รายงานวิจัยฉบับสมบูรณ์

โครงการ: การศึกษาเปรียบเทียบตัวรับฮอร์โมนเอสโตรเจนและโปรเจสเตอโรนในสุกรนางหลังจากการผสมเทียมแบบสอดท่อเข้าคอมดลูก, แบบสอดท่อเข้ามดลูก และแบบสอดท่อเข้าปีกมดลูก

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กรกฎาคม 2555

สัญญาเลขที่ MRG5380173

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สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย และจุฬาลงกรณ์มหาวิทยาลัย

บทคัดย่อ

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ในอุตสาหกรรมการผลิตสุกรได้มีความพยายามในการลดจำนวนของตัวอสุจิในการทำการผสมเทียม โดยทำการปล่อยน้ำเชื้อที่ตำแหน่งถัดจากของคอมดลูกทำให้สามารถปล่อยน้ำเชื้อได้ในบริเวณที่ลึกกว่าปกติคือที่ตำแหน่งมดลูก (การผสมเทียมแบบปล่อยน้ำเชื้อที่มดลูก, IUI) และที่ปีกมดลูก (การผสมเทียมแบบปล่อยน้ำเชื้อที่ปีกมดลูก, DIUI) ทำให้สามารถลดความเข้มข้นของจำนวนอสุจิในน้ำเชื้อลง อย่างไรก็ตามพบว่าวิธีเหล่านี้ทำให้จำนวนลูกต่อครอกลดลงเมื่อเปรียบเทียบกับการผสมเทียมแบบดั้งเดิม (การผสมเทียมแบบปล่อยน้ำเชื้อที่คอมดลูก, AI)ร วมทั้งพบจำนวนของตัวอสุจิที่ตำแหน่งรอยต่อระหว่างท่อนำไข่และมดลูกซึ่งเป็นที่กักเก็บอสุจิน้อยลงอีกด้วย จากการศึกษาก่อนหน้านี้พบว่าสเตียรอยด์ฮอร์โมนมีความเกี่ยวข้องกับการขนส่งตัวอสุจิ เซลล์ไข่ และตัวอ่อน ในท่อทางเดินสืบพันธุ์ของสุกรเพศเมียโดยมีความสัมพันธ์กับการแสดงออกของตัวรับสเตียรอยด์ฮอร์โมนที่จำเพาะนั่นคือ ตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่า และตัวรับฮอร์โมนโปรเจสเตอโรน ดังนั้นการทดลองในครั้งนี้มีจุดประสงค์เพื่อที่จะศึกษาการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่าและตัวรับฮอร์โมนโปรเจสเตอโรนหลังจากที่ทำการผสมเทียมด้วยวิธีที่ต่างกัน โดยผลการศึกษาจะถูกวัดด้วยสองวิธีคือวิธีการให้คะแนนจากภาพ และการใช้โปรแกรมวิเคราะห์ภาพ ผลการศึกษาพบว่าการแสดงออกของตัวฮอร์โมนเอสโตรเจนชนิดแอลฟ่าและตัวรับฮอร์โมนโปรเจสเตอโรนมีระดับต่ำในกลุ่มที่ใช้วิธีการผสมเทียมแบบปล่อยน้ำเชื้อที่มดลูกและปีกมดลูกเปรียบเทียบกับกลุ่มที่ผสมเทียมแบบดั้งเดิมที่ส่วนท่อนำไข่ มดลูกและคอมดลูกสำหรับการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่า และพบความแตกต่างในส่วนท่อนำไข่และคอมดลูกสำหรับการแสดงออกของตัวรับฮอร์โมนโปรเจสเตอโรน แต่ไม่พบความแตกต่างระหว่างการผสมเทียมด้วยวิธีต่าง ๆ สำหรับการแสดงออกของตัวรับฮอร์โมนโปรเจสเตอโรนในมดลูก จากการศึกษาก่อนหน้านี้พบว่าในน้ำเชื้อสุกรนั้นมีระดับของฮอร์โมนเอสโตรเจนในระดับสูงทำให้สามารถกระตุ้นให้เกิดการแสดงออกของตัวรับฮอร์โมนที่เพิ่มขึ้นได้ในอวัยวะสืบพันธุ์ของสุกร ดังนั้นการผสมเทียมด้วยวิธีปล่อยน้ำเชื้อที่ตำแหน่งมดลูกและปีกมดลูกซึ่งใช้ปริมาณของน้ำเชื้อและตัวอสุจิลดลงอย่างมากอาจจะมีผลทำให้การแสดงออกของตัวฮอร์โมนน้อยกว่าในกลุ่มที่ผสมเทียมแบบดั้งเดิม ในส่วน

ของมดลูกสุกรพบว่าเยื่อบุต่อมมดลูกมีการแสดงออกตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่าในระดับสูงในกลุ่มที่ผสมเทียมด้วยวิธีดั้งเดิม ซึ่งอาจเกี่ยวข้องกับอัตราการเกิดการปฏิสนธิและตั้งท้องที่สูงกว่าเมื่อเปรียบเทียบกับการผสมเทียมด้วยวิธีอื่น ๆ ทั้งนี้อาจเนื่องมาจากเยื่อบุต่อมมดลูกทำหน้าที่หลักในการหลั่งสารที่ช่วยในการปรับสภาพแวดล้อมของอวัยวะสืบพันธุ์ให้เหมาะสมต่อการเกิดการปฏิสนธิและตั้งท้องโดยมีการควบคุมผ่านทางการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่าในส่วนของเยื่อบุต่อมมดลูก ในส่วนของคอมดลูกพบว่ากล้ามเนื้อคอมดลูกเป็นเนื้อเยื่อที่มีการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่าในระดับสูงเมื่อเปรียบเทียบกับเยื่อบุคอมดลูก ในทางตรงกันข้ามกับเนื้อเยื่อมดลูกและท่อนำไข่ พบการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่าในระดับสูงในสุกรนางกลุ่มที่ทำการผสมเทียมแบบปล่อยน้ำเชื้อที่ปีกมดลูกทั้งนี้แสดงให้เห็นว่ากล้ามเนื้อคอมดลูกหลังจากผ่านการผสมเทียมแบบปล่อยน้ำเชื้อที่ปีกมดลูก นั้นต้องการการกระตุ้นเพื่อให้เกิดการบีบตัวจากการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่าที่มากกว่าการผสมเทียมแบบดั้งเดิม จากผลการศึกษาครั้งนี้สรุปได้ว่าการผสมเทียมด้วยวิธีต่าง ๆ มีอิทธิพลต่อการแสดงออกของตัวรับสเตียรอยด์ฮอร์โมนในระบบทางเดินสืบพันธุ์ของสุกรนาง ซึ่งอาจมีผลต่อการเกิดการปฏิสนธิที่สมบูรณ์ได้

คำสำคัญ การผสมเทียมแบบดั้งเดิม การผสมเทียมแบบปล่อยน้ำเชื้อที่มดลูก การผสมเทียมแบบปล่อยน้ำเชื้อที่ปีกมดลูก ตัวรับฮอร์โมนเอสโตรเจนชนิดแอลฟ่า ตัวรับฮอร์โมนโปรเจนเตอโรน สุกรนาง

