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Flutamide alters β-catenin expression and distribution, and its interactions with E-cadherin in the porcine corpus luteum of mid- and late pregnancy

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Summary. This study examined whether flutamide-induced androgen deficiency during mid- and late pregnancy in pigs affected luteal expression of adherens junction protein, β-catenin, and its interactions with E-cadherin. Flutamide (50 mg/kg body weight) was administered into pregnant gilts between days 43-49 (GD50F), 83-89 (GD90F) or 101-107 (GD108F) of gestation. Corpora lutea (CLs) were obtained on day 50, 90 or 108 of pregnancy (n=8-11 per each group). Total β-catenin and E-cadherin expression was examined at mRNA (real-time PCR) and protein (Western blot) level. Moreover, subcellular β-catenin fractions were extracted and immunoblotted. Immunohistochemistry was used for β-catenin localization. To determine whether flutamide disturbs β-catenin/E-cadherin mutual interactions, coimmunoprecipitation using anti-β-catenin antibody was performed. Furthermore, phosphorylation of E-cadherin was assessed. Flutamide exposure led to decreased β-catenin mRNA expression in all examined groups ($p<0.001$ or $p<0.01$), but protein level was lower only in the GD90F and GD108F groups ($p<0.05$). E-cadherin mRNA ($p<0.05$ or $p<0.01$) and protein ($p<0.05$) levels were up-regulated in all flutamide-treated groups when compared to controls. β-catenin was predominantly found in membranes of luteal cells with no significant changes after antiandrogen treatment. β-catenin/E-cadherin complexes were more abundant in the GD90F ($p<0.05$) and GD108F ($p<0.01$) groups than in controls due to enhanced E-cadherin phosphorylation at serine 838/840 in those animals ($p<0.05$). Overall, although androgen deficiency affected β-catenin expression in the CL of pregnancy in pigs, a compensatory mechanism by enhanced interactions with E-cadherin is possible. Thus, androgen signaling via androgen receptors appears to be crucial in the regulation of luteal cells cross-talk.

Key words: β-catenin, E-cadherin, Flutamide, Corpus luteum, Pig

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Introduction

The corpus luteum (CL) is a transient endocrine gland that produces progesterone, a hormone required for the establishment and maintenance of pregnancy (Stjernholm, 2012). In some domestic animals, such as pig, the CL is functionally active during the entire gestation, and insufficient progesterone secretion leads to pregnancy loss or premature parturition (Taverne, 2001). Luteal synthesis of progesterone is under the control of numerous factors (e.g. LH, prolactin, prostaglandin $F_{2\alpha}$); however, the exact mechanism of this regulation is not completely understood (Niswender et al., 2000). Because there are two distinct types of steroidogenic luteal cells (large and small luteal cells) in the CL of pigs and most other mammalian species, cell-to-cell interactions are crucial for both the CL structural integrity and effective luteal steroidogenesis, predominantly progesterone production (Grazul-Bilska et al., 1997; Borowczyk et al., 2007; Durlej et al., 2011).

The cross-talk between luteal cells is very complex and involves the formation of gap junctions, tight junctions, and adherens junctions (Khan-Dawood et al., 1996a-c; Groten et al., 2006; Shirasuna et al., 2007). Evidence collected thus far suggested an association between progesterone secretion and gap junctional connexin 43 in the ovine (Borowczyk et al., 2007) and porcine (Durlej et al., 2011) CLs. Apart from that, recent data implicated the involvement of β-catenin, a protein of adherens junctions, in the LH-induced progesterone synthesis in bovine luteal cells *in vitro* (Roy et al., 2009). Furthermore, in other steroidogenic tissues β-catenin was shown to participate in the regulation of estrogen production in rat ovarian follicles (Stapp et al., 2014) and inhibit steroidogenesis in mice adrenal glands (Walczak et al., 2014). Herein, we have examined for the first time wether β-catenin may be involved in luteal cell steroidogenic capacity, particulary in progesterone biosynthesis, in pigs during pregnancy.

