of the reproductive organs. The cervix consisted of 3 compartments: surface epithelium (SE), subepithelial layer of the stroma (STR) and muscular layer (M). The uterus was classified into 4 compartments: surface epithelium (SE), glandular epithelium (GE), subepithelial layer of the stroma (STR) and myometrium (Myo). The results of the immunostaining were evaluated semi-quantitatively by a manual scoring method. The scoring of ERα and PR positive cells was done by classification into three different levels of intensity: weak, 1; moderate, 2 and strong, 3. Since not all cells stained positively in some compartments of the tissue, the proportion of positive to negative cells was also included for these tissues. The proportions were estimated into four different levels (marked 1-4): low proportion (< 30% of positive cells, 1); moderate proportion (30-60% of positive cells, 2); high proportion (> 60-90% of positive cells, 3) and almost all cells positive (more than 90%, 4) (Sukjumlong et al., 2005). The total scores were calculated by the summary of intensity and proportional scores of each compartment of the reproductive tissues.

Evaluation of the results and statistical analysis: The result of immunohistochemical staining was evaluated semiquantitatively by using a staining score. Data were analysed using SAS (Statistical Analysis System, SAS Inst. V. 9.1, Cary, NC., USA.).

Descriptive statistics including the mean and the standard deviations (SD) of all parameters were calculated. The total score of each compartment of the reproductive organs were compared between groups, and $p<0.1$ were regarded to have statistical significance.

Results

Culling reason and post mortem examination: The present study revealed that of 20 culled gilts examined, the common culling reason was anoestrus. Upon necropsy revealed that 8 gilts had pathological lesions which were cystic ovary (luteal cyst, n = 4), edema and/or congestion of the uterine and/or cervical epithelium (n = 4) while 12 culled gilts showed no pathological lesion from macroscopic examination at all.

Immunohistochemistry: In general, positive immunostaining of both ERα and PR were observed in the nuclei of different cell types in all reproductive organs examined. In the cervix and uterus, the positive cells could be found in the surface epithelium, the glandular epithelium (only in the uterus), the stroma and the muscular layer (myometrium for the uterus). The immunostaining results were summarized in Tables 1 and 2. In the cervix, (Tables 1.1 and 1.2), higher PR score was significantly observed in the SE and muscular layer of the cervix of normal prepubertal gilts than gilts with reproductive lesion; while ERα scores showed no significant difference though ERα score seemed to be higher in normal prepuberty gilts. In the uterus (Tables 2.1 and 2.2), higher ERα and PR scores were found in all compartments of the uterus in normal prepuberty group. However, the differences were observed only in the stroma for ERα immunostaining and in the myometrium for both ERα and PR immunostaining.

Table 1.1 The immunohistochemical score of ERα in each compartment of the cervix of culling gilts

Groups of gilts	ERα-SE	ERα-STR	ERα-M
Prepuberty-normal finding	4.54±1.58	3.50+1.38	4.83±1.57
Prepuberty-pathological lesions	3.31±1.90	2.88±1.35	3.69±2.21

Different letters within the same column represent significant differences.
NS: not significant, SE: surface epithelium, STR: cervical stroma, M: muscular layer of the cervix

Table 1.2 The immunohistochemical score of PR in each compartment of the cervix of culling gilts

Groups of gilts	PR-SE	PR-STR	PR-M
Prepuberty-normal finding	4.5±1.29[a]	3.00±1.47	4.50±1.66[a]
Prepuberty-pathological lesions	2.18±2.60[b]	2.00±1.54	2.56±1.87[b]

Different letters within the same column represent significant differences.
NS: not significant, SE: surface epithelium, STR: cervical stroma, M: muscular layer of the cervix

Table 2.1 The immunohistochemical score of ERα in each compartment of the uterus of culling gilts

Groups of gilts	ERα-SE	ERα-STR	ERα-GE	ERα-Myo
Prepuberty-normal finding	3.0±1.89	2.90+1.80[a]	3.09±2.38	4.55±1.19[a]
Prepuberty-pathological lesions	2.36±2.40	1.97±1.70[b]	2.40±2.32	3.8±1.18[b]