Abstract

Project code: MRG5380173

Project title: Comparative Studies of oestrogen and progesterone receptors in reproductive organs of artificial inseminated (AI), Intrauterine inseminated (IUI) and deep intrauterine inseminated (DIUI) sows

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Project period: 1 December 2006-30 November 2008

In pig production, there are efforts to reduce the number of spermatozoa by insemination into the posterior region of the cervix. By using special device that can be passed through the cervix allowing the deposition of sperm in the uterine body (Intrauterine insemination, IUI) or uterine horn (Deep intrauterine insemination, DIUI), the number of spermatozoa per dose could be reduced. However, it was found that these techniques resulted in a small litter size compared with conventional artificial insemination (AI) as well as smaller number of spermatozoa at the uterotubule junction. The earlier studies reported that steroid hormones influenced the transportation of spermatozoa, ovum and embryos in the sow reproductive tract which related to the presence of their specific receptors; oestrogen receptor alpha (ERα) and progesterone receptor (PR). Therefore, the present study aims to evaluate the immunolocalization of ERα and PR in the sow oviducts, uterus and cervix after different artificial insemination techniques. The percentage of ERα and PR immunostaining was evaluated by manual scoring and image analysis system and the results showed significant lower percentage of positive staining in IUI and DIUI groups compared with AI group in the oviduct, uterus and cervix for ERα and only in the oviduct and cervix for PR. In the oviduct, significant higher immunostaining of both ERα and PR was observed in AI group compared to the others. It has been demonstrated that oestrogen (E_2) in boar semen can up-regulate steroid receptors in the pig reproductive tract, a small volume of semen used for IUI and DIUI groups might also influence the lower expression of these steroid receptors due to the lower amount of E_2. In the uterus, significant higher ERα immunolocalization found in the glandular epithelium of AI groups may indicate the higher successful fertilization as glandular epithelium was known for secretory activity mediated through ERα in order to facilitate fertilization and pregnancy. On the other hand, the prominent staining was found in the muscular layer of the cervix in which higher ERα staining was found in DIUI group and it was suggested that the cervix in DIUI group needs more level of ERα in order to mediate cervical muscular contraction compared to the others. In conclusion, the methods of insemination regarding the volume of semen can have the effects on the expression of steroid receptors in the sow reproductive tracts and this may influence the successful fertilization in sows.

Keywords: Artificial insemination, Intrauterine insemination, Deep intrauterine insemination, Reproductive tract, Oestrogen receptor alpha, Progesterone receptor, Sow

Comparative Studies of oestrogen and progesterone receptors in reproductive organs of artificial inseminated (AI), Intrauterine inseminated (IUI) and deep intrauterine inseminated (DIUI) sows

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Abstract

In pig production, there are efforts to reduce the number of spermatozoa by insemination into the posterior region of the cervix. By using special device that can be passed through the cervix allowing the deposition of sperm in the uterine body (Intrauterine insemination, IUI) or uterine horn (Deep intrauterine insemination, DIUI), the number of spermatozoa per dose could be reduced. However, it was found that these techniques resulted in a small litter size compared with conventional artificial insemination (AI) as well as smaller number of spermatozoa at the uterotubule junction. The earlier studies reported that steroid hormones influenced the transportation of spermatozoa, ovum and embryos in the sow reproductive tract which related to the presence of their specific receptors; oestrogen receptor alpha (ERα) and progesterone receptor (PR). Therefore, the present study aims to evaluate the immunolocalization of ERα and PR in the sow oviducts, uterus and cervix after different artificial insemination techniques. The percentage of ERα and PR immunostaining was evaluated by manual scoring and image analysis system and the results showed significant lower percentage of positive staining in IUI and DIUI groups compared with AI group in the oviduct, uterus and cervix for ERα and only in the oviduct and uterus for PR. In the oviduct, significant higher immunostaining of both ERα and PR was observed in AI group compared to the others. It has been demonstrated that oestrogen (E_2) in boar semen can up-regulates steroid receptors in the pig reproductive tract, a small volume of semen used for IUI and DIUI groups might also influence the lower expression of these steroid receptors due to the lower amount of E_2. In the uterus, significant higher immunolocalization was found in the GE of AI groups may indicate the higher successful fertilization as GE was known for secretory activity in order to facilitate fertilization and pregnancy. On the other hand, the prominent staining was found in the muscular layer of the cervix in which higher ERα staining was found in DIUI group and it was suggested that the cervix in DIUI group needs more level of ERα in order to transport the lower number of semen compared to the others. In conclusion, the methods of insemination regarding the volume of semen can have the effects on the expression of steroid receptors in the sow reproductive tracts and this may influence the successful fertilization in sows.

Keywords: Artificial insemination, Intrauterine insemination, Deep intrauterine insemination, Reproductive tract, Sow

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Executive summary

Introduction and objectives

In Thailand, artificial insemination (AI) has an important roles in swine industry in order to increase the efficiency in swine production. Regarding conventional artificial insemination, the diluted fresh semen was used and released at the cervix of female pigs. This can reduce the numbers of spermatozoa for 5-10 folds compared to natural mating. However, Sumransap et al. (2007) found that this technique caused the loss of more than 90% of spermatozoa before they can reach the fertilization site which caused by the drawback of the semen(Steverink et al., 1998). In order to overcome this problem, the newer technique of artificial insemination was introduced by insertion the insemination cohet into the female reproductive tracts and release the semen in the uterus which was called "intrauterine insemination" (IUI) and deeper into the uterine horn which was called "deep intrauterine insemination" (DIUI) without any surgical treatment. There was early study on the IUI technique by Watson and Behan (2002) showed that the number of spermatozoa used was only 1x 10^9 per dose which was not affected the number of fertilization rate. In addition, the study by Martinez et al. (2001; 2002) reveal that the DIUI technique which release the semen at the uterine horn can reduce the concentration of the semen to 60 folds compared to convention AI. In Thailand, the number of 150 million spermatozoa was used for DIUI technique which resulted in the number of fertilization rate in 5 from 8 gilts. Moreover, the embryos was also found in both sides of uterine horn with the average of 11.4 embryos per gilts Tumaruk et al. (2007). This certify that this new techniques of artificial insemination may be able to use with freezing semen, sex determination semen and may also be able to develop to use for embryonic transfer in the future.

The mechanism of sperm transportation in the female reproductive tracts is complicated and regulated by several biological factors both from the female as well as the quality and concentration of semen (Rodriguez-Martinez et al., 2005). After insemination, spermatozoa will be transported through the reproductive tract to sperm reservoir. There are studies revealed that ovulation can affect sperm distribution and transportation, i.e. sperm distribution from sperm reservoir by the changes in hormonal levels during ovulation (Hunter, 1984). In addition, the contraction of female reproductive tract was also needed in order to transport sperm to sperm reservoir (Langendijk et al., 2002a). Also, the contraction of the oviduct takes part in the transportation of ovum to the fertilization site(Orihuela et al., 2001).

