Androgen Deficiency During Mid- and Late Pregnancy Alters Progesterone Production and Metabolism in the Porcine Corpus Luteum

Reproductive Sciences
2014, Vol. 21(6) 778-790
© The Author(s) 2014
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1933719113518991
rs.sagepub.com

Malgorzata Grzesiak, PhD¹, Katarzyna Knapczyk-Stwora, PhD¹,
Renata E. Ciereszko, PhD^{2,3}, Aniela Golas, PhD⁴,
Iwona Wieciech, MSc¹, and Maria Slomczynska, PhD¹

Abstract

We determined whether androgen deficiency induced by flutamide treatment during mid- and late pregnancy affects the functions of the porcine corpus luteum (CL). Pregnant gilts were injected with flutamide between days 43 and 49 (gestation day [GD] 50F), days 83 and 89 (GD90F), or days 101 and 107 (GD108F) of gestation. Antiandrogen treatment increased the luteal progesterone concentration in the GD50F group and decreased progesterone content in the GD90F and GD108F groups. Luteal levels of side-chain cleavage cytochrome P450 (CYP11A1) mRNA and protein were significantly downregulated in the GD90F and GD108F groups as compared with the respective controls. The 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase (HSD3B) mRNA and protein expression were significantly reduced only in the GD108F group as compared with the control. Decreased luteal 20 α -hydroxysteroid dehydrogenase (AKR1C1) mRNA and protein levels were observed in the GD50F group. Thus, androgen deficiency during pregnancy in pigs led to CL dysfunction that is marked by decreased progesterone production. Furthermore, exposure to flutamide during late pregnancy downregulated steroidogenic enzymes (CYP11A1 and HSD3B) in pigs. We conclude that androgens are important regulators of CL function during pregnancy.

Keywords

corpus luteum, pregnancy, progesterone, flutamide, pig

Introduction

The corpus luteum (CL) is an ephemeral endocrine gland that secretes progesterone (P4). The CL of pregnancy, as well as the CL of the estrous cycle, is comprised of distinct cell types: parenchymal (steroidogenic) cells, fibroblasts, endothelial cells, pericytes, and immune cells. The parenchymal component is subdivided into large and small luteal cells that are derived from their corresponding counterparts in ovarian follicles: granulosa and theca interna cells, respectively. Crucial differences in steroidogenic activity between large and small luteal cells have been observed (for review see¹⁻³).

The pig is a particularly interesting model for examining luteal function during pregnancy because the porcine CL is the major source of P4 during the entire gestational period.⁴ Although the porcine placenta is able to synthesize small amounts of this hormone, its functional significance is negligible because of immediate P4 secretion into the fetal circulatory system.^{5,6} Because an ovariectomy performed at any time during the gestational period invariably leads to abortion, the presence of a functional CL is essential for the maintenance of pregnancy.⁷

The precise timing of both the synthesis and the degradation of P4 is crucial for reproductive success. The expression of enzymes involved in P4 synthesis and metabolism is regulated during pregnancy.³ Briefly, steroidogenic acute regulatory protein (STAR) transports cholesterol to the inner mitochondrial membrane, where the conversion of cholesterol into pregnenolone by side-chain cleavage cytochrome P450 (CYP11A1) takes place. Subsequently, 3 β -hydroxysteroid dehydrogenase/

¹ Department of Endocrinology, Institute of Zoology, Jagiellonian University in Krakow, Krakow, Poland

² Department of Animal Physiology, University of Warmia and Mazury, Olsztyn, Poland

³ Research and Educational Center, Laboratory of Molecular Diagnostics, University of Warmia and Mazury, Olsztyn, Poland

⁴ Department of Genetics and Evolution, Institute of Zoology, Jagiellonian University in Krakow, Krakow, Poland

Corresponding Author:

Malgorzata Grzesiak, Department of Endocrinology, Institute of Zoology, Jagiellonian University in Krakow, Gronostajowa 9, 30-387 Krakow, Poland.
Email: malgorzata.durlej@uj.edu.pl

$\Delta 5\text{-}\Delta 4$ isomerase (HSD3B), which is located in the smooth endoplasmic reticulum, is responsible for the conversion of pregnenolone to P4.⁸ Both enzymes are likely constitutively expressed in luteal tissue and remain highly expressed in the CL throughout pregnancy.^{9,10} In rodents^{11,12} and domestic animals,^{13,14} luteal 20 α -hydroxysteroid dehydrogenase (AKR1C1) converts P4 into its biologically inactive form, 20 α -hydroxyprogesterone, and plays a crucial role in the completion of pregnancy and the initiation of parturition.² Despite its importance, the complex mechanisms controlling P4 synthesis and metabolism in the porcine CL during pregnancy are not completely understood.

In addition to P4, the porcine CL also produces estradiol and androgens.¹⁵ Mounting evidence has revealed significant changes in the expression of steroid hormone receptors and steroidogenic enzymes within porcine CL during mid- and late pregnancy. Of particular importance, cytochrome P450 17 α -hydroxylase/c17, 20-lyase is found in small luteal cells beginning on day 50 of gestation, indicating a potential site of androgen synthesis.¹⁶ The expression of androgen receptors (ARs) in the porcine CL has been observed until late pregnancy, and luteal cells show a nuclear pattern of AR staining up to day 71.¹⁷ After day 71, cytoplasmic staining is also observed in those cells, and almost all luteal cells show AR staining exclusively in their cytoplasm on day 90.¹⁷ The shift from nuclear to cytoplasmic immunolocalization of AR suggests changes in androgen action. Furthermore, a very low level of cytochrome P450 aromatase (CYP19A1) protein is characteristic of CLs by day 90 of pregnancy and then the level of CYP19A1 protein getting higher (our unpublished data). In the porcine CL during this stage of pregnancy, androgens likely serve predominantly as a substrate for CYP19A1 to synthesize estrogens. All of these observations indicate that androgens are involved either directly via AR or indirectly via conversion to estrogens in the regulation of CL maintenance and function during pregnancy in pigs.

Androgens modulate luteal function by stimulating P4 production. In the rat, a nonaromatizable androgen, dihydrotestosterone, was shown to promote P4 production in incubated luteal cells obtained on day 15 of pregnancy.¹⁸ A subsequent elegant study performed on mice further showed the critical role of androgens in the stimulation of luteal P4 release during pregnancy.¹⁹

In the present study, we tested the hypothesis that androgen deficiency during mid- and late pregnancy leads to CL dysfunction marked by impaired P4 production in pigs. Androgen deficiency was experimentally attained using flutamide, a nonsteroid antiandrogen that blocks ARs.²⁰ Our motivation to undertake this endeavor is justified by an increasing number of reports concerning negative effects of environmental compounds, including antiandrogens, which interfere with the action of endogenous hormones.²¹ Therefore, the specific objectives of this study were to determine the effects of flutamide treatment on (1) the concentration of P4 in plasma and luteal tissue; (2) luteal expression of STAR, CYP11A1, HSD3B, and AKR1C1 mRNA and protein; and (3)

immunolocalization of CYP11A1 and HSD3B in luteal tissue on days 50, 90, and 108 of pregnancy in pigs.