Different letters within the same column represents significant differences.
NS: not significant, SE: surface epithelium, STR: uterine stroma, GE: glandular epithelium, Myo: myometrium

Table 2.2 The immunohistochemical score of PR in each compartment of the uterus of culling gilts with different pathological finding

Groups of gilts	PR-SE	PR-STR	PR-GE	PR-Myo
Prepuberty-normal finding	2.59±2.05	2.45+1.75	2.27±2.70	4.36±1.16[a]
Prepuberty-pathological lesions	1.33±1.69	1.30±1.56	1.79±1.70	3.35±1.53[b]

Different letters within the same column represents significant differences.
NS: not significant, SE: surface epithelium, STR: uterine stroma, GE: glandular epithelium, Myo: myometrium

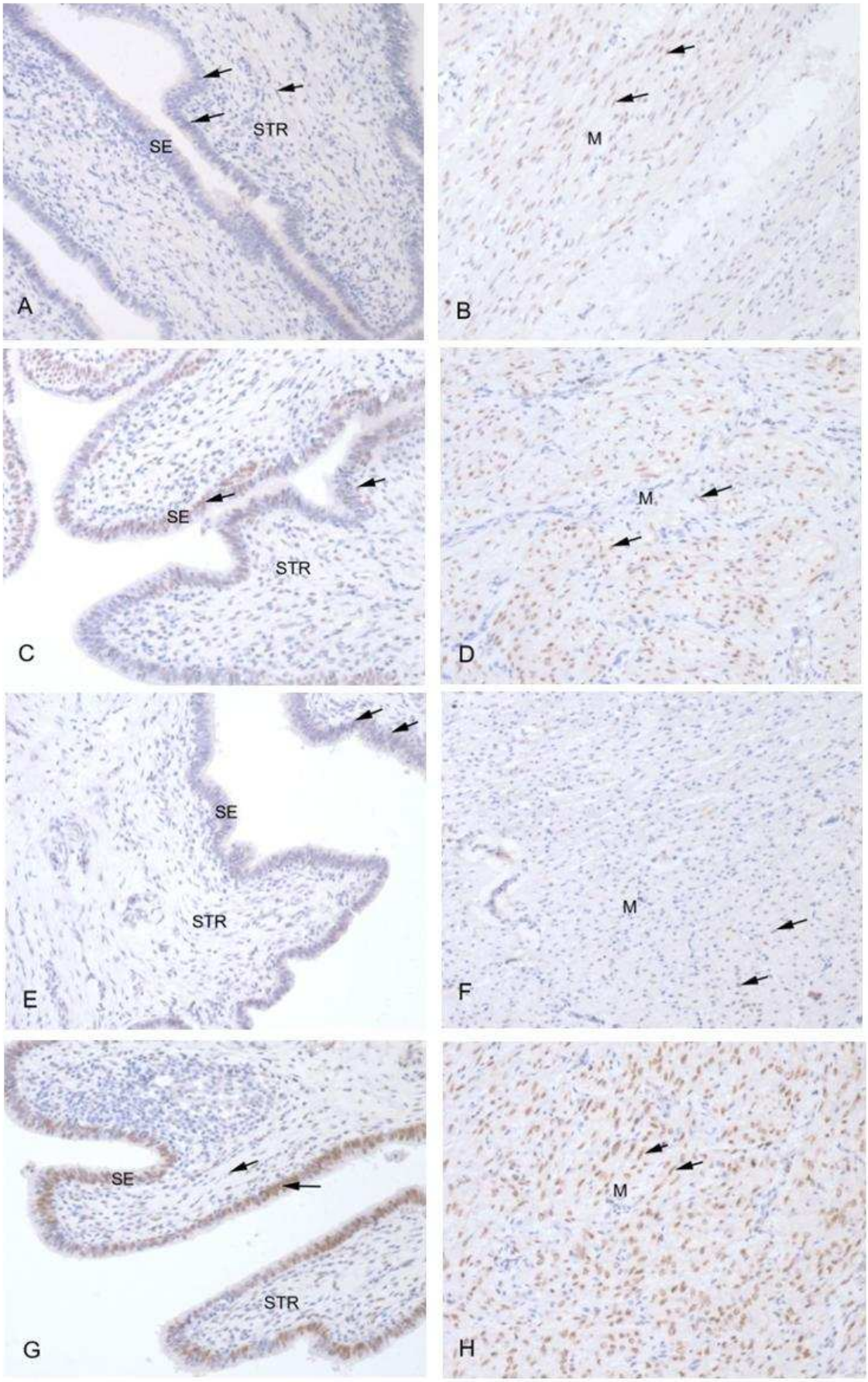

Figure 1 Immunohistochemical staining of ERα (A-D) and PR (E-H) in the cervix of culling gilts. A-B and E-F demonstrated ERα and PR immunostaining respectively in reproductive disturbance gilts, C-D and G-H demonstrated ERα and PR immunostaining respectively in normal prepubertal gilts. SE: surface epithelium, STR: stroma, M: muscular layer of cervix, arrows represent positive cells.

