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Original article

Synthesis of steroid hormones in the porcine oviduct during early pregnancy

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ABSTRACT

Past studies of the oviducts have documented oviductal steroid production during the oestrous cycle in pigs. The present study examined whether the pig oviducts are the source of steroid hormones during early pregnancy. In the ampulla and isthmus, the expression of 3β-hydroxysteroid dehydrogenase (3βHSD) and aromatase cytochrome P450 (CYP19) mRNA by real-time PCR, cellular localization and quantities of the studied proteins by immunofluorescence and Western blot analysis, and concentration of steroid hormones in oviductal flushings by radioimmunoassay, were studied. The expression of *3βHSD* in the ampulla and isthmus was correlated (r = 0.89) and higher on Days 2–3 and 15–16 than on Days 10–11 and 12–13. *CYP19* expression was elevated in the ampulla on Days 2–3, 10–11 and 15–16 and in the isthmus on Days 2–3 *vs.* the other days studied. The studied proteins were localized in oviductal epithelial cells. In the ampulla, the quantity of 3βHSD protein did not change, and was greater in the isthmus on Days 2–3 *vs.* Days 12–13 of pregnancy. The P450arom protein quantity increased in the ampulla on Days 2–3 *vs.* Days 10–11 and 15–16 and *vs.* Days 10–11 and 12–13 in the isthmus. The concentrations of progesterone and androstenedione in oviductal flushings were lowest on Days 12–13 and on Days 2–3 and 15–16, respectively, while oestradiol-17β and oestrone levels did not change. Porcine oviducts are the sources of steroid hormones during early pregnancy. The expression of steroidogenic enzymes primarily increases during the embryos presence in the oviduct, i.e., on Days 2–3 of pregnancy.

1. Introduction

The steroid hormones oestradiol-17β (E_2) and progesterone (P_4) play a key role in the regulation of female reproduction in pigs [1–5]. The physiological effect of these steroids is mediated by their binding to specific receptors localized in the tissues of the maternal tract, including the oviduct, as well as in early embryos. The expression of nuclear ER was determined in oviductal epithelial cells and the whole oviducts of pigs [5], cows [6], and rodents [7] during the oestrous cycle and in the oviducts of early pregnant rabbits [8]. Similarly, the expression of nuclear or membrane PRs was determined in oviductal epithelial cells and the whole oviducts of pigs [5], bovine [6,9,10], mares [11], and rodents [7,12] during the oestrous cycle and in the oviducts of early pregnant rabbits [13] and cows [10].

In the course of the oestrous cycle in pigs, E_2 and P_4 regulate the oviductal motility [14–16], differentiation and functions of oviduct epithelial cells [1,3,4,17–19] and affect the volume and composition of oviductal fluid [1,4,20]. The actions of steroids in the oviduct throughout the regulation of synthesis and secretion of specific oviductal factors support fertilization and early embryonic development [20–22]. During the peri-conceptional and early pregnancy periods, oviductal fluid contains ions, nutrients, oviduct-specific glycoprotein 1, growth factors, prostaglandins and steroid hormones that regulate the activity of the organ [1–4,21–25].

E_2 increases the binding of sperm to the oviductal epithelium and, in turn, may affect sperm reservoir formation [26]. During the peri-conceptional period, the P_4 gradient between the ampulla and isthmus acts as a significant molecular signal that modifies sperm flagellar activity [22,25]. This process enables the transport of sperm towards the ampullary-isthmic junction for oocyte fertilization [3,25]. An increasing level of P_4 in the oviductal microenvironment (up to 35 ng/ml) results in epithelial cell regression in both the ampulla and isthmus [17]. Both, E_2 and P_4 may control the formation of an appropriate intra-oviductal milieu, which is necessary for gamete maturation and embryo development [22,27]. Moreover, these hormones could directly affect porcine embryos localized in the oviduct, as ER-alpha and PGR mRNA and proteins were detected in porcine zygotes and embryos at the 2- and 4-cell stages [28,29]. In response to oestrogens, porcine ampulla oviductal epithelial cells produce specific proteins, and the rate of zygote cleavage is significantly increased *in vitro* [30].

The changes in the steroid hormone concentrations in oviductal fluid during the oestrous cycle, including the peri-ovulatory period, were determined in cows [31], mares [32] and pigs [33,34]. A local increase in the ovarian steroid concentration in the peripheral blood

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Table 1
Forward and reverse primer sequences, amplicon lengths and GenBank accession numbers of the genes used during the real-time PCR analysis.

Name of the gene	Primer sequence forward/reverse	Amplicon lengths, bp	Accession Number
Ubiquitin C (*UBC*)	5′ GGAGGAATCTACTGGGGCGG 3′ 5′ CAGAAGAAACGCAGGCAAACT 3′	103	XM_003483411.3
Beta-actin (*ACTB*)	5′ CACGCCATCCTGCGTCTGGA 3′ 5′ AGCACCGTGTTGGCGTAGAG 3′	380	XM_003357928.2
3β-hydroxysteroid dehydrogenase (3β*HSD*)	5′ GTGAAAGGTACCCAGCTCCTGC 3′ 5′ GCGTTCTGGATGACCTCCCT 3′	119	NM_001004049.1
Cytochrome P450 aromatase (*CYP19*)	5′ TGAGGTACCAGCCTGTCGTGGA 3′ 5′ ATTTGGCTTTGGGCCCCGGG 3′	216	NM_214431.1

supplying the oviduct was found in early pregnant pigs [35]. Therefore, different sources of steroid hormones in oviductal fluid in pigs are assumed [3,4,27,33,35]. These steroids could diffuse into the oviduct from follicular fluid after ovulation to the bloodstream and subsequently to the oviductal lumen, from blood and lymphatic vessels using a counter-current mechanism, from the granulosa cells of the *corona radiata* of oocytes appearing in the oviduct, and from local steroidogenesis occurring in oviductal tissues. A local increase in the ovarian steroid concentration in blood supplying the oviduct was found in early pregnant pigs [35].