From the study of Langendijk et al. (2002b), it was showed that during estrus, the myometrium contraction was activated under the influence of steroid hormones. Similarly, another study reported that estrogen addition in the semen can activate sperm transportation and reduced the loss of semen from artificial insemination (Willenburg et al., 2003). In female rabbit, exogenous estradiol can increase the efficiency of artificial insemination by increasing the number of spermatozoa at the oviducts, uterus and cervix (Hawk et al., 1982). In pig, there was an evidence that the amount of estrogen found in the boar semen has an effect on activating the myometrial function for spermatozoa transportation (Claus, 1990) and this was involved with the influence of estradiol acting through the expression of oestrogen receptor subtype alpha (ERα) in the uterus (Sukjumlong et al., 2004b). However, the mechanism of oestrogen acting on myometrial contraction was still unclear as there was a study report that exogenous hormone can cause lower fertilization rate from inappropriate myometrial contraction (Langendijk et al., 2005). In addition to oestrogen influencing on muscular contraction in female reproductive tract, progesterone which was found highly increased after ovulation may involve with sperm transportation as well as embryonic migration after fertilization as well (Mburu et al., 1996). The injection of exogenous progesterone into the pig oviduct cause polyspermy from the changes of reproductive tract environment under the influence of progesterone (Hunter, 1972). The changes in the levels of hormones as well as their receptors may involve with sperm transduction in the female reproductive tracts, thus the mechanism of these events still not clearly understood.

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; Spencer and Bazer, 2002; Lessey, 2003; Drummond, 2006). These steroid hormones elicit their functions by binding through specific receptor proteins in target tissues (Jensen and DeSombre, 1973; Jensen, 1991; Yamashita, 1998), therefore the presence of steroid receptors is as important as the levels of steroid hormones as 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 sows 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., 2004a). Moreover, the presence of ER in the gene level was involved with the reproductive performance of the pigs (van Rens et al., 2000; Isler et al., 2002). Though the studies of steroid receptors in normal reproductive tracts are widely documented, the data of these receptors localization in the sows after different artificial insemination techniques is still lacking. Therefore, the present study aims to investigate the presence of steroid receptors, oestrogen receptor subtype alpha (ERα) and progesterone receptor (PR) in the sow reproductive tracts after different artificial insemination techniques.

2. Material and Methods

Animals

Twelve crossbred Landrace×Yorkshire multiparous sows were purchased from a commercial swine herd and were brought to the department of Obstetrics, Gynaecology and Reproduction, Nakhon Pathom province, Thailand on the day of weaning. The sows were kept in individual pen and were fed twice a day (approximately 4.0-5.0 kg per day) with a commercial feed (Starfeed176® BP Feed Co. Ltd, Saraburi, Thailand) containing 15.0% protein, 2.0% fat and 10.0% fiber. Water was provided *ad libitum* via water nipples. The sows were carefully detected for the onset of standing estrus twice a day (am/pm) after weaning.

Detection of estrus and ovulation

Estrus detection was initially performed on the day after weaning (Day 1), by allowing the sows to have nose-to-nose contact with a mature boar and applying the back pressure test. Sows with a standing reflex were considered in estrus. The onset of estrus was defined as the first time the sow showed standing reflex minus 6 h. At standing estrus, hCG (Chorulon®, Intervet Ltd., Boxmeer, The Netherlands) 750 IU was administrated intramuscularly to the sows in order to induce ovulation (Wongkaweewit et al., 2012). The time of ovulation was determined by monitoring an appearance of the follicles every 8 h using transrectal realtime B-mode ultrasonography adjusted to a 5-MHz linear transducer (Honda Electronics Co., Ltd., Tokyo, Japan). The ovulation time was defined as 4 h before the first time when no follicle was visible.

Artificial insemination

All sows were divided into 3 groups according to the technique of insemination which were conventional artificial insemination (n = 4), Intrauterine insemination (n=4) and Deep intrauterine insemination (n=4). The sows were inseminated with a single dose of diluted semen during the second oestrus after weaning. The time of ovulation during the first oestrus was used to determine the timing of insemination, which was carried out at 6–8 h prior to the expected time of ovulation. Semen with a motility of $\geq$70%, a concentration of $\geq$150 x10^6 spermatozoa/ml and with normal sperm $\geq$85%, was extended with Beltsville thawing solution (Pursel and Johnson, 1976). The sperm dose contained 3000x10^6 spermatozoa in 100 ml for AI, 1000 x10^6

spermatozoa in 50 ml for IUI and 150 x10^6 spermatozoa in 5 ml for DIUI. Both the IUI and the DIUI techniques have been adapted from Sumransap et al. (2007). Briefly, after cleaning the perineal area of the sows, a commercial AI catheter (Goldenpig®;Minitube, Tiefenbach, Germany) was inserted through the vagina into the cervix where the diluted semen was deposited in AI group. In IUI group, the IUI device (Magaplus®, Magapor, Ejea de los Caballeros, Spain) was inserted through the vagina into the cervix. Thereafter, the inner tube extended about 20 cm beyond the tip of the outer catheter and resided in the uterine body or the posterior uterine horn in order to deposit the diluted semen. In DIUI group, the long flexible catheter (1.8 m) was inserted through the conventional AI catheter. This long catheter was moved forward and deposited diluted semen in the uterine body along one uterine horn (unknown side) for its full length. The diluted fresh semen with 150 x10^6 motile sperm in 5.0 ml was deposited in the proximal third of one side of the uterine horn. Subsequently, a warm BTS, 2.5 ml in volume was used to flush the semen into the uterine horn after insemination.

Tissue collection

Approximately 12 h after insemination, all sows were slaughter and the reproductive tracts were removed, placed in an ice box and transferred to the laboratory within 30 min. Post-mortem examination was performed on each part of the reproductive organs. Three different parts of reproductive tract which were the oviduct, uterus and cervix were collected and kept in 4% paraformaldehyde for 24-36 h. Thereafter, they were dehydrated, 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 PR primary antibody can recognized both PR-A and PR-B, so the results shown in the present study was the accumulation of PR-A and PR-B. The incubation time for both primary antibodies was 1 h at room temperature.

Negative controls were obtained by replacing the primary antibodies to ERα or PR with normal mouse IgG (sc-2025, Santa Cruz Biotechnology Inc., USA) in a dilution of 1:200.

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 reproductive tissues

The classification of positively stained cells was done separately in each compartment of the reproductive organs. The oviduct were divided into 3 parts which were isthmus, ampulla and infundibulum. The uterus was classified into 3 compartments: surface epithelium (SE), glandular epithelium (GE), and myometrium (Myo). The cervix will be classified into 2 compartments: surface epithelium (SE), and muscular layer (M). The results of the immunostaining were evaluated semi-quantitatively by a manual scoring method as well as image analyses by computer software (Image-pro plus version 6.0, Media Cybernetics, Inc., MD, USA).

Evaluation of the results and statistical analysis

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.

In addition to manual scoring method, the image analysis was performed by using image analysis software (Image Proplus 6.0). Quantification of the immunostaining was performed on five randomly selected fields in each compartment for the uterus and the cervix. Since the mesosalpinx was very thin in the infundibulum, therefore the evaluation was done in selected fields which comprised of the surface epithelium and the mesosalpinx. using colour discrimination software, the nuclear stainining could be distinguished from negative staining. However, it is difficult to exclude the cytoplasmic staining in some cells, therefore it was included in the results in some reproductive organs. The results from image analysis are presented as mean percentage of total area of positive staining per total are of cell nuclei.