Materials and Methods

Animals and Experimental Design

All experiments were approved by the Local Ethics Committee at Jagiellonian University in Krakow (approval no. 122/2009). Surgical procedures were performed by a veterinarian. Animals used in the experiment were obtained from the Center of Applied Biotechnology and Basic Sciences (University of Rzeszow, Kolbuszowa, Poland). Twelve sexually mature crossbred gilts (Large White $\times$ Polish Landrace; weighing 109.5 ± 7.5 kg) of similar age (~ 10 months) and genetic background were kept in the same farm conditions with food and water ad libitum. Detection of estrus was based on typical estrus symptoms (swelling of the vulva and enlargement of the clitoris, the presence of the sticky discharge, increased activity and vocalization, elevation of the ears and immobilization in response to manual back pressure). Animals with at least 1 estrus symptom were checked daily for other estrus signs. After 2 consecutive estrous cycles, gilts were mated with a fertile boar at the onset of estrus and again 12 and 24 hours later. The day on which mating took place was considered the first gestation day. Pregnant animals were fed according to nutritional recommendations for pregnant pigs. Randomly assigned pregnant gilts were injected with the antiandrogen flutamide (Sigma-Aldrich, St Louis, Missouri) between days (1) 43 and 49 of gestation (gestation day [GD] 50F), (2) 83 and 89 of gestation (GD90F), or (3) 101 and 107 of gestation (GD108F). Flutamide was suspended in corn oil and injected subcutaneously daily for 7 days at a dose of 50 mg/kg body weight. The dose was chosen based on data published by our group^{22,23} and other researchers^{24,25} to antagonize androgen action via ARs, without exerting toxic effects on the gilts or their offspring. For each flutamide-treated group, a control group was established (GD50C, GD90C, and GD108C). Control animals were injected only with vehicle (corn oil) in a manner similar to the flutamide-treated pigs. The days of pregnancy chosen for flutamide treatments reflect the periods of midpregnancy (GD50), late pregnancy (GD90), and around parturition (GD108).

Collection of CLs and Blood

Bilateral porcine ovaries were obtained from pregnant gilts by an ovariectomy that was performed under thiopental anesthesia on day 50 (GD50), 90 (GD90), or 108 (GD108) of gestation. Fresh CLs were excised from the right and left ovaries of control (C; $n = 8\text{--}11$ per group) and flutamide-treated (F; $n = 8\text{--}11$ per group) animals. Each CL was cut into 4 pieces that were either snap frozen in liquid nitrogen for real-time PCR, Western blotting, and radioimmunoassay (RIA) analyses or fixed in 10% (v/v) neutral-buffered formalin for immunohistochemistry.

Blood samples from control and flutamide-treated pregnant gilts were collected on GD50, GD90, and GD108 by jugular

Figure 1. Representative images of *AKR1C1* and *GAPDH* mRNA expression in porcine tissues used as positive controls (placenta, endometrium, and CL from the estrous cycle) and in selected control (gestation day 50: GD50C) and flutamide-exposed (GD50F) CLs during pregnancy. The 141-bp *AKR1C1* product was observed in all investigated tissues. *GAPDH* was used as an internal control. *AKR1C1* indicates 20 α -hydroxysteroid dehydrogenase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; CL, corpus luteum M, molecular weight marker; B, blank (no template in PCR); –RT, no reverse transcriptase; bp, base pairs.

venipuncture and placed in heparinized test tubes. Thereafter, plasma was separated by centrifugation at 2000 $\times g$ for 10 minutes at 4°C and stored at –20°C.

Radioimmunoassay for P4

The P4 concentration in CL homogenates and plasma of control and flutamide-treated pregnant pigs was measured with ³H-radioimmunoassay after extraction. Briefly, CLs were dissected from the ovary, weighed, and homogenized in phosphate buffer (pH 7.2). Appropriate aliquots of homogenates were extracted with 2.5 mL hexane (Sigma-Aldrich). After extraction (45 minutes, 4°C), samples of supernatants (10 μ L) were used for RIA. Analysis of plasma samples was conducted without the extraction step. Validation of the P4 assay in porcine tissues has been reported.²⁶ The specificity of the antiprogesterone (SO/91/4) antibodies is described elsewhere.²⁷ Intra- and interassay coefficients of variation for P4 were 3.75% and 2.45%, respectively. The lower limit of detection of P4 was 6 pg/tube. Serial dilutions of luteal homogenate and plasma samples showed parallelism with the standard curves of examined steroids. All analyses were performed in duplicate.

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from collected CLs using TRI Reagent solution (Ambion, Austin, Texas), according to the manufacturer's instructions, resuspended in water, and stored frozen at –80°C. The concentration of the total RNA was determined by measuring the absorbance at 260 and 280 nm with a NanoDrop ND2000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware). Electrophoresis on a 1% (w/v) denaturing agarose gel was used to evaluate the quality of RNA samples based on the appearance of the 18S and 28S ribosomal RNA bands. Complementary DNA (cDNA) was synthesized from 1 μ g total RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, California). Reverse transcriptase reaction mixtures were prepared in a

volume of 20 μ L using random primers, dNTP mix, RNase inhibitor, and Multi Scribe Reverse Transcriptase. Reactions were performed in a Veriti Thermal Cycler (Applied Biosystems) with the following parameters: 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. Amplification of contaminating genomic DNA was checked periodically with control experiments in which reverse transcriptase was omitted during the reverse transcription step. Samples were stored at –20°C until further analysis.

Real-Time PCR for *STAR*, *CYP11A1*, and *HSD3B*

Real-time PCR was performed with a StepOne Real-Time PCR System (Applied Biosystems) utilizing TaqMan Gene Expression Assays (Ss03381250_u1 for *STAR*, Ss03384849_u1 for *CYP11A1* and Ss03391751_m1 for *HSD3B*; Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified as an endogenous control (Ss03373286_u1). All real-time PCR experiments were performed in duplicate. Real-time PCR reactions were performed in a total reaction volume of 20 μ L and included 10 μ L TaqMan Gene Expression Master Mix, 1 μ L 20 $\times$ TaqMan Gene Expression Assay, 8 μ L water, and 2 μ L analyzed sample. The real-time PCR conditions were as follows: preheating at 50°C for 2 minutes, denaturation at 95°C for 10 minutes, and 40 cycles of amplification and quantification (15 seconds at 95°C and 60 seconds at 60°C).

The expression of *STAR*, *CYP11A1*, and *HSD3B* relative to *GAPDH* was determined using the $2^{-\Delta C_t}$ method.²⁸ Briefly, the cycle threshold (C_t) of *CYP11A1* and *HSD3B* (defined as the cycle number at which the fluorescence exceeds the threshold level) was determined for each sample. The C_t value of the reference gene *GAPDH* was subtracted from the C_t value of the gene of interest (ΔC_t) and relative expression was presented as $2^{-\Delta C_t}$. These $2^{-\Delta C_t}$ values were used to calculate statistical differences.

Real-Time PCR for *AKR1C1*

To conduct real-time PCR experiments with Power SYBR green PCR master mix (Applied Biosystems), cDNA (DQ474065) and DNA (NM 001044569) sequences of *AKR1C1* were used. Primers for *AKR1C1* were located in 2 consecutive exons and were designed with Primer3 software v.4.0 (National Institutes of Health, Bethesda, Maryland) as follows: forward, 5'-CCAAAAGCCAGCGTCTTC-3'; reverse, 5'-CGATATGACGGAACCCAACT-3'. Primer sequences were checked for specificity with the Primer-BLAST database (NCBI) and the expected PCR product of 141 bp was obtained only for *Sus scrofa AKR1C1* mRNA. Polymerase chain reaction with 1 U DyNAzyme II polymerase (Finnzymes, Espoo, Finland) showed no product in the negative control (reverse transcription without reverse transcriptase, –RT). Products of 141 bp were observed (Figure 1) from cDNAs obtained from positive control tissues (endometrium, placenta, CL from the estrous cycle)²⁹ as well as from representative luteal tissues of control and flutamide-treated pregnant animals.

