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Immunohistochemical studies on distribution of ERα in the uterus of sows with reproductive disturbances

Birutė Karvelienė[1],

Henrikas Žilinskas[2],

Aloyzas Januškauskas[2],

Vita Riškevičienė[1]

[1] Department of Infectious Diseases, Lithuanian Veterinary Academy, Tilžės 18, Kaunas, Lithuania
E-mail: birutek@lva.lt; vitarisk@lva.lt

[2] Department of Non-infectious Diseases, Lithuanian Veterinary Academy, Tilžės 18, Kaunas, Lithuania
E-mail: rektorius@lva.lt; janusalo@lva.lt

The aim of the present study was to examine the distribution of oestrogen receptor alpha (ERα) in the uterus of sows with reproductive disturbances as well as to study the expression of ERα in relation to plasma oestradiol-17β (E_2) and progesterone (P_4) concentrations in the blood. Crossbred 3–4 parity Lithuanian White x Danish Landrace breed sows presenting various reproductive disturbances were recruited for the present study. Immunohistochemical investigation of sows' uterus horns was carried out by the streptavidin–biotin peroxidase method using antibodies against ERα. Blood samples were collected from the jugular vein 1 h prior to slaughter for analysis of E_2 and P_4 levels.

Examination revealed that ovaries in 78.3% of sows remained active during the supposed anoestrus. 5% of the sows with active ovaries, as it became evident after post-mortem examination, were in oestrus despite the fact that no visual signs of oestrus were observed before slaughtering. The ERα were distributed in all uterine tissue compartments: surface epithelium, subepithelial connective tissue, glandular epithelium and in muscle cells of the myometrium. The lowest number (<30%) and lowest staining intensity (+) of positively stained for ERα nuclei found in uterine wall compartments were observed at oestrus, though the E_2 concentration in blood was high (43.03 pmol/l). Glandular epithelial nuclei stained positively for ERα in >80% of cells at late dioestrus, but staining intensity differed through the rest of the oestrus cycle stages. The most prominent staining of ERα in the myometrial cells was found at pro-oestrus when E_2 was high (350.79 pmol/l) and P_4 was low (15.45 nmol/l), and at late dioestrus when the concentration of E_2 and P_4 was low (3.42 and 0.19 nmol/l, respectively). The concentration of P_4 was high (49.01 nmol/l) at dioestrus and low at late dioestrus, while the concentration of E_2 was low at both stages. The intensity of nuclear positive staining varied not only among the stages of the oestrus cycle, but also among the different uterine compartments.

In conclusion, we determined that sows with reproductive disturbances had cyclic active ovaries, but the levels of P_4 and E_2 in their plasma did not fully fit the physiological norm of these hormones during their oestrus cycle. Similarly, ERα expression in different uterine compartments of sows is the very low.

Key words: reproductive disturbances, oestrus cycle, ovaries, hormone receptors, sow

INTRODUCTION

The structure and functions of female reproductive organs are influenced by steroid hormones. Both E_2 and P_4 express their biological effects on the development, differentiation, and maintenance of reproductive tract and other target tissues through gene regulation by nuclear steroid receptors that function as ligand-dependent transcription factors [1–4]. The E_2 and P_4 are released from the developing follicle and the corpus luteum (CL), respectively. The E_2 stimulates the preovulatory surge of the luteinizing hormone (LH) which in turn stimulates the final maturation of the follicle and ovulation. Plasma P_4 level is high during dioestrus, in non-pregnant animals the CL tends to regress and the production of P_4 drops. The follicles start to grow, the concentration of E_2 increases and a new oestrus cycle begins. When the concentration of E_2 is too low, oestrus in sows

becomes unidentified or "silent". These hormonal changes may cause repeated oestrus, failure to conceive and infertility.

Many of the known physiological actions of oestrogens are considered to be mediated within the target cells primarily by two nuclear oestrogen receptors, ERα and ERβ. Although both isoforms are expressed in the uterus [5], ERα has a broad spectrum of expression [4, 6]. ERα is the isoform that predominantly regulates reproductive behavior and physiology in females [4, 7] and is known to be an important regulator through its ligand-dependent and -independent mechanisms in the uterus [8]. As a result, the studies on the steroid receptor proteins in the sows' uterus with reproductive disturbances may explain some causes of the regulatory mechanisms carried out by the steroid hormones during the oestrus cycle. Most of the studies on ERα in the reproductive organs have been done in gilts [9, 10] and sows with a normal reproductive performance [8, 11].