There are multiple pools of β-catenin protein within the cell. That works with its function as a structural component of adherens junctions and the key nuclear effector of canonical Wnt signalling pathway (Valenta et al., 2012). Most β-catenin is located in the cell membrane, where it is linked to the cytoplasmic domain of E-cadherin and participates in cell adhesion (Papkoff, 1997). A smaller pool of β-catenin is distributed in the nucleus and together with transcription factors of the lymphocyte enhancer-binding factor 1 (LEF)/T cell factor (TCF) family activates transcription of Wnt responsive genes (Gottardi and Gumbiner, 2001). The cytoplasmic β-catenin fraction is bound to the adenomatous polyposis coli (APC)-axin complex, which targets β-catenin for degradation in the absence of the Wnt signal (MacDonald et al., 2009). As the main binding partner of β-catenin, E-cadherin plays a crucial role in β-catenin stabilization and function via formation of mutual complexes.

Androgens are important regulators of luteal steroidogenesis in mammals. They were recognized to have a stimulatory effect on luteal progesterone secretion in pregnant rats (Carrizo et al., 1994; Goyeneche et al., 2002). Furthermore, our previous study revealed that antiandrogen flutamide decreased luteal production and metabolism of progesterone (Grzesiak et al., 2014) during late pregnancy in pigs. Interestingly, flutamide treatment down-regulated β-catenin mRNA and protein expression in various porcine reproductive organs, such as fetal ovary and testis (Knapczyk-Stwora et al., 2013), testis and epididymis of adult boar (Hejmej et al., 2012; Gorowska et al., 2014). Taken together, the question arises whether previously shown disrupted CL functioning marked by altered progesterone production (Grzesiak et al., 2014) might be accompanied by flutamide-induced changes in β-catenin expression? To further investigate this relationship, the objectives of the study were to determine whether flutamide administration during mid- and late pregnancy influences: (1) total luteal expression of mRNAs and proteins for β-catenin and E-cadherin; (2) localization of β-catenin within luteal tissue; (3) β-catenin distribution in subcellular fractions, and (4) formation of complexes between β-catenin and E-cadherin in the CLs on days 50, 90, and 108 of pregnancy in pigs.

Material and methods

Animals and experiment design

Porcine CLs of pregnancy used in the present investigation were derived from the same experimental animals that were examined in our previous study (Grzesiak et al., 2014). Briefly, twelve sexually mature crossbred gilts (Large White × Polish Landrace) of similar body weight (bw; 109.5±7.5 kg), age (~10 months) and genetic background were housed at the same farm conditions. Animals with at least one normal estrus symptom were checked daily for other estrus signs. After two consecutive estrous cycles, gilts were mated to a fertile boar at the onset of estrus and again 12 and 24 h later. The gestation day (GD) was estimated from the first mating day. Randomly assigned pregnant gilts were divided into three groups (two per each goup) and injected with the antiandrogen flutamide (Sigma-Aldrich, St. Louis, MO, USA) between days (i) 43 and 49 of gestation (GD50F), (ii) 83 and 89 of gestation (GD90F), or (iii) 101 and 107 of gestation (GD108F). Flutamide was suspended in corn oil administered by subcutaneous injections daily for 7 days at a dose of 50 mg/kg bw. For each flutamide-treated group, a respective control group (two animals per each group) given a vehicle only (corn oil) was established (GD50C, GD90C, GD108C). The days of pregnancy chosen for flutamide treatments correspond with the periods of midpregnancy (GD50), late pregnancy (GD90), and time around parturition (GD108).

All experimental procedures were performed by a veterinarian in accordance with the national guidelines approved by the Local Ethics Committee at the Jagiellonian University in Krakow, Poland (approval no. 122/2009).