To optimize real-time PCR, 100 nmol/L, 200 nmol/L, and 300 nmol/L starters' concentrations were tested with 20× diluted cDNA (StepOne Real-Time PCR System). Subsequently, five 1:2 serial dilutions of cDNA were prepared, and real-time PCR efficiency for the target and endogenous control gene with 300 nmol/L starters was tested in triplicate. Standard cycling conditions were used, that is, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute and melting curve analysis (ramp +0.5°C). These reaction conditions were used to analyze the expression level of *AKR1C1* and *GAPDH* in control and flutamide-exposed CLs on 50, 90, and 108 days of pregnancy. The *GAPDH* primers used were according to Cheng et al.³⁰: forward, 5'-GGACTCAT-GACCACGGTCCAT-3'; reverse, 5'-TCAGATCCACAACCGACACGT-3'. The expression of *AKR1C1* was normalized to *GAPDH* and determined using $2^{-\Delta C_t}$ as described earlier for *STAR*, *CYP11A1*, and *HSD3B*.

Protein Isolation and Western Blotting

Equal amounts of frozen luteal tissue (30 mg) were homogenized on ice-cold Tris/EDTA buffer (50 mmol/L Tris, 1 mmol/L EDTA, pH 7.5) containing 10 µL/mL Protease Inhibitor Cocktail (Sigma-Aldrich), sonicated, and centrifuged at 10 000 ×g for 20 minutes at 4°C to remove nonhomogenized tissue debris. Protein content was determined with the Bradford method.³¹ Samples containing 50 µg protein were denatured for 5 minutes in Laemmli sample buffer (Bio-Rad Laboratories Inc, GmbH, Munich, Germany) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12% for *STAR*, *HSD3B*, and *AKR1C1*, 10% for *CYP11A1*) under reducing conditions according to Laemmli.³² Separated proteins were electroblotted onto a nitrocellulose membrane in transfer buffer (20 mmol/L Tris, 150 mmol/L glycine in 20% [v/v] methanol, pH 8.4). After overnight blocking in 5% (w/v) nonfat milk in Tris-buffered saline (TBS; 0.05 M Tris-HCl, pH 7.4) containing 0.1% (v/v) Tween 20 (TBST) at 4°C with shaking, the membranes were incubated with primary antibodies as follows: polyclonal rabbit anti-*STAR* (1:1000, sc-25806; Santa Cruz Biotechnology Inc, Santa Cruz, California), polyclonal goat anti-*CYP11A1* (1:500, sc-18040; Santa Cruz Biotechnology Inc), polyclonal rabbit anti-*HSD3B* (1:10 000; a generous gift from the late Professor Anita H. Payne, Stanford University School of Medicine, Stanford, California), or polyclonal rabbit anti-*AKR1C1* (1:1000, LS-C135732; LifeSpan Biosciences Inc, Seattle, Washington) for 1.5 hours at room temperature. Subsequently, the membranes were washed in TBST buffer and incubated with specific secondary antibodies linked to horseradish peroxidase: anti-goat IgG (1:4000; Vector Laboratories, Burlingame, California) or anti-rabbit IgG (1:4000; Vector Laboratories) for 1 hour at room temperature. To control for variable amounts of protein, the membranes were stripped and reprobed with monoclonal mouse anti-β-actin (ACTB) antibody (1:3000; Sigma-Aldrich) followed by horseradish peroxidase-conjugated anti-mouse IgG (1:4000; Bio-Rad Laboratories Inc). Signals were detected

with chemiluminescence using Luminol Reagent (Santa Cruz Biotechnology Inc) and visualized using ChemiDoc XRS+ System (Bio-Rad Laboratories Inc). Analysis of images was performed using the public domain ImageJ program (developed at the National Institutes of Health) using the “Gel Analysis” functions. The bands were densitometrically quantified and normalized to their corresponding ACTB bands. Analysis was performed for 3 separate experiments from each control and flutamide-treated group.

Immunohistochemistry

Fragments of CLs from control and flutamide-treated animals were fixed, embedded in paraplast (Sigma-Aldrich), cut into 5-µm-thick sections, and mounted onto 3'/3'-aminopropyl-triethoxysaline-coated slides (Sigma-Aldrich). The sections were deparaffinized in xylene, rehydrated gradually through a series of ethanol dilutions, and rinsed in water. The slides were treated using an unmasking procedure with microwave heating in 0.01 mol/L citrate buffer (pH 6.0), followed by 30 minutes in 0.3% (v/v) H₂O₂ in TBS to quench endogenous peroxidase activity. Blocking of nonspecific binding sites was performed with 5% (v/v) normal horse serum (for *CYP11A1*) or 5% (v/v) normal goat serum (for *HSD3B*) prior to incubation with anti-*CYP11A1* (1:100) or anti-*HSD3B* (1:1000). After overnight incubation at 4°C in a humidified chamber, the sections were washed with TBST. The antigens were visualized using biotinylated secondary antibody: horse anti-goat IgG or goat anti-rabbit IgG (both 1:300, 1.5 hours at room temperature; Vector Laboratories), avidin–biotin–peroxidase complex (40 minutes at room temperature; StreptABComplex-HRP, Vector Laboratories), and 3,3'-diaminobenzidine (Sigma-Aldrich) as a chromogen. Sections were then dehydrated and mounted in DPX (Sigma-Aldrich). For the negative control reaction, sections were incubated with nonimmune goat or rabbit IgG instead of primary antibodies and processed as mentioned earlier. Selected sections were photographed using a Nikon Eclipse Ni-U microscope and a Nikon Digital DS-Fi1-U3 camera (Nikon, Tokyo, Japan) with corresponding software. The intensity of *CYP11A1* and *HSD3B* immunohistochemical staining was analyzed on each section. Slides were anonymized and coded until they had been examined. The captured digital images for 6 different luteal sections obtained from each CL of every control and flutamide-treated animal were examined using ImageJ software. The intensity of staining was expressed as the relative optical density (ROD) and was calculated using the following formula:

$$\begin{aligned} \text{ROD} &= \text{OD}_{\text{specimen}} / \text{OD}_{\text{background}} \\ &= \log(\text{GL}_{\text{blank}} / \text{GL}_{\text{specimen}}) / \log(\text{GL}_{\text{blank}} / \text{GL}_{\text{background}}), \end{aligned}$$

where GL is the gray level for the stained area (specimen) and the unstained area (background), and blank is the gray level measured after removing the slide from the light path.³³

Statistical Analysis

Statistical analyses were performed using Statistica v.10 program (StatSoft, Inc, Tulsa, Oklahoma). All data are presented as the overall mean $\pm$ standard error of the mean (SEM), and differences were considered statistically significant at the 95% confidence level ($P < .05$). Normal distribution of data was tested with the Shapiro-Wilk test. Then, the nonparametric Mann-Whitney U test was used to determine significant differences between the control and flutamide-treated groups. In addition, the differences among control groups (GD50C vs GD90C vs GD108C) and among flutamide-treated groups (GD50F vs GD90F vs GD108F) were assessed using the nonparametric Kruskal-Wallis test. In this study, each CL within the same ovary and from right and left ovaries was considered as an individual sample because of heterogeneity of porcine CLs.³⁴

Results

Effect of Flutamide on the P4 Concentration in Luteal Tissue and Peripheral Plasma

The P4 content in luteal tissues was greater (1.59-fold; $P = .027$) in the GD50F group as compared with the GD50C group (Figure 2A). In contrast, flutamide treatment reduced the P4 luteal content in both the GD90F (1.45-fold; $P = .015$) and GD108F (1.7-fold; $P = .023$) groups (Figure 2A). Moreover, the luteal P4 concentration in the GD90C and GD108C groups was higher than in the GD50C group (1.93-fold and 1.96-fold, respectively; $P < .05$).