In order to better understand changes in genital organs of pigs showing reproductive disturbances during the oestrus cycle, the aim of the present study was to investigate plasma oestradiol-17β and progesterone concentrations and changes in the localization of ERα in the uterus of sows with reproductive disturbances.

MATERIALS AND METHODS

Crossbred Lithuanian White x Danish Landrace (LW x DL) sows with reproductive disturbances and 3–4 were used. During the period of approximately three months in autumn (September, October, November) samples of reproductive organs of 150 culled sows with reproductive disturbances (unsuccessful oestrus manifestation) after weaning, conception failure after insemination) were collected from the local abattoir. Immediately after slaughter, genital organs were removed and examined for the number and size of CL and follicles. Based on ovarian appearance, the following stages of the oestrus cycle were assessed: pro-oestrus (19 to 21 day of the cycle), oestrus (0 to 1 day of the cycle), early dioestrus (2 to 7 day of the cycle), dioestrus (8 to 12 day of the cycle), late dioestrus (16to 18 day of the cycle) and anoestrus. For the immunohistochemical investigation, uterine samples were collected from sows (n = 3) at each stage of the oestrus cycle from the mesometrial side of each uterine horn approximately 30 cm from the tip of the horns. Thereafter the uterine samples were fixed in 10% buffered formaldehyde for 48 hours. The samples were then embedded in paraffin and stored for immunohistochemical labelling (labelled streptavidin–biotin (LSAB) reagents (DakoCytomation, CA, USA)).

Blood samples were collected from the jugular vein 1 h prior to slaughter. The plasma E_2 and P_4 levels were determined by radioimmunoassay using commercial E_2 and P_4 diagnostic kit (E2-RIA-CT and PROG-RIA-CT, BioSource Europe S. A., Belgium) according to the method described by Duchens et al. [12] and validated for E_2 analysis in pigs as described by Mwanza et al. [13].

At least two uterine samples were dissected from each animal. Each specimen was cut into 2.5 μm thick sections and mounted on Superfrost® Plus slides (Menzel-Glaser, Freiburg, Germany), deparaffinized in xylene and dehydrated in absolute and then in 95% ethanol. After washing with distilled water the staining procedure was carried out. The slides were placed in a plastic jar, overlaid with distilled water target retrieval solution (1:10) pH 6.1 (DakoCytomation, USA) and heated in a microwave oven at high power for 20 min (5 × 4 min). The slides were allowed to cool for 20 min and then rinsed in PBS. The subsequent procedures were performed at room (25 °C) temperature. Endogenous peroxidase activity was blocked with hydrogen peroxide, followed by rinsing in distilled water. The sections were then placed in a fresh PBS buffer bath. Taped off excess buffer and wiped slides were applied with the primary antibody or negative control reagent. The antibodies or negative control (supernatant mouse monoclonal IgG1 antibody) were added and the sections were incubated for 10 min. The primary mouse monoclonal antibodies used were ERα (Clone: 1D5, DakoCytomation, CA, USA). Following primary antibody binding, the sections were washed in PBS, incubated for 10 min with the link antibody (DakoCytomation, CA, USA) and for 10 min with streptavidin–peroxidase (streptavidin conjugated to horseradish peroxidase) and then incubated for 5 min with substrate–chromogen solution. The sections were washed with distilled water and placed in a bath of Mayer's hematoxylin for counterstaining, washed with ammonia water and mounted in glycerine–gelatine before investigation under a light microscope. For quality control, one known positive control for each set of test conditions was included in each staining run following the DakoCytomation (CA, USA) requirements.

The results are presented for different uterine compartments: surface epithelium, subepithelial connective tissue, glandular epithelium and myometrium. The results of immunohistochemical staining were evaluated in a semi-quantitative way, manually scoring of ERα in all tissue compartments.