The synthesis of the steroid hormones is catalysed by 3β-hydroxysteroid dehydrogenase (3βHSD), which converts pregnenolone into P_4, and aromatase cytochrome P450 (P450arom), which converts androgens into oestrogens [36,37]. Previously, we also detected the expression of enzymes of the steroidogenic pathway in the oviductal epithelium of pigs during the oestrous cycle, indicating local steroid hormone synthesis in the oviduct [33]. The mechanism/phenomenon of steroid hormone synthesis in the oviduct of early pregnant pigs is unknown. Therefore, the aim of the present study was to determine whether pig oviducts are sources of steroid hormones during early pregnancy. In detail, 1) the expression of *3βHSD* and *CYP19* mRNA, 2) cellular localization and abundance of 3βHSD and P450arom proteins in the ampulla and isthmus and 3) progesterone (P_4), androstenedione (A_4), oestradiol-17β (E_2) and oestrone (E_1) concentrations in oviductal flushings harvested from pigs on Days 2–3, 10–11, 12–13 and 15–16 of pregnancy were determined. We selected the period of early pregnancy as the presence (Days 2–3 of pregnancy) and absence (Days 10–11, 12–13 and 15–16 of pregnancy) of embryos in the oviducts. Therefore, we determined whether/how oviductal steroidogenic activity changes during the course of early pregnancy, when the embryos leave the organ to develop and implant in the uterus.

2. Materials and methods

2.1. Animals, oviducts and oviductal flushings collection

Crossbred gilts (Large White × Polish Landrace; 7–8 months old, 90–110 kg bw.) during early pregnancy (n = 20) were used in the present study. The gilts were observed throughout two consecutive cycles for oestrus behaviour in the presence of an intact boar, and these animals were naturally mated on the second day of the second oestrus. The animals were sacrificed in a commercial slaughterhouse on Days 2–3 (the presence of embryos inside the oviduct n = 5), 10–11 (the presence of the embryos inside the uterus, time before maternal recognition of pregnancy n = 5), 12–13 (maternal recognition of pregnancy n = 5), and 15–16 (the beginning of implantation n = 5) of pregnancy. On Days 2–3 of pregnancy, the stage of development and number of embryos occurring in the oviductal flushings were microscopically determined. Only oviducts in which embryos (from 2- to 4-cell stage of the development) were present were used for future analyses [2,38]. Days 10–11, 12–13 and 15–16 of pregnancy were confirmed by the recovery of embryos in uterine horn flushing with 20 ml of PBS (Sigma Aldrich, USA). The right and left oviducts of each studied gilt were flushed with 2 ml of PBS/each and subsequently cut into the ampulla and isthmus [2,26]. The flushings from the oviducts were placed on ice and then frozen at −20 °C for further determination of steroid hormone concentrations. Collected from pregnant gilts, compartments of the oviduct were frozen in liquid nitrogen, transported to the laboratory, and stored at −80 °C until further analysis of the mRNA and protein expression of steroidogenesis enzymes. The experiments were approved by the Animal Ethics Committee of the University of Warmia and Mazury in Olsztyn, Poland (17/2007/N and 52/2015/DTN).

2.2. RNA extraction, complementary DNA (cDNA) synthesis and determination of 3βHSD and CYP19 gene expression

The RNA isolation and reverse transcription have previously been described in detail [33]. Total RNA was isolated from the ampulla and isthmus of the oviducts using the TRIzol (Sigma Aldrich, USA) method. Reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen, Germany) was followed by the quantity and quality analysis of the isolated RNA (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). After reverse transcription, each sample was diluted in nuclease-free water to a final concentration of 100 ng/μL and used for real-time PCR analysis. The expression of 3βHSD and CYP19 mRNA was analysed using the 7300 Real-time PCR System (Applied Biosystems, USA) and SYBR® Green PCR Master Mix (Life Technologies, USA). The specificity of the primers used in this experiment is presented in Table 1. The real-time PCR programme started with an initial denaturation (10 min/95 °C), followed by 40 cycles of denaturation (15 s/95 °C), elongation (1 min/72 °C) and primer annealing (60 min/60 °C). A melting curve was performed for specificity confirmation of the amplified product in each reaction. In each assay, a negative control, containing nuclease-free water (NTC, no template control) instead of the cDNA template, was added. Each reaction was performed in duplicate. The *ΔΔCt* method was used for data normalization. The quantity of the studied genes was divided by the amount of the selected housekeeping genes, β-actin (*ACTB)* and ubiquitin C (*UBC)*, as the most stable genes for 3βHSD and CYP19 mRNA determination in the porcine oviduct according to previous studies [33]. All data are presented in arbitrary units. DNA sequencing of randomly selected probes from 3BHSD and CYP19 mRNA was performed by the Genomed Poland Company.

2.3. Localization and abundance of 3βHSD and P450arom proteins

2.3.1. Immunofluorescence staining

Immunofluorescence staining was used to determine the abundance of 3βHSD and P450arom proteins in the oviducts of pregnant pigs (n = 5/per stage of pregnancy), as previously described [33]. Sections (7-μm thick) of the ampulla and isthmus were prepared on a cryostat (Leica, Germany), followed by fixation in paraformaldehyde, washing with PBS and blockage with 1% donkey serum. After washing with PBS, the sections were incubated overnight with rabbit anti-human 3βHSD and P450arom primary antibodies diluted in PBS, as described in