Plasma P4 concentration in individual pregnant gilts of all flutamide-treated groups (GD50F, GD90F, and GD108F) tended to be lower in comparison to the respective control groups (GD50C, GD90C, and GD108C; Figure 2B).

Effects of Flutamide on *STAR*, *CYP11A1*, *HSD3B*, and *AKR1C1* mRNA Expression

The expression of *STAR* mRNA was not altered by flutamide treatment (Figure 3A). However, this expression declined with pregnancy progression in both the control and flutamide-treated groups ($P < .05$). In the control groups, *STAR* mRNA expression was lower on GD108 than GD50 (0.41-fold). In treated animals, the expression was downregulated on GD90 and GD108 (0.54-fold and 0.41-fold, respectively) in comparison to GD50 (Figure 3A).

The *CYP11A1* mRNA was upregulated in the GD50F group (1.28-fold; $P = .03$) but was downregulated in the GD90F (0.77-fold; $P = .009$) and GD108F (0.73-fold; $P = .017$) groups as compared with their respective controls (Figure 3B). In control groups, *CYP11A1* mRNA expression increased with pregnancy progression (GD50C vs GD90C vs GD108C; $P < .05$). Within flutamide-treated groups, upregulation of *CYP11A1* mRNA was only found in the GD108F group when compared to the GD50F (1.51-fold; $P < .05$) and GD90F (1.48-fold; $P < .05$) groups (Figure 3B).

Figure 2. The effect of flutamide on luteal and plasma concentrations of P4 in pregnant gilts. (A), P4 concentration in homogenates of corpora lutea (CLs) retrieved from control (C) and flutamide-treated (F) gilts on days 50 (GD50), 90 (GD90), and 108 (GD108) of gestation (open bars, control groups; solid bars, flutamide-treated groups). Asterisks indicate significant differences between control and flutamide-treated groups for each gestational stage (Mann-Whitney U test; P values are presented in the Results' section). Different letter superscripts indicate differences (Kruskal-Wallis test; $P < .05$) among control groups (lowercase letters) and among flutamide-treated groups (uppercase letters). (B) Peripheral plasma P4 concentration of individual control and flutamide-treated gilts on days 50 (GD50), 90 (GD90), and 108 (GD108) of gestation. Black dots represent value of plasma P4 for each animal. Two gilts per each control and flutamide-treated group were used.

Luteal *HSD3B* mRNA expression was affected by flutamide treatment exclusively in the GD108F group; the mRNA level was lower (0.68-fold; $P = .007$) than in GD108C group (Figure 3C). The *HSD3B* mRNA level was higher in the GD90C and GD108C groups compared with the GD50C group (1.81-fold and 1.77-fold, respectively; $P < .05$). The *HSD3B* mRNA expression was higher in the GD90F group in comparison to the GD50F and GD108F groups (2.01-fold and 1.73-fold, respectively; $P < .05$; Figure 3C).

Flutamide administration resulted in decreased (0.53-fold; $P = .02$) *AKR1C1* mRNA expression only on GD50 (Figure 3D). In control animals, *AKR1C1* mRNA expression increased

Figure 3. Relative expression of *STAR* (A), *CYP11A1* (B), *HSD3B* (C), and *AKR1C1* (D) mRNA in corpora lutea (CLs) retrieved from control (C) and flutamide-treated (F) gilts on days 50 (GD50), 90 (GD90), and 108 (GD108) of gestation. The mRNA expression of each gene (quantitative real-time PCR) was expressed as the ratio relative to *GAPDH* (open bars, control groups; solid bars, flutamide-treated groups). Asterisks indicate significant differences between control and flutamide-treated groups for each gestational stage (Mann-Whitney *U* test; *P* values are presented in the Results' section). Different letter superscripts indicate differences (Kruskal-Wallis test; *P* < .05) among control groups (lowercase letters) and among flutamide-treated groups (uppercase letters). *STAR* indicates steroidogenic acute regulatory protein; *CYP11A1*, side-chain cleavage cytochrome P450; *HSD3B*, 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase; *AKR1C1*, 20 α -hydroxysteroid dehydrogenase.

on GD90 (1.61-fold; *P* < .05) and GD108 (1.89-fold; *P* < .05) when compared with GD50. Similarly, *AKR1C1* mRNA expression was elevated in the GD90F and GD108F groups (3.35-fold and 3.0-fold; *P* < .05; Figure 3D).

Effects of Flutamide on *STAR*, *CYP11A1*, *HSD3B*, and *AKR1C1* Protein Expression

The effect of flutamide treatment on protein expression of *STAR*, *CYP11A1*, *HSD3B*, and *AKR1C1* was determined in CLs obtained from control and experimental gilts on days 50, 90, and 108 of pregnancy (Figure 4). Specific single bands representing *STAR*, *CYP11A1*, *HSD3B*, and *AKR1C1* were detected at 30, 60, 32, and 36 kDa, respectively, in the control and flutamide-exposed CLs during all examined days of pregnancy (Figure 4E).

The expression of *STAR* was not altered by the flutamide treatment nor pregnancy progression (Figure 4A). The expression of *CYP11A1* was significantly decreased in the GD90F (0.52-fold; *P* = .029) and GD108F (0.77-fold; *P* = .021) groups as compared with the respective controls (GD90C and GD108C; Figure

4B). In control animals, *CYP11A1* protein expression was lower on GD90 than on GD50 (0.74-fold; *P* < .05).

The *HSD3B* protein level was significantly lower (0.74-fold; *P* = .039) in the GD108F group than that in the GD108C group (Figure 4C). Both in control and flutamide-treated animals, *HSD3B* expression did not differ among the examined pregnancy days (Figure 4C).

The *AKR1C1* protein expression was significantly lower (0.6-fold; *P* = .03) in the GD50F group than in the GD50C group (Figure 4D). In control animals, *AKR1C1* expression increased on GD108 in comparison to GD50 and GD90 (1.32-fold and 1.11-fold, respectively; *P* < .05). Within flutamide-treated animals, *AKR1C1* expression was higher on GD90 and GD108 than on GD50 (2.28-fold and 2.04-fold, respectively; *P* < .05; Figure 4D).

Effect of Flutamide on *CYP11A1* Immunolocalization

CYP11A1 was detected in CLs obtained from control and flutamide-treated gilts on days 50 (Figure 5A, A', and B), 90 (Figure 5D and E), and 108 (Figure 5G, G', and H) of

Figure 4. Relative expression of STAR (A), CYP11A1 (B), HSD3B (C), and AKR1C1 (D) proteins in corpora lutea (CLs) retrieved from control (C) and flutamide-treated (F) gilts on days 50 (GD50), 90 (GD90), and 108 (GD108) of gestation. (E) Representative Western blots are shown. The relative expression of each protein was evaluated with densitometry and expressed as the ratio relative to ACTB (open bars, control groups; solid bars, flutamide-treated groups). Asterisks indicate significant differences between control and flutamide-treated groups for each gestational stage (Mann-Whitney *U* test; *P* values are presented in the Results' section). Different letter superscripts indicate differences (Kruskal-Wallis test; *P* < .05) among control groups (lowercase letters) and among flutamide-treated groups (uppercase letters). STAR indicates steroidogenic acute regulatory protein; CYP11A1, side-chain cleavage cytochrome P450; HSD3B, 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase; AKR1C1, 20 α -hydroxysteroid dehydrogenase; ACTB, β -actin.

gestation, and the relative intensity of CYP11A1 staining was estimated for both the groups on the examined days (Figure 5C, F, and I).