The distribution and relative density of cells immunoreactive to the antibody were documented at x 400 magnification under an OLYMPUS BH-2 light microscope. The evaluation of positively stained cells was carried out as a blinded preparation by the same person. For ERα immunohistochemical staining, variation in the intensity of positively stained cell nuclei was present in different uterine horn tissue compartments. Therefore, the staining intensity is expressed as an average of all cells and described as weak, moderate or strong (represented as +, ++, +++, respectively). The proportion of cells staining for ERα was estimated by counting the number of positive cells in randomly selected five fields of view.

The nuclei of positive cells were stained reddish-brown and of negative cells blue. Average proportions of positively stained cells were estimated at four different levels as follows: low proportion (<30% of positive cells, A); moderate proportion (31–50% of positive cells, B); high proportion (51–80% of positive cells, C) and almost all cells positive (>81%, D).

Statistical analysis was performed using the SPSS statistical package [14]. Data included in the model were analyzed using descriptive statistics (means ± SD) and one-way ANOVA analysis. Pearson's rank correlations were used to calculate the relationship between the plasma level of E_2 and P_4 and the number of follicles and CL. Probability values at $p < 0.05$ were considered as statistically significant.

RESULTS

Visual observation of the ovaries revealed that in 78.3% of sows culled due to the reproductive disturbances ovaries remained active and only in 21.6% of sows they were in anoestrus stage (Figure); 5% of sows were in the stage of oestrus despite the fact that no visual signs of oestrus were observed before slaughtering.

The average count of follicles and CL are presented in Table 1. The size of follicles and CL and their color differed depending on the stage of the oestrus cycle. The diameter of follicles (9 to10 mm), as physiologically normal, was largest in the oestrus. The color of CL was pink and their diameter reached 9 to 10 mm during the dioestrus (8 to 12 days of the cycle). During the period of regression in late dioestrus CL tended to be smaller (8 to 5 mm) and yellowish-white in color. The counted CL numbers correlated with the plasma levels of P_4 ($R = 0.54$; $p \leq 0.5$). There was a significant effect of the different stages of oestrus cycle on the mean number of follicles ($p \leq 0.05$) and CL ($p \leq 0.001$) (Table 1).

Steroid hormone concentration in the blood plasma of sows corresponded to the stage of the oestrus cycle (Table 1). The E_2 level was the highest during pro-oestrus and eightfold lower at oestrus. The P_4 concentration in blood during pro-oestrus varied from 14.02 to 17.41 nmol/l and during late dioestrus averaged to 0.19 nmol/l, though at early dioestrus, dioestrus and late dioestrus P_4 and E_2 levels were close to physiological norm. The plasma E_2 and P_4 levels differed significantly during oestrus cycle stages ($p \leq 0.05$).

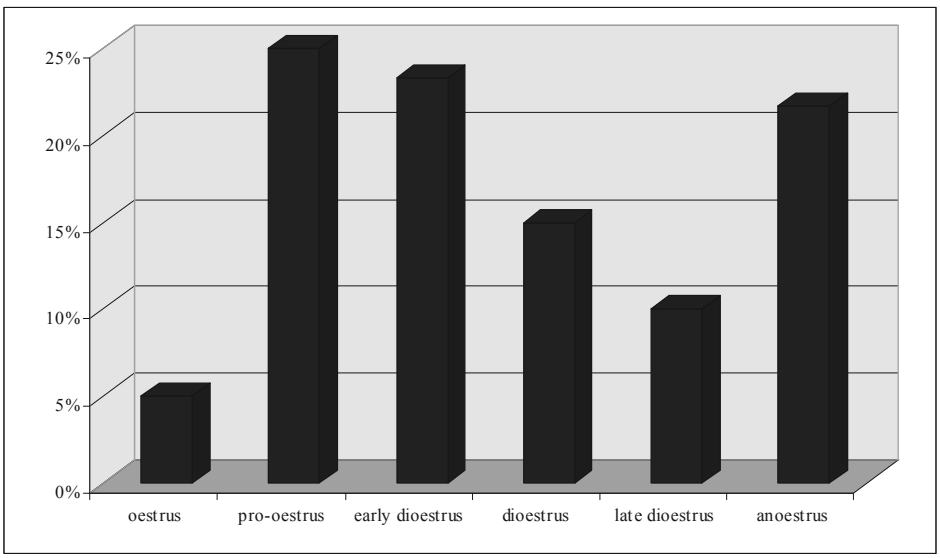

Figure. Stages of oestrus cycle defined in sows slaughtered because of reproductive disturbances