CLs collection

Bilateral porcine ovaries were retrieved from pregnant gilts by an ovariectomy 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=8-11 per group) and flutamide-treated (F; n=8-11 per group) animals. Each CL was cut into three pieces that were either snap frozen in liquid nitrogen for real-time PCR and Western blot analyses or fixed in 10% (v/v) neutral buffered formalin for immunohistochemistry.

RNA preparation and reverse transcription

Total RNA from collected CLs was extracted using TRI Reagent solution (Ambion, Austin, TX, USA)

according to the manufacturer's instructions as previously described (Knapczyk-Stwora et. al., 2013). The quantity and quality of the total RNA was ascertained by measuring the absorbance at 260 and 280 nm with NanoDrop ND2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Moreover, 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 μg total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reactions were performed in a Veriti Thermal Cycler (Applied Biosystems). Genomic DNA contamination was checked by 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 analysis for β-catenin and E-cadherin

Quantitative real-time PCR assays were performed with StepOne™ Real-Time PCR System (Applied Biosystems) utilizing porcine-specific TaqMan Gene Expression Assays (Applied Biosystems) for β-catenin (*CTNNB1*; assay ID: Ss02667776_m1) and E-cadherin (*CDH1*; assay ID: Ss03377287_u1). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified as an endogenous control (assay ID: Ss03373286_u1). All real-time PCR experiments were performed in duplicate. Real-time PCR reactions were performed in a total reaction volume of 20 μL and included 10 μL TaqMan Gene Expression Master Mix, 1 μL 20× TaqMan Gene Expression Assay, 8 μL water, and 2 μL analyzed sample. The real-time PCR conditions were as follows: pre-heating at 50°C for 2 min, denaturation at 95°C for 10 min, and 40 cycles of amplification and quantification (15 sec at 95°C and 60 sec at 60°C). Expression levels of *CTNNB1* and *CDH1* were determined using the $2^{-\Delta Ct}$ method. Briefly, the cycle threshold (Ct; defined as the cycle number at which the fluorescence exceeds the threshold level) was determined for each sample. All samples were normalized to the *GAPDH* gene (ΔCt value) and relative expression was presented as $2^{-\Delta Ct}$. These $2^{-\Delta Ct}$ values were used to calculate statistical differences.

Total protein extraction and western blot analysis for β-catenin and E-cadherin

Equal amounts of frozen luteal tissue (30 mg) were homogenized, sonicated, and centrifuged as previously described (Grzesiak et al., 2014). Samples containing 20 μg protein were separated by 8% (v/v) SDS-PAGE under reducing conditions according to Laemmli (1970). Separated proteins were electroblotted onto a PVDF membrane. After overnight blocking in 5% (w/v) non-fat 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 diluted in TBST with 1% (w/v) BSA: polyclonal rabbit anti-β-catenin (1:500; cat. No. 71-2700, Invitrogen, Camarillo, CA, USA) and or polyclonal rabbit anti-E-cadherin (1:500; cat. No. sc-7870, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1.5 h at room temperature (RT). Subsequently, the membranes were incubated with specific secondary anti-rabbit IgG antibody linked to horseradish peroxidase (1:4000; Vector Laboratories, Burlingame, CA, USA) for 1 h at RT. To control for variable amounts of protein, the membranes were stripped and reprobed with monoclonal mouse anti-β-actin 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 (Bio-Rad Laboratories Inc.) and visualized using ChemiDoc XRS+ System (Bio-Rad Laboratories Inc.). Analysis of images was performed using the public domain ImageJ program (NIH, MD, USA) using the "Gel Analysis" functions. The bands were densitometrically quantified and normalized to their corresponding β-actin bands. Semi-quantitative analysis was performed for three separately repeated experiments from each control and flutamide-treated group.