CYP11A1 immunoexpression was present in the cytoplasm of small (Figure 5A, D, and G') and large (Figure 5A', D, and G) luteal cells in all control groups. After flutamide treatment, CYP11A1 was detected in both luteal cell types in the GD50F group (Figure 5B), and the staining intensity was higher

(1.21-fold; *P* = .01) as compared with the control group (Figure 5C). In the GD90F group, CYP11A1 was found only in small luteal cells (Figure 5E), with significantly decreased (0.89-fold; *P* = .04) overall staining intensity (Figure 5F). Only sparse luteal cells demonstrated CYP11A1 expression in the GD108F group (Figure 5H). In these cells, lower (0.89-fold; *P* = .04) staining intensity was observed as compared with the GD108C group (Figure 5I).

Figure 5. Representative micrographs of side-chain cleavage cytochrome P450 (CYP11A1) immunostaining in corpora lutea (CLs) retrieved from control (C; A, A', D, G, G') and flutamide-treated (F; B, E, H) gilts on days 50 (GD50; A, A', B), 90 (GD90; D, E), and 108 (GD108; G, G', H) of gestation. The CYP11A1 staining was observed in the cytoplasm of large (arrows) or small (open arrowheads) luteal cells. Control sections in which the primary antibody was replaced by goat IgG did not exhibit any positive staining (E inset). Charts (C, F, I) represent the intensity of CYP11A1 immunostaining expressed as the relative optical density (ROD) in CLs from all examined days of pregnancy (open bars, control groups; solid bars, flutamide-treated groups). *P* values were calculated with the Mann-Whitney *U* test. All scale bars represent 50 μ m.

Effect of Flutamide on HSD3B Immunolocalization

HSD3B was detected in CLs obtained from control and flutamide-treated gilts on days 50 (Figure 6A and B), 90 (Figure 6D and E), and 108 (Figure 6G and H) of gestation. The relative intensity of HSD3B staining was estimated for both groups on the examined days (Figure 6C, F, and I).

Intense HSD3B expression was detected in the cytoplasm of large luteal cells in all control (Figure 6A, D, and G) and flutamide-treated groups (Figure 6B, E, and H). However, flutamide administration affected the relative intensity of HSD3B immunostaining. The intensity of staining was greater (1.21-fold; $P = .004$) in the GD90F group (Figure 6F) and lower (0.87-fold; $P = .018$) in the GD108F group (Figure 6I) as compared with their respective controls.

Discussion

This study provides the first evidence that androgen deficiency may lead to dysfunction in the porcine CL during late pregnancy that is marked by diminished P4 production. We also showed that in addition to the decreased luteal P4 concentration, exposure of pigs to the antiandrogen flutamide downregulated luteal expression of related steroidogenic enzymes, that is, CYP11A1 and HSD3B. Animal models that mimic androgen deficiency are helpful in elucidating the impact of antiandrogenic endocrine disruptors on reproductive functions.^{21,35} An understanding of the functional effects of antiandrogen treatment during pregnancy is important because of the potential risk from endocrine-disrupting chemicals.³⁶

Figure 6. Representative micrographs of 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase (HSD3B) immunostaining in corpora lutea (CLs) retrieved from control (C; A, D, G) and flutamide-treated (F; B, E, H) gilts on days 50 (GD50; A, B), 90 (GD90; D, E), and 108 (GD108; G, H) of gestation. The HSD3B staining was found in the cytoplasm of large luteal cells (arrows). Control sections in which the primary antibody was replaced by rabbit IgG did not exhibit any positive staining (A, inset). Charts (C, F, I) represent the intensity of HSD3B immunostaining expressed as the relative optical density (ROD) in CLs from all examined days of pregnancy (open bars, control groups; solid bars, flutamide-treated groups). *P* values were calculated with the Mann-Whitney *U* test. All scale bars represent 50 μ m.

Effect of Flutamide on Luteal and Plasma P4 Concentration

The reported presence of ARs in the CL of pregnant rats³⁷ and pigs¹⁷ suggests that androgens affect luteal function via the receptor-mediated pathway. In the current study, reduced luteal P4 content was found in GD90F and GD108F groups (late pregnancy) as compared with control animals. However, the P4 content was higher in the GD50F group than in the GD50C group (midpregnancy). Our findings concerning late pregnancy are consistent with direct stimulatory effects of androgens on luteal P4 production in pregnant rodents.^{18,19,38,39} For example, androstendione prevents a decrease in the plasma P4 concentration in rats treated with the antiprogesterin RU486 during late pregnancy,³⁸ and dihydrotestosterone was shown to stimulate

P4 synthesis in the presence of placental luteotropins in pseudopregnant rats.³⁹ Similarly, androgens markedly increase in vitro P4 production by luteal cells from pregnant rats and mice.^{18,19} Previously, we have reported that flutamide administration diminishes the P4 concentration in the CLs of cycling pigs.⁴⁰ Thus, the reduced luteal P4 content in the GD90F and GD108F groups indicates that the stimulatory effect of androgens on P4 synthesis in the CL may be inhibited by anti-androgens. On the other hand, the flutamide-induced increase in luteal P4 on GD50 (midpregnancy) may result from the fact that the endogenous androgen level to be overcome by flutamide is different in mid- and late pregnancy. Similar explanation of data revealed in sheep was suggested by Luense et al.⁴¹ In pigs, the midpregnancy period corresponds to the time of follicle assembly in fetal ovaries.⁴² Previously, we demonstrated

that in utero flutamide treatment delayed fetal folliculogenesis.²³ According to Nilsson and Skinner,⁴³ in vitro treatment of fetal bovine ovaries with high concentration of P4 decreased the assembly of primordial follicles. Therefore, the observed increase in P4 level in the GD50F group may contribute to delayed folliculogenesis in fetal porcine ovaries.

The partial cessation of luteal P4 production during late pregnancy in the flutamide-treated pigs led us to assume that a decrease in subsequent plasma P4 concentrations would be found. Plasma P4 level tended to decrease in the GD50F, GD90F, and GD108F groups. The relatively small number of pregnant animals in this study needs to be taken into account when considering the study and its contributions. However, in the present study, the responses of the individual animals to experimental conditions within each experimental group were very homogeneous. To date, the arterial and umbilical fetal plasma P4 levels are higher than those in the maternal circulation,⁵ and such a P4 transfer from fetus to mother has been observed in ovariectomized pigs during late pregnancy.⁴⁴ The experimental design of our study may have precluded us from recording a decrease in plasma P4 because the blood samples in this study were collected just after flutamide administration, and its effect on P4 metabolism may not yet have been apparent. In such a case, the gradual decrease in plasma P4 concentration would induce the onset of myometrial contractions and premature parturition.^{45,46} However, in the present study the time of parturition has not been determined.