The data obtained from both manual scoring and image analysis 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 or percentage of positive staining from each compartment of the reproductive organs were compared between groups using Kruskal-Wallis's test and Wilcoxon rank sum test (NPAR1WAY procedure of SAS), and $P < 0.05$ were regarded to have statistical significance.

3. Results

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 oviduct, uterus and cervix, 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) but with different proportion and intensity. Furthermore, cytoplasmic staining for ERα was observed periodically whereas it was not observed for PR immunostaining. The immunostaining results were summarized according to different artificial insemination techniques in Tables 1-3 for image analysis and in Tables 4-6 for manual scoring

Image analysis

Regarding different insemination techniques, higher percentage of both ERα and PR was found in the AI groups compared to the others in the oviduct (ampulla and infundibulum part for ERα, isthmus and infundibulum for PR) (Table 1.1 and 1.2) while no difference was observed in any compartment of the uterus for PR immunostaining (Table 2.2). The same pattern was noticed for ERα immunostaining in the epithelia (surface and glandular epithelia) (Table 2.1) as well as in the cervical muscle that higher localization was found in AI groups (Table 3). On the other hand, significant higher staining was found in the DIUI group for PR in the surface epithelium of the cervix (Table 3).

When comparing between ERα and PR, the results showed higher positive staining for PR comparing to ERα especially in the cervix. In the uterus, positive ERα and PR immunostaining could be found in all compartments by using image analysis system. The prominent staining of ERα was found in the glandular epithelium while PR was expressed at a high level in all compartments of the uterus. In the cervix, only low immunostaining was

observed for both ERα and PR except those for PR immnostaining in the muscular layer of the cervix

Manual scoring

The manual scoring results showed that higher ERα and PR score was found in AI groups compared to the other insemination techniques though it was not always significantly different (Tables 4-6). Similar to image analysis results, the most prominent ERα score in the uterus was found in the glandular epithelium while it was almost negative in the surface epithelium (Table 5.1). For PR, high immunostaining score was found in all compartments of the uterus. In the cervix, immunostaining score for both ERα and PR was very low in the surface epithelium with no positive immunostaining score for ERα in all groups of insemination techniques (Table 6). On the other hand, high PR immunostaining score was observed in the muscular layer of the cervix but no significant difference was observed among insemination techniques (Table 6).

4. Discussion

From the present study, the immunohistochemical results showed that the localization of steroid receptors ERα and PR varied among different genital organs (oviduct, uterus and cervix). This indicated the differences in physiological status among these different reproductive organs as described by other studies (Couse et al., 1997; Wang et al., 2000; Pfaffl et al., 2001; Okada et al., 2005).

Regarding the different insemination techniques the present results showed higher immunostaining (for both image analysis and manual scoring) in AI group compared to the others. This demonstrates that different insemination techniques may have the influence on the expression of these steroid receptors in different reproductive organs of sows. It has been demonstrated that oestrogen (E_2) in boar semen can up-regulates steroid receptors in the pig reproductive organs, a small volume of semen used for IUI and DIUI groups might also influence the lower expression of these steroid receptors due to the lower amount of E_2. However, the influence of E_2 in boar semen should be different in different reproductive organs and in different compartments of the reproductive tissues.

In the oviducts, the present study supported the results from our earlier study which demonstrated the higher PR staining in the oviductal part of uterotubule junction (UTJ) of AI sows comparing to IUI and DIUI sows (Tummaruk et al., 2010). The suggestion from that study was that DIUI may influence the lower expression of PR in the UTJ from lower number of spermatozoa in the UTJ which served as sperm reservoir (Tummaruk and Tienthai, 2008). Similar to our present results, the lower PR was found in the isthmus part of the oviduct which located near the UTJ as well as in the infundibulum part. As progesterone can influence the transportation of spermatozoa both before and after fertilization (Mburu et al., 1996), this mechanism may mediate through the expression of PR which showed lower expression when the number of sperm was lower by IUI and DIUI technique.

In addition to sperm transportation, oviductal cilia are believed to have the critical role in ovum transport from the oviduct to the uterus in cyclic and pregnant rats (Halbert et al., 1989). Oestrogen may have roles in ovum transport by regulating oviductal ciliogenesis in rats indirectly via ERα in the epithelium (Okada et al., 2003). Moreover, a single injection of 17β-estradiol (E2) on day 1 of the reproductive cycle or pregnancy can shorten oviductal transport of eggs (Croxatto, 2002) and this mechanism was believed to mediate via ERα in the oviductal epithelium as well (Croxatto, 2002; Orihuela et al., 2003). From the results of the present study, we found positive ERα in all parts of the oviducts (isthmus, ampulla and infundibulum) which may involve in the possible mechanism of ciliogenesis regulation via ERα in the oviductal epithelium. However, the oviductal epithelium consisted of two cell types which were ciliated cells and secretory cells thus, further study should be considered regarding the differentiation

between these two cell types. Comparing different insemination techniques in regards to gamete transportation, the higher ERα staining found in AI may indicate that more successful fertilization could result from the better transportation of ovum which may regulated by ERα in the oviductal epithelial cells as well.

Also in the uterus, marked positive staining for ERα was found in the glandular epithelium in all groups with the higher staining in AI groups. The glandular epithelium was known to have secretory activities in order to facilitate conceptus survival and development in pigs (Bazer and Roberts, 1983; Gray et al., 2001; Mathew et al., 2011). Therefore, the higher volume of spermatozoa and semen in AI technique may involve in the mechanism of high expression of ERα. On the other hand, in the uterine surface epithelium, high expression of ERα was found in DIUI group compared to AI group. The downregulation of ERα in the surface epithelium observed as soon as 5-6 h after AI might be the results from insemination as described before by sukjumlong et al.(2004b). In 1993, ott et al. suggested that the decrease in oestrogen receptor protein was the way the endometrium was able to remain responsive to progesterone and thereby maintain pregnancy. Therefore, the low presence of ERα may be the results of the increasing progesterone levels after ovulation. However, this low presence of ERα in the surface epithelium occurred already at 12 h after insemination. Therefore the amount of semen could also responsible for this as it is shown that oestradiol can down-regulate its own receptor in the endometrium of ovariectomized gilts (Sahlin et al., 1990). Supporting this concepts, the results of our study showed significant lower ERα staining in AI group which performed by using the higher volume of semen compared to DIUI group.

The similar results were observed in the myometrium that significant high expression of ERα was found in the DIUI group. In general, the myometrium has a crucial function as it is the compartment which undergoes contraction in order to transport sperms (Kunz et al., 1997; Bulletti et al., 2000) and/or embryo if fertilization occurs (Nathanielsz et al., 1995; Bulletti and de Ziegler, 2006). The presence of ERα was important for myometrial contractions in the influence of increasing oestrogen levels as has been shown in women by Li et al (1995). During estrus, uterine contraction are needed for semen transportation (Langendijk et al. 2002) and that oestrogen in boar semen can result in the increase of myometrial contraction (Claus, 1990). Therefore, the deep intrauterine insemination technique which released the semen at the uterine horn may directly activate the expression ERα in the myometrium for uterine contraction than other methods. However, when compared ERα in the myometrium with ERα in other compartments such in the uterine glands, low expression of ERα should be found in the myometrium of all inseminated sow as the uterus should be quiescent after fertilization and during early pregnancy as described before in the earlier study (Sukjumlong et al., 2004b).