To examine phosphorylated E-cadherin (S838/S840) Western blot analysis was conducted with the use of rabbit monoclonal anti-E-cadherin (phospho S383/S840) antibody (1:10000; cat. No. ab76319, Abcam, Cambridge, UK) followed by horseradish peroxidase-conjugated anti-rabbit IgG (1:4000; Vector Laboratories). The bands were densitometrically quantified and normalized to their corresponding total E-cadherin bands as described above.

Subcellular protein extraction and western blot analysis for β-catenin

Subcellular protein extraction (cytoplasmic, membranous, nuclear and cytoskeletal fractions) was performed with equal amounts of frozen CLs (30 mg) using the ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's instruction. The kit contains four extraction buffers, protease inhibitor cocktail to prevent protein degradation, and benzonase nuclease to remove contaminating nucleic acid. To verify the quality of extracted protein fractions the ProteoExtract® S-PEK Antibody Control Kit (Novagen, Darmstadt, Germany) was used. The kit provides four monoclonal antibodies (anti-HSP90α, anti-calnexin, anti-PARP-1, anti-vimentin) that recognize proteins specific for each of the four subcellular fractions, respectively. In the present study only cytoplasmic, membranous and nuclear protein fractions were used for further analysis. Protein content in all samples was determined using Bradford assay, the samples were separated by 8% (v/v) SDS-PAGE, and β-catenin was

detected as described above. The obtained results were expressed as the percentage of β-catenin protein within each subcellular fraction relative to total β-catenin content in the control and flutamide-exposed groups.

β-catenin immunohistochemistry

Immunohistochemistry was performed as previously described (Grzesiak et al., 2012). Briefly, CL sections from control and flutamide-treated animals were deparaffinized in xylene and rehydrated gradually through a series of ethanol dilutions. The slides were treated with 0.01 M citrate buffer (pH 6.0), followed by 30 min in 0.3% (v/v) H_2O_2 in TBS to quench endogenous peroxidase activity. Blocking of non-specific binding sites was performed with 5% (v/v) normal goat serum prior to incubation with anti-β-catenin antibody (1:400). After overnight incubation at 4°C in a humidified chamber, the sections were washed with TBST. The antigens were visualized using biotinylated secondary antibody goat anti-rabbit IgG (1:300, 1.5 h at RT; Vector Laboratories), avidin-biotin-peroxidase complex (1:100, 40 min at RT; StreptABComplex-HRP, Vector Laboratories), and 3,3'-diaminobenzidine (Sigma-Aldrich) as a chromogen. Sections were then counterstained with Mayer hematoxylin, dehydrated and mounted in DPX (Sigma-Aldrich). For the negative control reaction, sections were incubated with non-immune rabbit IgG (cat. No. NI01, Calbiochem) instead of primary antibody and processed as above. 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.

Precipitation with anti-β-catenin antibody

The precipitation procedure was conducted with the use of an Immunoprecipitation Kit (Protein G) (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, the clarified lysates of luteal tissues extracted previously from the control and flutamide-treated groups (200 μg of total protein) were precleaned with Protein G and incubated with 20 μl (5 μg) of anti-β-catenin antibody overnight at 4°C on an orbital rotator (Biosan, Riga, Latvia). Then the samples were mixed with Protein G and incubated for another 3 h at 4°C on an orbital rotator. Next the precipitated materials were washed two times with Wash Buffer 1 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, detergent), two times with Wash Buffer 2 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, detergent), and once with Wash Buffer 3 (50 mM Tris-HCl, pH 7.5, detergent). The precipitated proteins were eluted by boiling the Protein G complexes for 3 min in Laemmli sample buffer in the presence of reductant. The samples were separated by 8% (v/v) SDS-PAGE and transferred onto the PVDF membrane. Afterwards, membranes were sequentially incubated with specific antibodies diluted in TBST with 1% (w/v) BSA (1:250 for anti-β-catenin antibody and 1:500 for anti-E-cadherin antibody) for 1 h at RT in accordance with the abovementioned description. E-cadherin bands in precipitated material from the control and flutamide-treated groups were densitometrically quantified and normalized to their corresponding E-cadherin bands in the whole homogenates (expressed as arbitrary units).