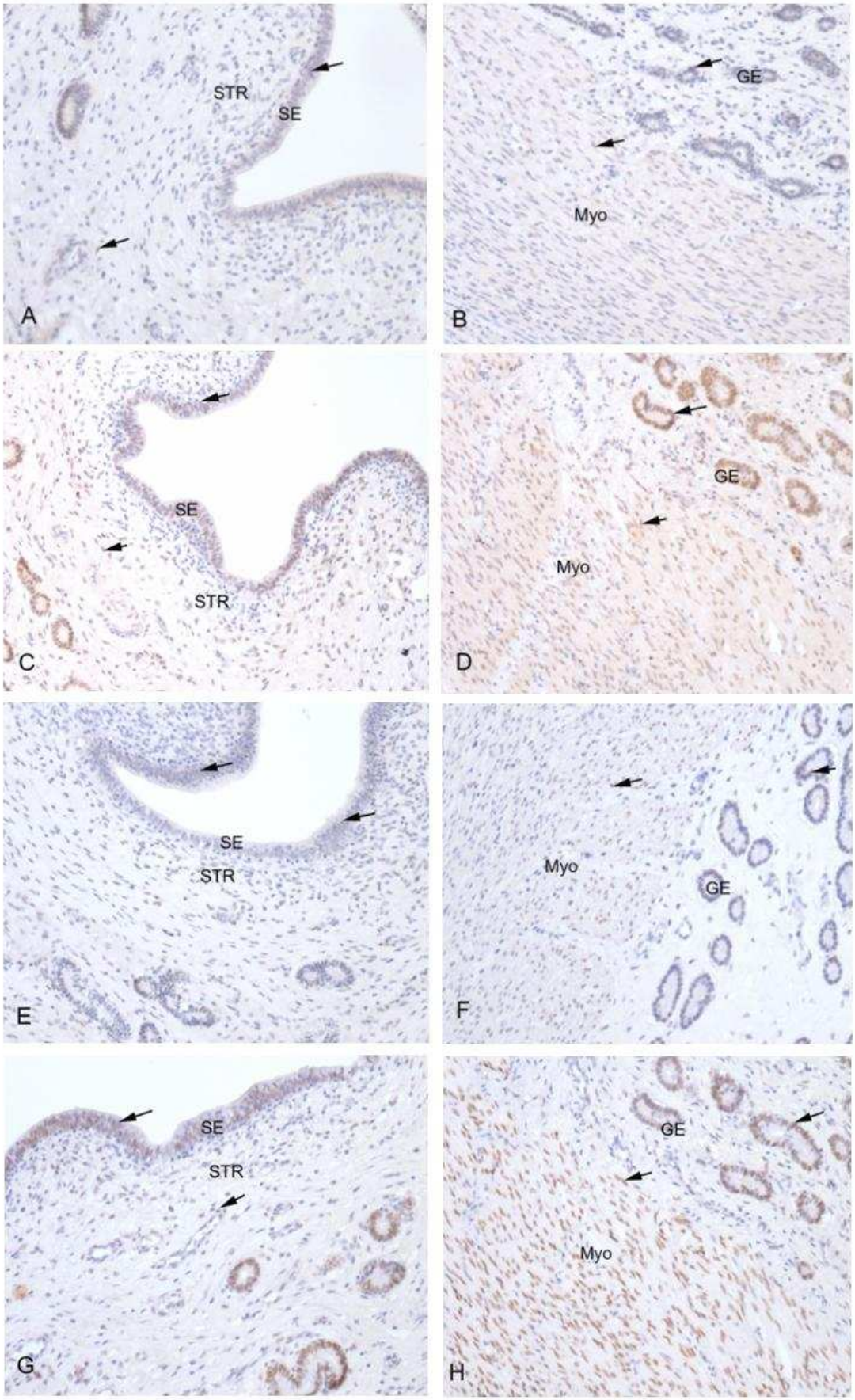

Figure 2 Immunohistochemical staining of ERα (A-D) and PR (E-H) in the uterus of culling gilts. A-B and E-F demonstrated ERα and PR immunostaining respectively in gilts with reproductive disturbance, C-D and G-H demonstrated ERα and PR immunostaining respectively in prepubertal normal gilts. SE: surface epithelium, STR: stroma, GE: glandular epithelium, Myo: myometrium, arrows represent positive cells.

Discussion

In the cervix of culling gilts, the ERα scores showed no difference between gilts demonstrated reproductive lesions and normal gilts. This demonstrates that the cervix may not be the main target of changes by the expression of steroid receptors, as suggested by Cano et al., (1990) that the capacity of response to the steroid hormones of cervical cells is limited compared with other target tissues, such as endometrium. However, significant difference was observed for PR immunostaining in the muscular layer of the cervix with higher scores in normal prepubertal gilts. As suggested in other studies that uterine and cervical quiescence is maintained by elevated progesterone acting through progesterone receptor (PR) (Boos et al., 2006; Brown et al., 2004; Mesiano and Welsh, 2007). On the other hand, the increased inflammatory response for uterine contraction at term and preterm was involved with the impairment of PR to mediate cervical and uterine quiescence (Mendelson, 2009). This may explain our results on the decrease of PR in the cervical muscle of the culling gilts with reproductive lesion that the lower expression of PR may relate to the inhibition of cervical and uterine quiescence in order to react to the inflammatory response which occurred in the gilts with reproductive problems. For the presence of PR in the surface epithelium of the cervix, similar pattern was observed that lower PR was found in the gilts with pathological lesions. This may suggested that the lower expression of PR in the surface epithelium may involve with the impairment of reproductive function in this group of gilts. However, more studies were need in order to explain the mechanism of PR in the cervix of culling gilts which cause the impairment of reproductive function as there is a limited data on the expression of steroid receptors in the porcine cervix especially there is none in gilts with reproductive disturbance.