For PR immunostaining in the uterus, high expression was observed in all insemination groups and in all compartments of the uterus which is in accordance with other studies on PR in pigs (Koziorowski et al., 1984; Kotwica, 1986; Stanchev et al., 1990; Geisert et al., 1994; Sukjumlong et al., 2005) as well as in other species (Dhaliwal et al., 1997; Ing and Tornesi, 1997; Bouchard, 1999). Though it was shown by our present results that insemination technique may have the effects on ERα localization, these effects may not involve in the presence of PR in these groups of inseminated sows. In addition, from the earlier study, the downregulation effect on the presence of PR from insemination was clearly observed from 20-25 h after ovulation (Sukjumlong et al., 2005). Therefore, the changes and differences from insemination techniques could be expected at the later period. Furthermore in the myometrium, high PR expression was observed at all groups of insemination techniques. The speculation from this result was that PR in the myometrium was upreguated by high plasma levels of oestradiol during oestrus in order to prepare for the progesterone regulation via PR at the following stages of early pregnancy.

In general, there are two isoforms of PR; PR-A and PR-B which were arisen 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 the results of the present study was the accumulation of PR-A and PR-B and therefore the difference between these two isoforms of PR could not be demonstrated. On the other hand, there may be some difference in the expression of PR-A in these inseminated sows, 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 sows. However, the different localization of PR-A should be further studied as it may reveal or explain the possible effects on the localization of PR in different inseminated sows.

In the muscular layer of the cervix, which serves as the major compartment for cervical constriction or dilation under the influence of hormones (van Engelen et al., 2009), it was shown that higher score was observed in DIUI groups for ERα. This may because the cervix in DIUI group needs more level of ERα in order to mediate muscular constriction to prevent the drawback of the lower number of semen compared to the others. However, the mechanisms of cervical constriction or dilation are complex and need more than only the regulation from the expression of ERα. When considering PR immunostaining in the cervical muscle, high expression was observed in all groups with no difference, this may result from preparation of cervical muscular quiescent after fertilization by PR as it was documented in the earlier study (Sukjumlong et al., 2005).

In the cervical epithelium the result of PR from image analysis was vice versa that high PR expression was found in DIUI group. In inseminated sows, a significant lower of PR immunostaining could be observed in the surface epithelium of the uterus at 20-25 h after ovulation which indicated the downregulation effect from insemination (Sukjumlong et al., 2005). The downregulation effect on PR localization was also observed in the surface epithelium of the cervix in the present study. However, the higher PR presence in DIUI group compared to AI group may be the results from prolonged downregulation effect as the semen was deposited deeply in the uterine horn in DIUI group. However, from that earlier study, the presence of PR increased at the later stages of inseminated sows, thus it was not clear whether the temporary downregulation of PR was caused only by insemination or other regulatory mechanisms (Sukjumlong et al., 2005). Further, there is a limited data on the expression of steroid receptors in the porcine cervix and thus, the normal value of steroid receptor expression during different reproductive status should be documented.

From the result of the present study it was also demonstrated that ERα immunostaining was found in the cytoplasm of several reproductive cells i.e. in the epithelia of the uterus and oviduct. This is in agreement with previous studies reporting localization of ERα in non-nuclear sites of reproductive cells (Welshons et al., 1984; Marquez and Pietras, 2001; Monje and Boland, 2001; Monje et al., 2001). Moreover the study in rat demonstrated that mating can increase the number of ERα in non-nuclear compartments. The increase in ERα in non-nuclear compartment involved with the changes from non-genomic pathway in cyclic rat to genomic pathway in mating rat (Okada et al., 2003). The changes in these pathways has been designated "intracellular path shifting, or IPS" (Parada-Bustamante et al., 2007). Therefore, the marked ERα cytoplasmic staining could be involve with the changes of IPS induced by mating or insemination in the present study since no cytoplasmic staining was observed in cyclic sows (Sukjumlong et al., 2005) nor in epithelial cells for PR immunostaining.

In the present study, immunostaining of both ERα and PR was evaluated by two different methods, a manual scoring and image analysis. The results from these two methods were in agreement with each other, though minor differences could be observed in some groups

of reproductive organs. The explanation of the difference was that the manual scoring showed variations in patterns of immunostaining with regard to both proportion and intensity while the image analysis quantified the total amount of positive staining in randomly selected area. Moreover cytoplasmic staining could be excluded from manual scoring method but still be detected by image analysis system as it was shown in the present results.

In conclusion, the present study showed the differences in steroid receptor expression among different insemination methods and among different reproductive organs as well as in different tissue compartments. The methods of insemination regarding the volume of semen can have the effects on the expression of steroid receptors in the sow reproductive tracts and this may also influence the successful fertilization in sows

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Table 1.1 Percentage of ERα positive staining from image analysis software in the different parts of the oviduct

Insemination	Isthmus	Ampulla	Infundibulum
AI	37.78 ± 9.4	31.95 ± 13.82 a	45.33 ± 26.01a
IUI	31.08 ± 16.54	37.66 ± 23.58a	36.15 ± 21.89a
DIUI	33.89 ± 18.5	15.36 ± 10.63b	25.19 ± 19.26b
Overall significant	NS	P <0.05	P <0.05

Table 1.2 Percentage of PR positive staining from image analysis software in the different parts of the oviduct

Insemination	Isthmus	Ampulla	Infundibulum
AI	51.97 ± 11.95a	41.72 ± 8.6	43.59 ± 24.12a
IUI	27.89 ± 26.07b	33.07 ± 25.01	23.45 ± 18.65b
DIUI	30.27 ± 16.42b	46.63 ± 15.23	26.61 ± 24.08b
Overall significant	P <0.05	NS	P <0.05

AI = conventional artificial insemination
IUI = intrauterine insemination
DIUI= deep intrauterine insemination
Different letters within the same column represent significant difference

Table 2.1 Percentage of ERα positive staining from image analysis software in the different compartment of the uterus

insemination	SE	GE	Myo
AI	0.19 ± 0.45a	67.18 ± 6.68a	1.95 ± 1.54
IUI	5.72 ± 5.12b	22.63 ± 33.92b	2.14 ± 2 .42
DIUI	4.69 ± 0.74b	36.20 ± 16.76b	7.48 ± 4.90
Overall significant	P <0.05	P <0.05	NS

Table 2.2 Percentage of PR positive staining from image analysis software in the different compartment of the uterus

insemination	SE	GE	Myo
AI	99.89 ± 0.12	99.65 ± 0.14	96.58 ± 2.82
IUI	87.17 ± 14.1	91.82 ± 5.07	94.71 ± 3.35
DIUI	84.62 ± 11.51	89.76 ±5.93	98.06 ± 0.79
Overall significant	NS	NS	NS

AI = conventional artificial insemination
IUI = intrauterine insemination
DIUI= deep intrauterine insemination
SE = surface epithelium, GE = glandular epithelium, Myo = myometrium
Different letters within the same column represent significant difference

Table 3
Percentage of ERα and PR positive staining from image analysis software in the different compartment of the cervix