Statistical analysis

Statistical analyses were performed using Statistica v.10 program (StatSoft, Inc, Tulsa, OK, USA). All data are presented as the overall mean ± standard error of the mean (SEM), and differences were considered statistically significant at the 95% confidence level ($p<0.05$). To verify the normal distribution of data the Shapiro-Wilk and the Lilliefors tests were applied. Because of the lack of normality in data, the nonparametric Mann-Whitney *U*-test was used to determine significant differences between the control and flutamide-treated groups. In real-time PCR and Western blot analyses involving whole homogenates and subcellular fractions, 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 was considered as an individual sample because of heterogeneity of porcine CLs (Rao and Edgerton, 1984).

Results

Effect of flutamide on total β-catenin mRNA and protein expression

Real-time PCR and Western blot analyses were performed to assess the effect of flutamide on total β-catenin expression at mRNA and protein levels in CLs obtained from control and flutamide-exposed gilts on days 50, 90, and 108 of pregnancy (Fig. 1). Within control groups, β-catenin mRNA level was greatest on day 50 of pregnancy and markedly decreased ($p<0.05$) on days 90 and 108 of gestation (Fig. 1A). On the contrary, β-catenin protein expression was lowest in the GD50C and GD90C groups, but it increased ($p<0.05$) in the GD108C group (Fig. 1B). Flutamide treatment resulted in decreased β-catenin mRNA expression in all examined groups ($p<0.001$, $p<0.01$, and $p<0.01$ respectively) in comparison to the control groups (Fig. 1A). Western blot results showed significant down-regulation of β-catenin protein only in the GD90F ($p<0.05$) and GD108F groups ($p<0.05$), when compared with their controls (Fig. 1B).

Effect of flutamide on β-catenin immunolocalization

Localization of β-catenin within CLs from control (Fig. 2A,C,E) and flutamide-treated (Fig. 2B,D,F) gilts on days 50, 90, and 108 of gestation was assessed by immunohistochemistry. β-catenin showed predominantly membranous staining in large luteal cells in the control

groups (Fig. 2A,C,E), as well as in those exposed to flutamide (Fig. 2B,D,F). Membranous, cytoplasmic and nuclear β-catenin immunoexpression was observed in small luteal cells of control (Fig. 2A,C,E) and flutamide-treated (Fig. 2B,D,F) animals. Replacement of the primary antibody with non-immune rabbit IgG was performed to obtain negative control (Fig. 2A inset).

Effect of flutamide on β-catenin protein expression in subcellular fractions

Subcellular fractionation followed by Western blot analysis was performed to examine β-catenin distribution within CLs from control and flutamide-treated gilts on days 50, 90, and 108 of gestation (Fig. 3A-C, respectively). Results are expressed as a percentage of β-catenin in subcellular fraction to total β-catenin content. As shown in Fig. 3, β-catenin protein was mainly present in the membranous fraction ($p<0.05$) of luteal tissue of control and flutamide-exposed gilts on each day of pregnancy (Fig. 3A-C). In the GD108F group nuclear distribution of β-catenin was higher ($p<0.05$) than in the GD108C group (Fig. 3C).