Immunohistochemical staining of ERα in the uteri showed a positive (reddish brown) nuclear staining in all tissue compartments: surface epithelium, subepithelial connective tissue, glandular epithelium, and also in muscle cells of the myometrium. No specific staining was found in the negative controls. The number and intensity of nuclei positively stained for ERα in the all uterine horn wall compartments were lowest at oestrus (A/+), though blood E_2 level was high (43.03 pmol/l). The highest staining intensity of the positive

Table 1. **Differences in the count of follicles, corpora lutea and plasma concentrations of P_4 and E_2 in different stages of sexual cycle**

Oestrus cycle stages	Count of follicles	Count of corpora lutea	Progesterone nmol/l	Oestradiol-17β pmol/l
	Mean ± SD			
Pro-oestrus	25.21 ± 13.02*	0.64 ± 2.41***	15.45 ± 1.75*	350.79 ± 158.73*
Oestrus	22.00 ± 2.83*	3.00 ± 4.24***	0.46 ± 0.03*	43.03 ± 19.94*
Early dioestrus	14.86 ± 15.17*	16.86 ± 2.77***	20.8 ± 5.67*	9.99 ± 1.45*
Dioestrus	31.22 ± 14.03*	21.00 ± 3.60***	49.01 ± 17.86*	5.5 ± 1.12*
Late dioestrus	29.83 ± 10.44*	22.00 ± 8.39***		3.42 ± 0.74*

Differences among the rows are significant; * p £ 0.05; *** p £ 0.001.

cells was at late dioestrus when blood E_2 levels was low (3.42 pmol/l). The intensity of the positive nuclear staining varied not only among the stages of the oestrus cycle, but also among the different uterine compartments. All the immunohistochemical positively stained cells (mean number) and the immunostaining intensity of ERα at different stages of the oestrus cycle are shown in Table 2 according to different tissue compartments. The lowest number of the positive nuclear staining for ERα was determined in the surface epithelium at all oestrus cycle stages (A/+). In the subepithelium, 30 to 50% of positively stained cells were observed at late dioestrus, which was the highest number compared with the other stages of oestrus cycle.

In the endometrial glands, the staining intensity always appeared to be slightly stronger in the deep-laying than in the superficial glands. In the glandular epithelium, more than 80% of cells were positively (light-brown) stained for ERα, and the strongest intensity (+++) staining was consistently observed at late dioestrus, while less than 30% of positive cells were observed at oestrus.

In the myometrium, the strongest intensity and the highest number were found at late dioestrus and pro-oestrus, and the staining intensity was weakest at oestrus and dioestrus (Table 2).

Table 2. **Positively stained cells (mean) / staining intensity of oestrogen receptor alpha (ERα) of sows according to uterine compartments (manual scoring)**

Stage of the oestrus cycle	Surface epithelium	Subepithelium	Glandular epithelium	Myometrium
Pro-oestrus	A/+	A/+	B/++	C/++
Oestrus	A/+	A/++	A/+	A/+
Early dioestrus	A/+	A/+	C/+++	B/++
Dioestrus	A/+	A/+	C/+++	A/+
Late dioestrus	A/+	B/++	D/+++	C/++

A = low proportion (<30%), B = moderate proportion (30–50%), C = high proportion (>50–80%), D = almost all cells are positive (>81%)

Staining intensity: + = weak, ++ = moderate, +++ = strong.

DISCUSSION

Reproductive organs and particularly the ovarian physiology of sows highly depend upon a complex sequence of events of both endocrinological and neuroendocrinological nature. In the organism of sows, there functions a system of regulation among the hypothalamus, hypophysis and genital organs, which ensures sows' sexual development, maturation, cycle, fertility and other phenomena [15–18]. In order to evaluate changes in sows' genital organs and the stage of oestrus cycle and to overview ERα distribution in different uterus compartments, post-mortem examination of sows with reproductive disturbances was carried out.