Insemination	**ERα-SE**	**ERα-M**	**PR-SE**	**PR-M**
AI	0.92 ± 0.64	3.90 ± 2.9 a	0.19 ± 0.56 a	27.33 ± 11.93
IUI	2.44 ± 2.56	0.27 ± 0.17 b	0.93 ± 0.82 a	21.90 ± 4.10
DIUI	1.89 ± 1.22	0.14 ± 0.19 b	7.91 ± 5.10b	28.79 ± 15.98
Overall significant	NS	P <0.05	P <0.05	NS

AI = conventional artificial insemination
IUI = intrauterine insemination
DIUI= deep intrauterine insemination
SE= surface epithelium, M=muscular layer of the cervix
Different letters within the same column represent significant differences. NS= not significant;

Table 4.1 ERα positive staining score from manual scoring in the different parts of the oviduct

Insemination	Isthmus	Ampulla	Infundibulum
AI	4.0 +2.0	4.16 +2.0a	3.6 +2.08a
IUI	2.16 + 0.28	2.16 + 0.28b	1.34 +1.15b
DIUI	2.83 +0.76	2.16 +0.28b	0.67 +1.15b
Overall significant	NS	P <0.05	P <0.05

Table 4.2 PR positive staining score from manual scoring in the different parts of the oviduct

Insemination	Isthmus	Ampulla	Infundibulum
AI	6.67 +0.28	5.33 +0.28	4.83 +0.57a
IUI	4.5 + 0.86	4.67 + 0.76	2.0 + 0b
DIUI	6.3 + 0.29	6.16 +0.28	2.5 +1.0b
Overall significant	NS	NS	P <0.05

Table 5.1 ERα positive staining score from manual scoring in the different compartment of the uterus

Insemination	SE	GE	Myo
AI	2.6 ± 1.15a	8.3 ± 3.6a	3.3 ± 1.5
IUI	0.6 ± 1.1b	5.6 ± 0.2b	3.0 ±1.0
DIUI	2.3 ± 0.5a	5.8 ± 0.2b	2.0 ±0.0
Overall significant	P <0.05	P <0.05	NS

Table 5.2 PR positive staining from manual scoring in the different compartment of the uterus

Insemination	SE	GE	Myo
AI	6.5 ± 0.5	6.5 ± 0.5	6.30 ± 0.57
IUI	5.8 ± 0.2	4.5 ± 1.3	3.60 ± 0.5
DIUI	6.16 ± 1.04	6.83 ± 0.28	6.66 ± 0.57
Overall significant	NS	NS	NS

Table 6
ERα and PR positive staining score from manual scoring in the different compartment of the cervix

Insemination	**ERα-SE**	**ERα-M**	**PR-SE**	**PR-M**
AI	0	2.67 ± 1.15	0	5.67 ± 2.75
IUI	0	1.83 ± 0.29	0.67 ± 1.15	3.60 ± 1.15
DIUI	0	2.0 ± 0	1.83 ± 0.28	4.16 ± 0.76
Overall significant	NS	NS	NS	NS

AI = conventional artificial insemination
IUI = intrauterine insemination
DIUI= deep intrauterine insemination
SE= surface epithelium, M=muscular layer of the cervix
Different letters within the same column represent significant differences. NS= not significant

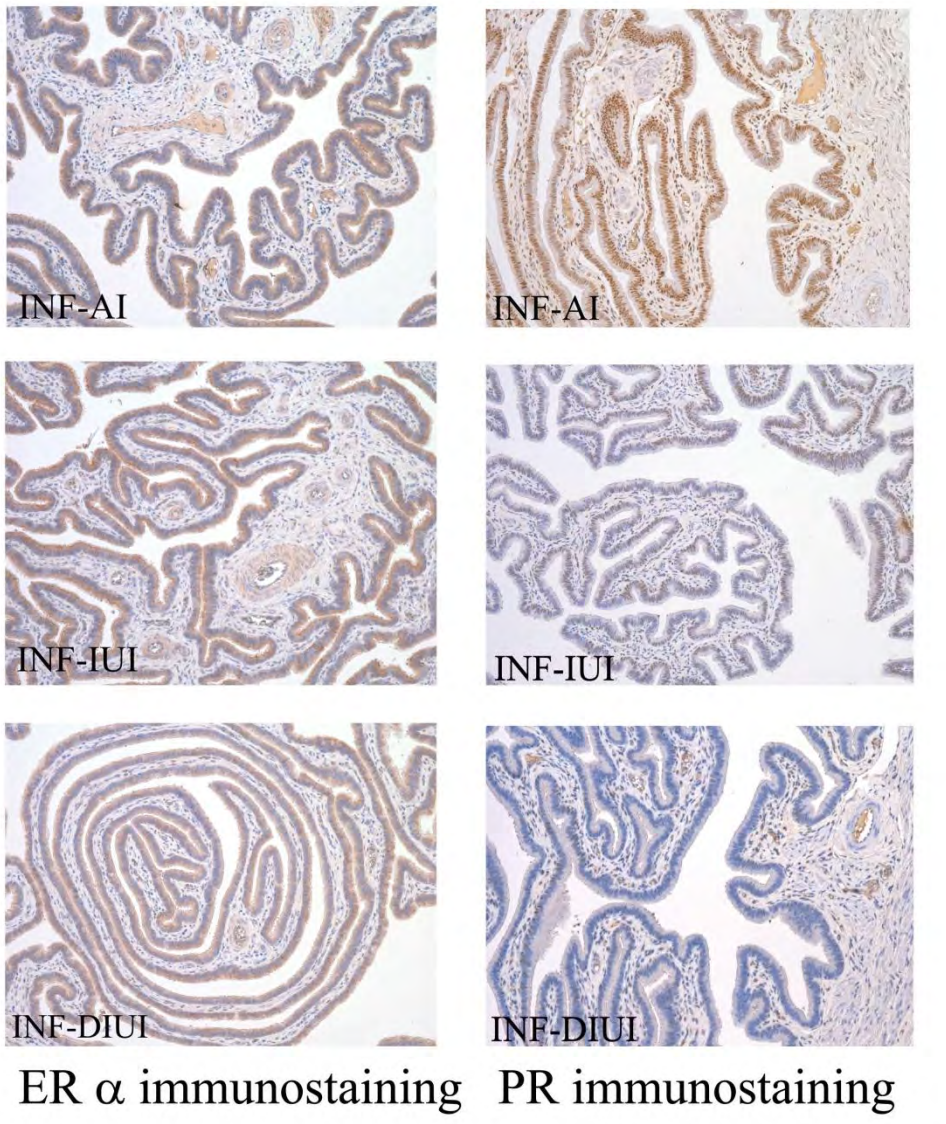

Figure 1 ERα immunostaining (left column) and PR immunostaining (right column) in the infundibulum part of the oviduct. INF = Infundibulum, AI= Conventional artificial insemination, IUI = Intrauterine insemination, DIUI = Deep intrauterine insemination.