In the current study, we also examined the effect of flutamide on the expression of protein responsible for mitochondrial cholesterol transport (STAR) as well as enzymes involved in P4 production (CYP11A1 and HSD3B) and metabolism (AKR1C1). We did not observe any changes in luteal STAR mRNA and protein expression following the flutamide treatment, which is consistent with previous reports on ovine fetal ovaries and rat fetal testes.^{41,47} These results indicate that flutamide does not affect mitochondrial cholesterol transport.

Effect of Flutamide on CYP11A1 Luteal Expression

We found a significant decrease in CYP11A1 expression during late pregnancy. On GD50, the flutamide treatment increased *CYP11A1* mRNA but not the protein level. Recently, maternal androgenization was found to downregulate fetal ovarian *CYP11A1* mRNA in sheep.⁴⁸ In another study, flutamide prevented a testosterone-induced decrease in *CYP11A1* mRNA in ovine fetal ovaries on day 90, but not on day 65, of pregnancy, suggesting the presence of a regulatory mechanism that is sensitive to developmental changes.⁴¹ This diverse pattern of CYP11A1 expression following exposure to flutamide also appears to be dependent on the gestation day. The environmental antiandrogen, methoxychlor (for review see⁴⁹), inhibited CYP11A1 activity in granulosa, theca, and luteal cells of rodents and decreased follicular and luteal P4 synthesis.^{50,51} It is possible that the downregulation of CYP11A1 expression in the GD90F and GD108F groups reduced the conversion of

cholesterol to pregnenolone and, consequently, affected P4 production in the porcine CL during pregnancy.

In the current study, CYP11A1 protein was exclusively expressed in small luteal cells of the GD90F CL, whereas in the CL of the GD90C group, CYP11A1 was found in both large and small luteal cells. The absence of the enzyme in large cells of CLs treated with flutamide may partially account for the decreased P4 concentration in the GD90F group. The P4 production in small and large luteal porcine cells is stimulated by luteinizing hormone (LH).⁵² Jimenez et al.⁵³ demonstrated that the LH level is significantly decreased following the administration of testosterone or dihydrotestosterone plus flutamide in gilts. An indirect action of androgens via changes in LH rather than a direct effect may be responsible for the observed changes in the GD90F large luteal cells. This notion, however, requires further investigation.

Effect of Flutamide on HSD3B Luteal Expression

Consistent with our earlier report, the presence of HSD3B protein was demonstrated in the cytoplasm of large luteal cells.⁵⁴ Significantly reduced HSD3B transcript and protein levels were found only in the GD108F group as compared with controls. Recently, Kostic et al.⁵⁵ demonstrated that in vivo and in vitro testosterone treatments increase HSD3B expression in the rat testis. An AR-dependent mechanism of HSD3B upregulation involving functional cooperation between STAT5a/b and AR was suggested.⁵⁶ These results indicate that androgens stimulate HSD3B expression in males. In contrast, two different antiandrogens, flutamide and vinclozolin, either elevate or do not affect *HSD3B* mRNA levels in rat testis, respectively.⁵⁷ In utero testosterone treatment⁴⁸ or testosterone and flutamide cotreatment⁴¹ does not affect ovarian *HSD3B* mRNA expression in sheep. The latter results are consistent with the unaltered HSD3B expression in the GD50F and GD90F groups in the current study. However, the downregulation of HSD3B in the GD108F group may have resulted from a direct action of flutamide on luteal HSD3B. Downregulation of *Hsd3b1* mRNA in mouse ovaries caused by antiandrogenic environmental disruptors such as methoxychlor⁵⁸ and bisphenol A⁵⁹ supports this concept.

Effect of Flutamide on AKR1C1 Luteal Expression

Our study is the first to demonstrate AKR1C1 mRNA and protein expression in luteal tissue during the second half of pregnancy in pigs. The AKR1C1 is detected in rat,¹¹ mouse,¹² bovine,¹³ and goat⁶⁰ CL during pregnancy, although its biological function has been determined predominantly in rodents.⁶¹ We found markedly diminished AKR1C1 mRNA and protein expression in the GD50F group as compared with the GD50C group. To our knowledge, no information has been published regarding androgen involvement in the regulation of luteal AKR1C1 expression. One study reported that androgens do not affect the activity of AKR1C1 in the CL of pregnant rats.³⁸ Of particular interest, patients with polycystic ovarian syndrome

demonstrate enhanced *AKR1C1* expression in subcutaneous adipose tissue,⁶² which suggests a positive correlation between androgens and *AKR1C1* levels. Therefore, the possible regulation of *AKR1C1* expression by androgens/antiandrogens cannot be ruled out. Alternatively, one study suggests that a decrease in luteal P4 synthesis and content may be necessary for the induction of *AKR1C1* expression in pregnant rats.⁶¹ In addition, P4 prevents the prostaglandin $F_{2\alpha}$ -induced *AKR1C1* activity in rat⁶³ and pig CL⁶⁴ and downregulates *AKR1C1* expression in cultured rat luteal cells.⁶⁵ Therefore, a local inhibitory action of increased luteal P4, rather than a direct androgen action, may have been responsible for the decrease in *AKR1C1* expression in the GD50F group.

Time-Related Differences in Flutamide-Induced Effects on P4 and Steroidogenic Enzymes

It is known that developmental changes in luteal P4 level and steroidogenic enzyme expression are crucial for normal pregnancy and fetal development.^{7,36,46} The presented results clearly showed that the effect of flutamide on P4 concentration and *CYP11A1*, *HSD3B*, and *AKR1C1* expression in porcine CL of pregnancy depends on the day of gestation. The prominent differences between C and F animals were observed in luteal P4 concentration, luteal mRNA expression of *STAR*, *CYP11A1*, and *HSD3B* as well as luteal protein level of *CYP11A1* and *AKR1C1*, and they altered with pregnancy progression. This suggests that androgen action may vary among different gestational periods and is especially critical for CL function during late pregnancy.

In summary, we demonstrated that an experimentally induced androgen deficiency altered the expression of key luteal steroidogenic enzymes (*CYP11A1*, *HSD3B*, and *AKR1C1*) in a time-dependent manner and was especially critical during late pregnancy and preparturient period. Flutamide-induced changes contributed to luteal disruption and decreased P4 production was found in the CL of GD90 and GD108 groups. The observed differences between control and flutamide-exposed luteal tissues indicate that androgens are at least partly involved in the complex network regulating luteal function during pregnancy in pigs. In addition, our data suggest a direct effect of flutamide on luteal cells. Moreover, since our experimental model included the use of antiandrogen flutamide, the obtained results may contribute to better understanding the action of environmental antiandrogens. Further research leading to the assessment of the flutamide-induced alterations during subsequent stages of steroidogenesis (synthesis of androgens and estrogens) in porcine luteal tissue during pregnancy are warranted.

Acknowledgments

We would like to thank Professor Marek Koziorowski (Department of Physiology and Reproduction of Animals, University of Rzeszow, Rzeszow, Poland) for the valuable assistance in the handling of animals and flutamide injections.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was financially supported by the National Science Center, Poland (Grant SONATA No. 2011/03/D/NZ4/00303 dedicated to M.G.). M.G. is supported by funding from the Jagiellonian University within the SET project. The SET project is co-financed by the European Union within the European Social Fund. Presented in part at the 9th International Conference on Pig Reproduction (ICPR), 9-12 June 2013, Olsztyn, Poland.