In the uterus, the most prominent staining of ERα and PR was always observed in the myometrium compared to other compartments. In general, the myometrium has a crucial function as it is the compartment which undergoes contraction in order to transport sperms (Bulletti et al., 2000; Kunz et al., 1997) and/or embryo if fertilization occurs (Bulletti and de Ziegler, 2006; Nathanielsz et al., 1995). However, in normal gilts, the expression of ERα was high during those periods in order to maintain the function of the myometrium (Sukjumlong et al., 2003; Sukjumlong et al., 2004[b]). In contrast, in reproductive disturbance gilts, lower presence of ERα was significantly found and this may be the cause of the impairment of reproductive activities in these gilts. In human, uterine contractility participated in the emptying of the uterine content (de Ziegler et al., 2001). The changes of ERα expression may cause the inability of the uterus to clear the secretion, other waste substances or infectious agents and finally was the cause of the impairment of the uterus. For PR immunostaining in the uterus, the lower PR presence in pathological culled gilts before puberty compared to the others may due to the excessive imbalance levels of progesterone from pathological lesions and cause a lower presence of PR. From the study in human about the inflammatory response in the uterus, it was shown that progesterone receptor has a major role in anti-inflammatory in the myometrium (Hardy et al., 2006). Therefore, the lower PR in the myometrium may involve with the lower inflammatory response to pathological lesions as it was shown in the culling gilts with reproductive problems. However, according to different isoforms of PR in reproductive organs, it was shown that progesterone mediated myometrial quiescence was suppressed by an increased expression of the type A PR (PR-A) (Mesiano et al., 2002). Unfortunately that the antibody to PR used in the present study could not differentiate the expression of PR-A and PR-B, so the result showed was the total score of both isoforms of PR. Hence, the lower level of PR in the myometium of reproductive disturbance gilts may be the results of PR-A only.