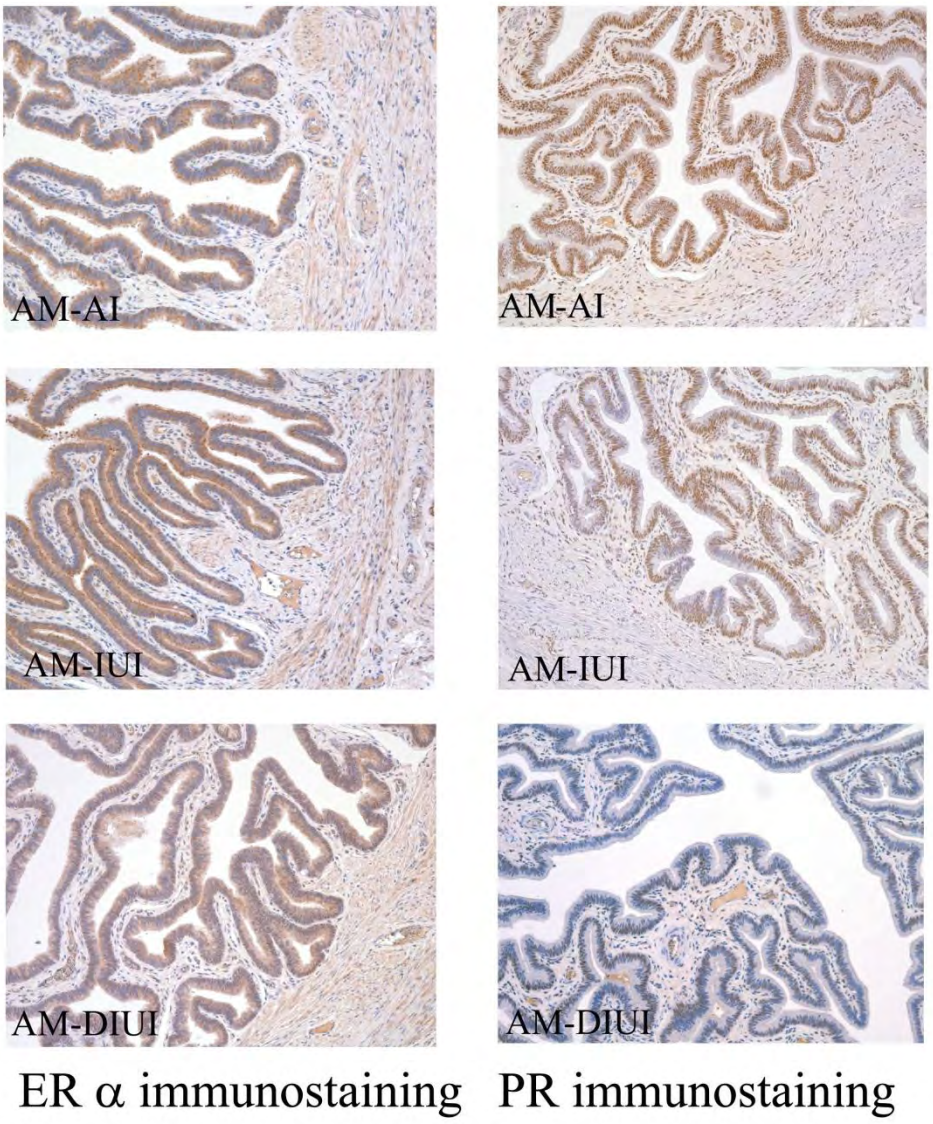

Figure 2 ERα immunostaining (left column) and PR immunostaining (right column) in the ampulla part of the oviduct. AM = Ampulla, AI= Conventional artificial insemination, IUI = Intrauterine insemination, DIUI = Deep intrauterine insemination.

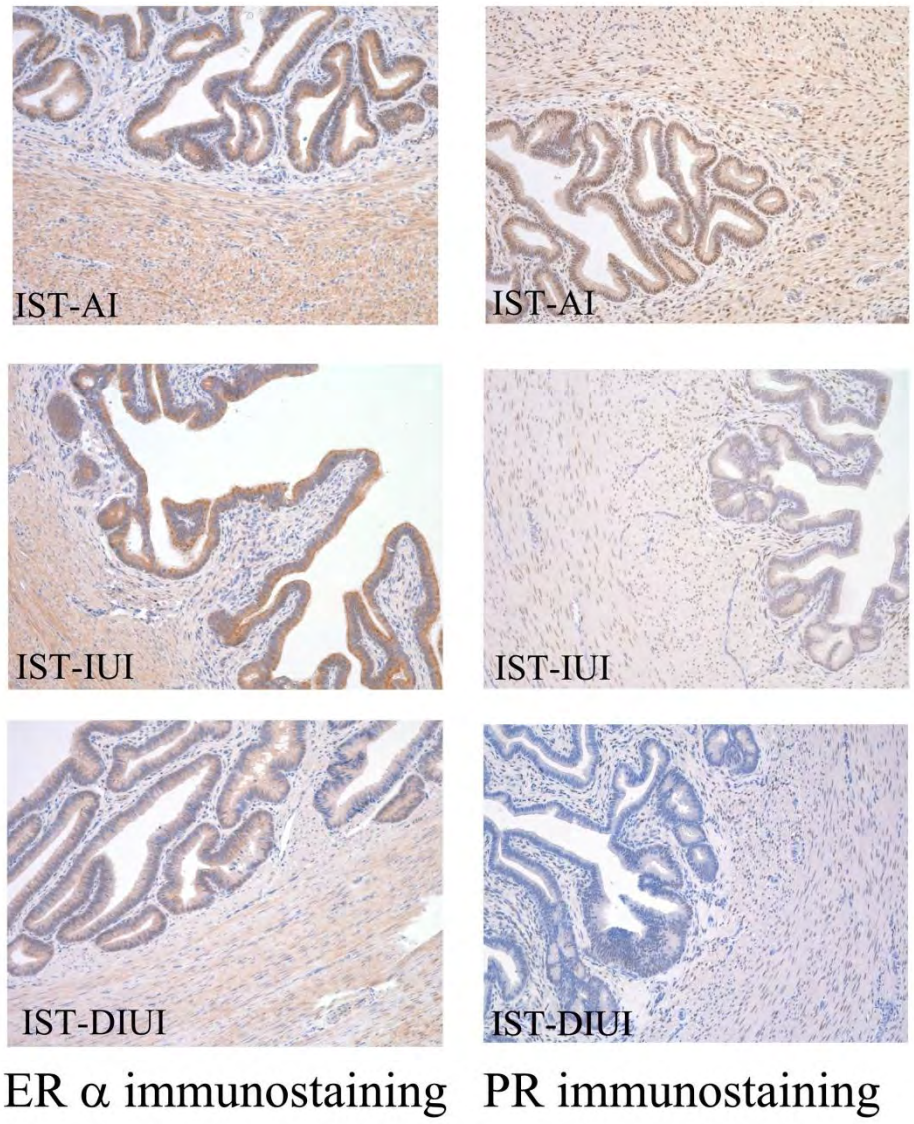

Figure 3 ERα immunostaining (left column) and PR immunostaining (right column) in the ampulla part of the oviduct. IST = Isthmus, AI= Conventional artificial insemination, IUI = Intrauterine insemination, DIUI = Deep intrauterine insemination.