The present study showed that 78.3% of sows were cycling and their ovaries were active, though they presented no visible oestrus signs and were regarded as anoestrous or failing to conceive. Changes in the sow reproductive organs are governed by P_4 and E_2 and their receptor proteins [19, 20]. According to Cooke et al. [3], E_2 effects on uterine epithelium are modified directly by the epithelial ERα or indirectly by the stromal ERα. Sukjumlong et al. [21] also found that the presence of ERα in the uterine compartments was generally related to the plasma levels of ovarian steroid hormones. We, however, found differences with respect to the uterine compartment and physiological status of the uterus in pigs with reproductive disturbances. The amount of ERα in the uterus of sows with reproductive disturbances at pro-oestrus showed the lowest number and intensity of positively stained cells in the surface epithelium and subepithelium, while in the glandular epithelium and myometrium cells they were higher. Possibly the amount of P_4 was suitable (15.45 nmol/l) to antagonize the upregulatory effect of E_2 on the ERα level both in the surface epithelium and subepithelium. That E_2 are influenced by interactions (cross-talk) with other gonadal steroids like P_4 has also been found by Cardenas [22].

The ERα were observed in the nuclei of all tissues of sow uterus, but the staining intensity of positive cells in it was different (Table 2). The highest staining intensity of ERα, was detected in the nuclei of glandular epithelium and myometrium at all oestrus cycle stages in sow uterus. Sukjumlong [23], who investigated normal cyclic sows, showed that in cyclic sows almost all surface epithelial cells were positively stained for ERα with the highest intensity at dioestrus and early dioestrus, while in our study of sows with reproductive disturbances the weakest immunostaining and the lowest proportion of stained cells were observed in surface epithelium at all oestrus cycle stages. Many reproductive processes in the uterus are mediated in a paracrine manner via receptors in the stroma [24]. The presumptive factor of that phenomenon could be the low content of ERα protein in surface epithelium or disturbed stromal ERα and stromal–epithelial interactions. Stromal cells may be of higher importance than epithelial cells as regards cyclic endometrial changes during the oestrus cycle. At oestrus we also found the highest number of cells positively stained for ERα in the subepithelial layer of the connective tissue stroma compared with other compartments at this stage, but compared with a similar finding by Sukjumlong et al. [11] in an experiment with normally cycling sows the number of positively stained cells was about four times lower. In our study, glandular epithelial nuclei stained positively more than 80% of cells at late dioestrus, but with a different intensity for ERα at all oestrus cycle stages, which may indicate an essential participation of ERα in gland secretary function changes during the oestrus cycle. Similar findings were described by others authors [9, 25, 26] for normally cycling sows and

gilts. In the present study, the most prominent staining of ERα in the myometrium cells was found at pro-oestrus when the concentration of E_2 was high (350.79 pmol/l) and of P_4 low (15.45 nmol/l), and at late dioestrus when the concentration of E_2 and P_4 was low (3.42 pmol/l and 0.19 nmol/l, respectively). One of the major functions of E_2 the regulation of cellular proliferation and growth of reproductive tissues as well as stimulation of endometrial glands and increase of uterine secretory and contractory activity. Uterine contractions in the sow myometrium have been shown to increase around oestrus, and ERα is suggested to mediate the oestrogenic influence that stimulates myometrial contractions [27]. The concentration of P_4 was high (49.01 nmol/l) at dioestrus and low at late dioestrus, while the concentration of E_2 was similarly low at both stages. It is known that P_4 opposes the effect of oestrogens. The high levels of P_4 have been shown to provide a quiescent uterus with a low myometrial activity [28].

The present experiment showed that the immunostaining pattern varied not only during the oestrus cycle and in the different tissue compartments of the uterus, but also among individual sows. In most animals E_2 induced the synthesis of ERα, although the target tissues usually maintain a constitutive receptor content probably controlled by genetic mechanisms [20]. However, the sex steroids, through interaction with different cognate nuclear receptors, regulate the expression of a cascade of local factors within the endometrium that act in an autocrine/paracrine and even intracrine manner [29].