In the study of endometrial pathology in human, it was shown that there was a decrease of the percent of positive cells and of the staining intensity of both ERα and PR such as in endometrial carcinomas (Sivridis et al., 2001; Yamauchi et al., 1996), endometrial hyperplasia (Nunobiki et al., 2003) and in luteal phase failure syndrome (Abd-el-Maeboud et al., 1997; Savchenko et al., 1990). In addition, there are studies showed considerable changes in steroid receptors, ER and PR in infertile women (Hirama and Ochiai, 1995; Thornburgh and Anderson, 1997) and in polycystic ovarian syndrome (Maliqueo et al., 2003; Villavicencio et al., 2006). Therefore, changes in hormonal status as well as the presence of their specific receptors may attribute to the pathological status which occurred in the target organs of these steroid hormones as shown by our present results.

In the endometrial stroma, there was similar pattern of expression to the myometrium. As ERα-positive stroma was essential for several reproductive phenomenon occurred in the oestrogen target tissues in a paracrine manner such as proliferation of uterine epithelium, uterine secretory function (Buchanan et al., 1999). Negative expression or lower presence of ERα may cause the impairment in these regulatory mechanisms of oestrogen and should be the cause of infertility or pathological lesions found in these culled gilts. In pigs, the mechanism of dihydrotestosterone (DHT) on the antagonism of estrogenic effects in the pig uterus was demonstrated by downregulation of the ERα mainly in the endometrial stroma and the myometrium (Cardenas and Pope, 2004). In the present study, the significant lower of ERα was observed mainly in uterine stroma and the myometrium as well. Therefore, it could be explained that uterine stromal and myometrial cells were more sensitive to the changes of circulating hormones and/or other factors which may consequently cause the uterine dysfunction from alteration of these hormone receptors

For PR expression in the endometrium, the present study failed to detect the difference between normal and gilts culled during different reproductive status. As mentioned before that there are two isoforms of PR; PR-A and PR-B which were arisen

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from single gene. It was well documented that the levels of PR-A and PR-B are differentially regulated during the reproductive cycle and therefore, may mediate different physiological responses to progesterone. In the ovary and uterus, the studies in mice revealed that ablation of PR-A results in severe abnormalities in ovarian and uterine function leading to female infertility but not for PR-B (Conneely et al., 2003). Furthermore, there is a recent study showed that PR-A has been absent in all compartment of the uterus in anoestrous sows (Karveliene et al., 2007). As it was shown in our results that the PR scores in this present study were the accumulation of PR-A and PR-B and therefore the difference in PR-A expression in culled gilts could not be demonstrated. On the other hand, there may be some difference in the expression of PR-A among these culled gilts, but it may also be balanced by the level of PR-B in the tissue compartments and therefore, cause the similar expression of PR in several compartments of the uterus among these culled gilts. However, the different localization of PR-A in reproductive disturbance gilts should be further studied as it may reveal or explain the cause of pathological lesions found from this present study.

In conclusion, these results show the expression of steroid receptors, ERα and PR in culled gilts with reproductive disturbance. The data obtained indicate that the changes of steroid receptors in some compartments of the cervix and uterus may involve with pathological status found in these culled gilts. As the regulation of steroid hormones through the expression of their receptors differed according to specific cell types of reproductive organs, therefore, the expression of steroid receptors in both normal and reproductive disturbance gilts should also vary. Moreover, the expression of ERα and PR in the reproductive organs of culled gilts indicated that there are responses to the influence of these steroid hormones and therefore, playing significant roles in reproductive physiology as well as pathology.

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