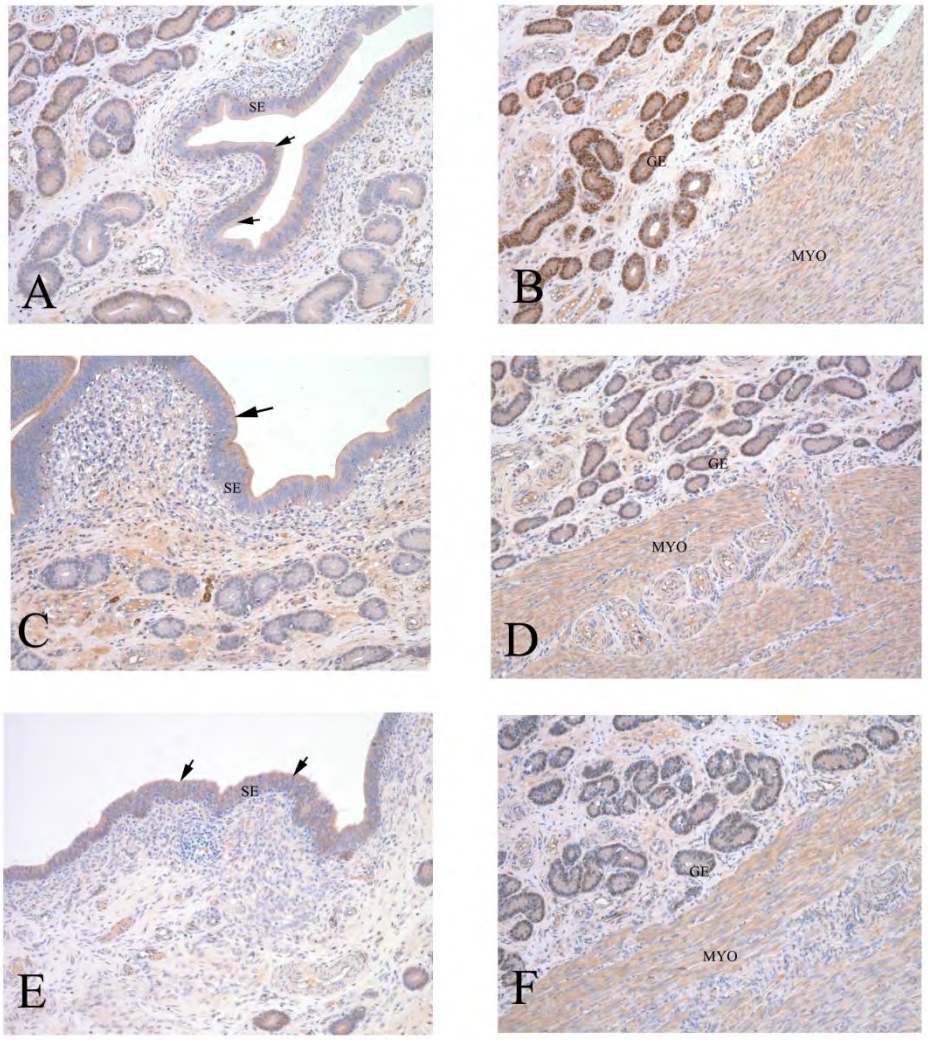

Figure 4 ERα immunostaining in the uterus according to different insemination techniques. A-B: conventional artificial insemination group (AI); C-D, Intrauterine insemination group (IUI); E-F, Deep intrauterine insemination group (DIUI). SE = Surface epithelium; GE = Glandular epithelium and Myo = Myometrium. Arrows show cytoplasmic staining in the uterine epithelium.

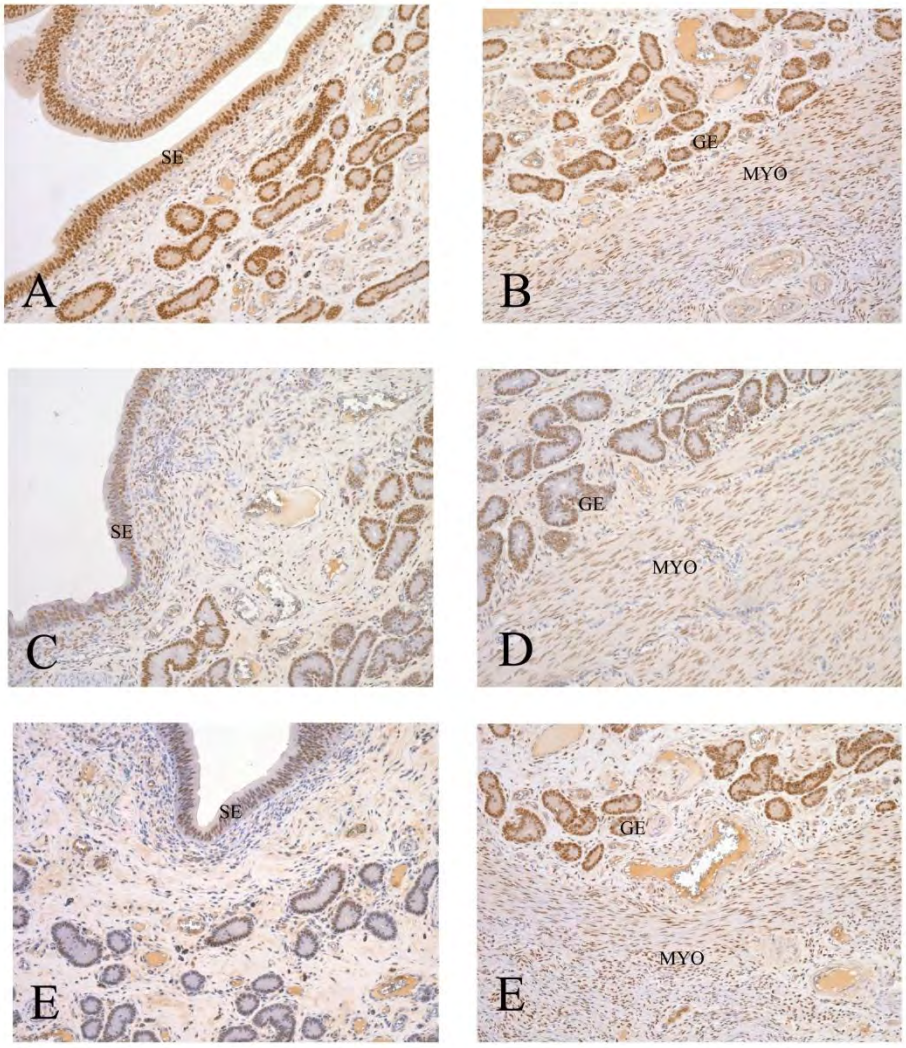

Figure 5 PR immunostaining in the uterus according to different insemination techniques. A-B: conventional artificial insemination group (AI); C-D, Intrauterine insemination group (IUI); E-F, Deep intrauterine insemination group (DIUI). SE = Surface epithelium; GE = Glandular epithelium and Myo = Myometrium.

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Suggestion for future research

In order to clarify more understanding on the localization of steroid receptors regarding the effect of insemination, further studies are planned as showed below

1. Comparing between insemination by using freeze-thaw semen and fresh diluted semen in order to study the effects of semen quality in relation to the expression of steroid receptors in the reproductive organs of female pigs
2. Expand the study of different insemination techniques regarding estradiol supplement in the boar semen and to evaluate the effects of this on steroid receptor expression.
3. Further studies on the localization of steroid receptors during the oestrous cycle and after early insemination in the cervix in order to have basic information on the level of these steroid receptors in the pig cervix which is still limited
4. Increase the number of sows in each experiment and design to collect the sample in a longer period than 12 h after insemination