In conclusion, though sows with reproductive disturbances have cyclic active ovaries, their neuroregulation and hormone secretion do not fully conform to the dynamics of the physiological norm. For that reason, the expression of ERα is too low as compared with normal oestrus cycle sows. We suggest that the number of expressed ERα receptors must be influenced by the level of steroid hormones circulating in blood, but irregular hormone secretion and synthesis and the lack of ERα may contribute to the low reproductive performance in sows.

Further studies are required to clarify the biological role of ERβ in sow uterus to provide insights to our understanding of the roles of ERs in sows' reproductive biology

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Birutė Karvelienė, Henrikas Žilinskas,
Aloyzas Januškauskas, Vita Riškevičienė

ESTROGENŲ RECEPTORIŲ ALFA NUSTATYMAS SUTRIKUSIOS REPRODUKCIJOS KIAULIŲ GIMDOJE

Santrauka

Mūsų darbo tikslas buvo nustatyti estrogenų receptorių alfa (ERα) kiekį ir pasiskirstymą sutrikusios reprodukcijos kiaulių gimdoje, taip pat įvertinti savitarpio priklausomybę tarp ERα receptorių kiekio ir steroidinių hormonų – estradiolio-17β (E_2) bei progesterono (P_4) – koncentracijos kraujyje. Tam tikslui buvo paskersta 3–4 vadų paršingumo 150 LB × DL (Lietuvos baltųjų ir danų landrasų) veislės sutrikusios reprodukcijos kiaulių. Imunohistocheminiai paršavedžių gimdos ragų tyrimai buvo atlikti streptavidino-biotino peroksidazės metodu naudojant antikūnus prieš ERα. Kraujo mėginiai E_2 ir P_4 koncentracijai nustatyti buvo imami iš Jungo venos likus 1 valandai iki skerdimo.

Įvertinus paskerstų paršavedžių kiaušides paaiškėjo, kad 78,3 proc. kiaulių kiaušidės buvo cikliškai aktyvios, nors prieš skerdimą joms buvo nustatyta *anestrus* būklė. Be to, 5 proc. kiaulių, kurių kiaušidės buvo aktyvios, buvo paskerstos rujos stadijoje, nors joms esant gyvoms būdingų išorinių rujos požymių nenustatyta. ERα rasti visuose gimdos sluoksniuose: paviršiniame epiteliniame, subepiteliniame, liaukiniame ir raumeniniame. Mažiausias ERα kiekis (<30 proc.) ir silpniausias nusidažymas (+) visuose keturiuose kiaulių gimdos audinių sluoksniuose nustatytas rujos stadijoje, nors tuo metu E_2 koncentracija kraujo plazmoje buvo aukšta (43,03 pmol/l). Kiaulių, buvusių porujo stadijoje, gimdos liaukinio epitelio ląstelėse ERα kiekis viršijo 80 procentų. Tuo tarpu branduolių nusidažymo intensyvumas šiame audinyje visose lytinio ciklo stadijose buvo skirtingas. Maksimalus raumeninio gimdos sluoksnio ląstelių branduolių nusidažymo intensyvumas nustatytas priešrujo stadijoje, kai E_2 koncentracija kraujo plazmoje buvo aukšta – 350,79 pmol/l, o P_4 koncentracija tesiekė 15,45 nmol/l, taip pat porujo stadijoje, kai E_2 ir P_4 koncentracijos kraujyje buvo žemos, atitinkamai 3,42 pmol/l ir 0,19 nmol/l. P_4 koncentracija kraujyje buvo aukšta (49,01 nmol/l) porujo stadijoje ir žema vėlyvojo porujo stadijoje, kai tuo tarpu E_2 koncentracija abiejų šių stadijų metu buvo žema. ERα ląstelių branduolių nusidažymo intensyvumas skyrėsi ne tik skirtingomis lytinio ciklo stadijomis, bet ir atskiruose gimdos audiniuose.

Tyrimais įrodėme, kad sutrikusios reprodukcijos kiaulių kiaušidės dažniausiai yra aktyvios, tačiau E_2 ir P_4 koncentracijos kraujo plazmoje žemos, o tai ir nulemia mažą ERα kiekį kiaulių gimdoje.

Raktažodžiai: sutrikusi reprodukcija, lytinis ciklas, kiaušidės, hormonų receptoriai, paršavedė