References

1. Sawyer HR. Structural and functional properties of the corpus luteum of pregnancy. *J Reprod Fert Suppl.* 1995;49:97-110.
2. Bowen-Shauver JM, Gibori G. The corpus luteum of pregnancy. In: Leung PCK, Adashi EY, eds. *The Ovary*. San Diego, CA: Elsevier Academic Press; 2004:201-230.
3. Stocco C, Telleria C, Gibori G. The molecular control of corpus luteum formation, function, and regression. *Endocr Rev.* 2007; 28(1):117-149.
4. Murphy BD, Gévry N, Ruiz-Cortés T, Côté F, Downey BR, Sirois J. Formation and early development of the corpus luteum in pigs. *Reprod Suppl.* 2001;58:47-63.
5. Barnes RJ, Comline RS, Silver M. Foetal and maternal progesterone concentration in the sow. *J Endocr.* 1974;62(2):419-420.
6. Barnes RJ, Nathanielsz PW, Rossdale PD, Comline RS, Silver M. Plasma progestagens and oestrogens in fetus and mother in late pregnancy. *J Reprod Fert Suppl.* 1975;(23):617-623.
7. Taverne MAM. Corpus luteum function and parturition in cattle and pigs. *Arch Tierz Dummerstorf.* 2001;44:37-50.
8. Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev.* 2004;25(6):947-970.
9. Goldring NB, Durica JM, Lifka J, et al. Cholesterol side-chain cleavage P450 messenger ribonucleic acid: evidence for hormonal regulation in rat ovarian follicles and constitutive expression in corpora lutea. *Endocrinology.* 1987;120(5):1942-1950.
10. Kaynard AH, Periman LM, Simard J, Melner MH. Ovarian 3 beta-hydroxysteroid dehydrogenase and sulfated glycoprotein-2 gene expression are differentially regulated by the induction of ovulation, pseudopregnancy, and luteolysis in the immature rat. *Endocrinology.* 1992;130(4):2192-2200.
11. Seong HH, Shiota K, Noda K, Ogura A, Asano T, Takahashi M. Expression of activities of two 20 α -hydroxysteroid dehydrogenase isozymes in rat corpora lutea. *J Reprod Fert.* 1992;96(2):573-580.
12. Ishida M, Chang K, Hirabayashi K, Nishihara M, Takahashi M. Cloning of mouse 20 α -hydroxysteroid dehydrogenase cDNA and its mRNA localization during pregnancy. *J Reprod Dev.* 1999; 45(5):321-329.
13. Naidansuren P, Park CW, Kim SH, et al. Molecular characterization of bovine placental and ovarian 20 α -hydroxysteroid dehydrogenase. *Reproduction.* 2011;142(5):723-731.

14. Seo KS, Naidansuren P, Kim SH, et al. Expression of aldo-keto reductase family 1 member C1 (AKR1C1) gene in porcine ovary and uterine endometrium during the estrous cycle and pregnancy. *Reprod Biol Endocrinol*. 2011;9(1):139.
15. Niswender GD, Juengel JL, Silna PJ, Rollyson MK, McIntush EW. Mechanism controlling the function and lifespan of the corpus luteum. *Physiol Rev*. 2000;80(1):1-29.
16. Knapczyk-Stwora K, Sternak M, Durlej M, Slomczynska M. Immunolocalization of cytochrome P450 17 α -hydroxylase/c17-20 lyase in the ovary of pregnant pigs and fetal gonads. *Reprod Biol*. 2011;11(2):71-82.
17. Duda M, Burek M, Galas J, Koziol K, Kozirowski M, Slomczynska M. Immunohistochemical localization of androgen receptor and aromatase in the ovary of the pregnant pig. *Reprod Biol*. 2004;4(3):289-298.
18. Carrizo DG, Rastrilla AM, Telleira CM, Aguado LI. Androstendione stimulates progesterone production in corpora lutea of pregnant rats: an effect not mediated by estrogens. *J Steroid Biochem Mol Biol*. 1994;51(3-4):191-197.
19. Thordarson G, Galosy S, Gundmundsson GO, Newcomer B, Sridaran R, Talamantes F. Interaction of mouse placental lactogens and androgens in regulating progesterone release in cultured mouse luteal cells. *Endocrinology*. 1997;138(8):3236-3241.
20. Tevell A, Lennernas H, Jonsson M, et al. Flutamide metabolism in four different species in vitro and identification of flutamide metabolites in human patient urine by high performance liquid chromatography/tandem mass spectrometry. *Drug Metab Dispos*. 2006;34(6):984-992.
21. Fowler PA, Bellingham M, Sinclair KD, et al. Impact of endocrine-disrupting compounds (EDCs) on female reproductive health. *Mol Cell Endocrinol*. 2012;355(2):231-239.
22. Durlej M, Kopera I, Knapczyk-Stwora K, et al. Connexin43 gene expression in male and female gonads of porcine offspring following *in utero* exposure to an anti-androgen, flutamide. *Acta Histochem*. 2011;113(1):6-12.
23. Knapczyk-Stwora K, Durlej-Grzesiak M, Ciereszko RE, Kozirowski M, Slomczynska M. Antiandrogen flutamide affects folliculogenesis during fetal development in pigs. *Reproduction*. 2013;145(3):265-276.
24. Williams K, McKinnell C, Saunders PT, et al. Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: evidence for importance of the androgen-oestrogen balance and assessment of the relevance to man. *Hum Reprod Update*. 2001;7(3):236-247.
25. Foster PM, Harris MW. Changes in androgen-mediated reproductive development in male rat offspring following exposure to a single oral dose of flutamide at different gestational ages. *Toxicol Sci*. 2005;85(2):1024-1032.
26. Ciereszko R, Petroff B, Ottobre A, Guan Z, Stokes B, Ottobre J. Assessment of the mechanism by which prolactin stimulates progesterone production by early corpora lutea of pigs. *J Endocrinol*. 1998;159(2):201-209.
27. Ciereszko R, Opalka M, Kamińska B, Wojtczak M, Okrasa S, Dusza L. Luteotrophic action of prolactin during the early luteal phase in pigs: the involvement of protein kinases and phosphatases. *Reprod Biol*. 2001;1(2):63-83.
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{- $\Delta\Delta C_t$} method. *Methods*. 2001;25(4):402-408.
29. Nonneman DJ, Wise TH, Ford JJ, Kuehn LA, Rohrer GA. Characterization of the aldo-keto reductase 1C gene cluster on pig chromosome 10: possible associations with reproductive traits. *BMC Vet Res*. 2006;2:28.
30. Cheng Y, Inoue N, Matsuda-Minehata F, Goto Y, Maeda A, Manabe N. Changes in expression and localization of connexin 43 mRNA and protein in porcine ovary granulosa cells during follicular atresia. *J Reprod Dev*. 2005;51(5):627-637.
31. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72(1):248-254.
32. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227(5259):680-685.
33. Smolen AJ. Image analytic techniques for quantification of immunocytochemical staining in the nervous system. In: Conn PM, ed. *Methods in Neurosciences*. New York, NY: Academic Press; 1990:208-229.
34. Rao CV, Edgerton LA. Dissimilarity of corpora lutea within the same ovaries or those from right and left ovaries of pigs during the oestrous cycle. *J Reprod Fertil*. 1984;70(1):61-66.
35. Craig ZR, Wang W, Flaws JA. Endocrine-disrupting chemicals in ovarian function: effects on steroidogenesis, metabolism and nuclear receptor signaling. *Reproduction*. 2011;142(5):633-646.
36. Bellingham M, Fiandanese N, Byers A, et al. Effects of exposure to environmental chemicals during pregnancy on the development of the male and female reproductive axes. *Reprod Dom Anim*. 2012;47(suppl 4):15-22.
37. Szoltys M, Slomczynska M, Galas J, Duda M, Sakiewicz A. Expression of androgen receptor and 3 β -hydroxysteroid dehydrogenase in corpora lutea during pregnancy in the rat. *Reprod Fertil Dev*. 2007;19(2):356-365.
38. Telleira CM, Stocco CO, Stati AO, et al. Dual regulation of luteal progesterone by androstendione during spontaneous and RU486-induced luteolysis in pregnant rats. *J Steroid Mol Biol*. 1995;55(3-4):385-393.
39. Takiguchi S, Sugino N, Kashida S, Yamagata Y, Nakamura Y, Kato H. Rescue of the corpus luteum and an increase in luteal superoxide dismutase expression induced by placental luteotropins in the rat: action of testosterone without conversion to estrogen. *Biol Reprod*. 2000;62(2):398-403.
40. Durlej M, Knapczyk-Stwora K, Duda M, et al. Prenatal and neonatal exposure to the antiandrogen flutamide alters connexin 43 gene expression in the adult porcine ovary. *Domest Anim Endocrinol*. 2011;40(1):19-29.
41. Luense LJ, Veiga-Lopez, Padmanabhan V, Christenson LK. Developmental programming: gestational testosterone treatment alters fetal ovarian gene expression. *Endocrinology*. 2011;152(12):4974-4983.
42. Bielanska-Osuchowska Z. Oogenesis in pig ovaries during the prenatal period: ultrastructure and morphometry. *Reprod Biol*. 2006;6(2):161-193.

43. Nilsson EE, Skinner MK. Progesterone regulation of primordial follicle assembly in bovine fetal ovaries. *Mol Cell Endocrinol.* 2009;313(1-2):9-16.
44. Craig VA. Placental steroid metabolism in late pregnancy. In: Cole DJA, Foxcroft GR, eds. *Control of Pig Reproduction.* London, England: Butterworths; 1982:405-418.
45. Ellicott AR, Dziuk PJ. Minimum daily dose of progesterone and plasma concentration for maintenance of pregnancy in ovariectomized gilts. *Biol Reprod.* 1973;9(3):300-304.
46. Gereš D, Žubčić D, Sabo D, et al. Changes of progesterone concentrations in blood plasma of sows during periparturient period. *Vet Arhiv.* 2000;70(1):47-57.
47. Adamsson A, Salonen V, Paranko J, Toppari J. Effects of maternal exposure to di-isononylphthalate (DINP) and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE) on steroidogenesis in the fetal rat testis and adrenal gland. *Reprod Toxicol.* 2009;28(1):66-74.
48. Hogg K, McNeilly AS, Duncan WC. Prenatal androgen exposure leads to alterations in gene and protein expression in the ovine fetal ovary. *Endocrinology.* 2011;152(5):2048-2059.
49. Ye L, Su ZJ, Ge RS. Inhibitors of testosterone biosynthetic and metabolic activation enzyme. *Molecules.* 2011;16(12):9983-10001.
50. Akgul Y, Derk RC, Meighan T, Rao KM, Muro EP. The methoxychlor metabolite, HPTE, directly inhibits the catalytic activity of cholesterol side-chain cleavage (P450_{scc}) in cultured rat ovarian cells. *Reprod Toxicol.* 2008;25(1):67-75.
51. Akgul Y, Derk RC, Meighan T, Rao KM, Muro EP. The methoxychlor metabolite, HPTE, inhibits rat luteal cell progesterone production. *Reprod Toxicol.* 2011;32(1):77-84.
52. Gregoraszczyk E. Steroid hormone release in cultures of pig corpus luteum and granulosa cells: effect of LH, hCG, PRL and estradiol. *Endocrinol Exp.* 1983;17(1):59-68.
53. Jiménez E, Cárdenas H, Pope WF. Effects of androgens on serum concentrations of gonadotropins and ovarian steroids in gilts. *Biol Reprod.* 2008;79(6):1148-1152.
54. Knapczyk K, Duda M, Czaplicki D, Slomczynska M. The presence of 3beta-hydroxysteroid dehydrogenase in the ovarian follicles and corpora lutea of pregnant swine. *Exp Clin Endocrinol Diabetes.* 2007;115(7):411-416.
55. Kostic TS, Stojkov NJ, Bjelic MM, Mihajlovic AI, Janjic MM, Andric SA. Pharmacological doses of testosterone upregulated androgen receptor and 3-beta-hydroxysteroid dehydrogenase/delta-5-delta-4 isomerase and impaired Leydig cells steroidogenesis in adult rats. *Toxicol Sci.* 2011;121(2):397-407.
56. Tan SH, Dagvadorj A, Shen F, et al. Transcription factor Stat5 synergizes with androgen receptor in prostate cancer cells. *Cancer Res.* 2008;68(1):236-248.
57. Kubota K, Ohsako S, Kurosawa S, et al. Effects of vinclozolin administration on sperm production and testosterone biosynthetic pathway in adult male rat. *J Reprod Dev.* 2003;49(5):403-412.
58. Basavarajappa MS, Craig ZR, Hernández-Ochoa I, Paulose T, Leslie TC, Flaws JA. Methoxychlor reduces estradiol levels by altering steroidogenesis and metabolism in mouse antral follicles in vitro. *Toxicol Appl Pharmacol.* 2011;253(3):161-169.
59. Peretz J, Gupta R, Singh J, Hernández-Ochoa I, Flaws J. Bisphenol A impairs follicle growth, inhibits steroidogenesis, and down-regulates rate-limiting enzymes in the estradiol biosynthesis pathway. *Toxicol Sci.* 2011;119(1):209-217.
60. Jayasekara WS, Yonezawa T, Ishida M, Yamanouchi K, Nishihara M. Molecular cloning of goat 20alpha-hydroxysteroid dehydrogenase cDNA. *J Reprod Dev.* 2004;50(3):323-331.
61. Stocco CO, Chedrese J, Deis RP. Luteal expression of cytochrome P450 side-chain cleavage, steroidogenic acute regulatory protein, 3beta-hydroxysteroid dehydrogenase, and 20alpha-hydroxysteroid dehydrogenase genes in late pregnant rats: effect of luteinizing hormone and RU486. *Biol Reprod.* 2001;65(4):1114-1119.
62. Wang L, Li S, Zhao A, et al. The expression of sex steroid synthesis and inactivation enzymes in subcutaneous adipose tissue of PCOS patients. *J Steroid Biochem Mol Biol.* 2012;132(1-2):120-126.
63. Telleria CM, Stocco CO, Stati AO, Deis RP. Progesterone receptor is not required for progesterone action in the rat corpus luteum of pregnancy. *Steroids.* 1999;64(11):760-766.
64. Kineman RD, Kraeling RR, Rampacek GB, Hausman GJ. Comparison of induced corpora lutea from prepubertal gilts and spontaneous corpora lutea from mature gilts: hydroxysteroid dehydrogenase activity. *J Anim Sci.* 1987;64(1):237-240.
65. Sugino N, Telleria CM, Gibori G. Progesterone inhibits 20alpha-hydroxysteroid dehydrogenase expression in the rat corpus luteum through the glucocorticoid receptor. *Endocrinology.* 1997;138(10):4497-4500.