Effect of flutamide on the formation of β-catenin/E-cadherin complexes

To examine whether flutamide influences the

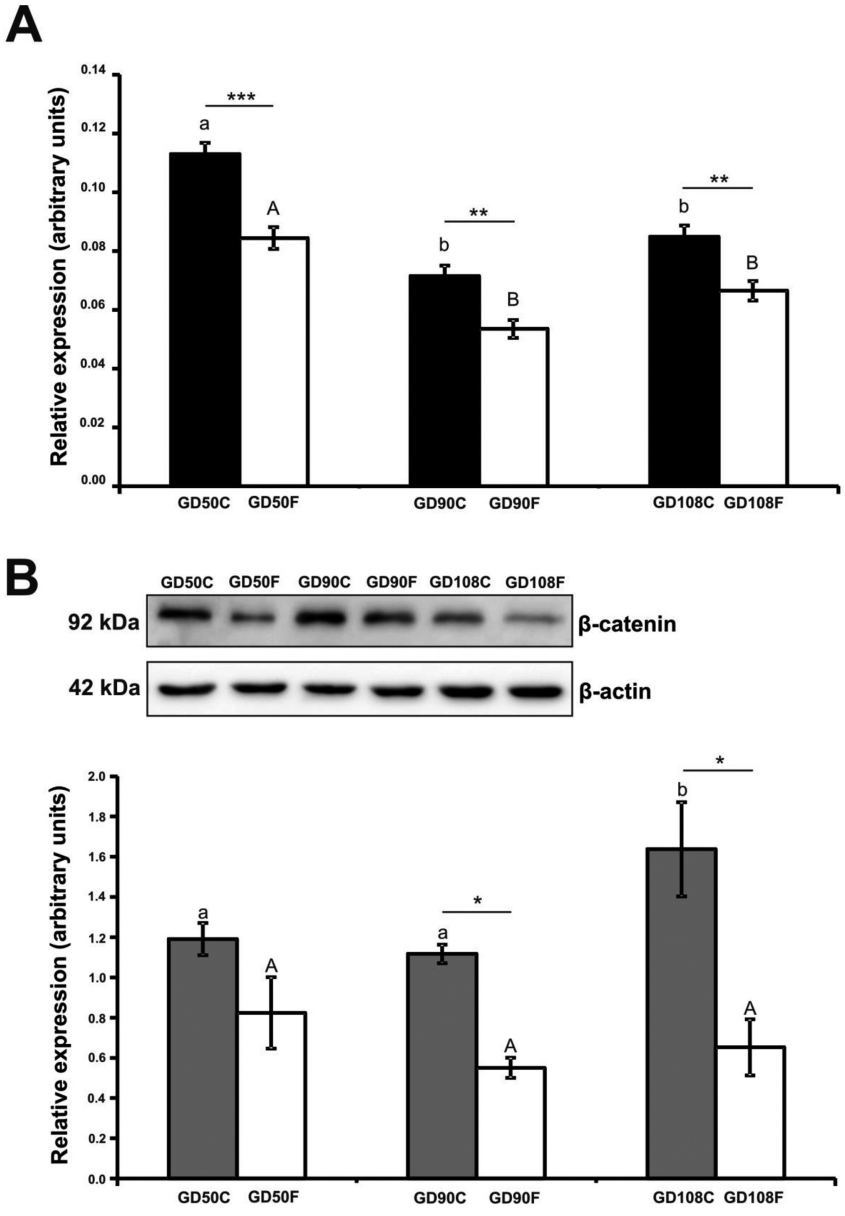


Fig. 1. Relative expression of mRNA and protein for β-catenin in corpora lutea obtained from control and flutamide-treated gilts on days 50 (GD50), 90 (GD90), and 108 (GD108) of gestation. **A.** The expression of β-catenin mRNA expressed as the ratio relative to *GAPDH*, and presented as $2^{-\Delta Ct}$ (solid bars, control groups; open bars, flutamide-treated groups). **B.** Representative blots of Western blot analysis and semi-quantitative densitometric analysis of β-catenin proteins are shown (solid bars, control groups; open bars, flutamide-treated groups). Asterisks indicate significant differences between control and flutamide-treated groups for each gestational stage (Mann-Whitney U-test; *$p<0.05$, **$p<0.01$, ***$p<0.001$). Different letter superscripts indicate differences (Kruskal-Wallis test; $p<0.05$) among control groups (lowercase letters) and among flutamide-treated groups (uppercase letters). Each value represents the mean ± SEM. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; GD, gestational day; C, control; F, flutamide.

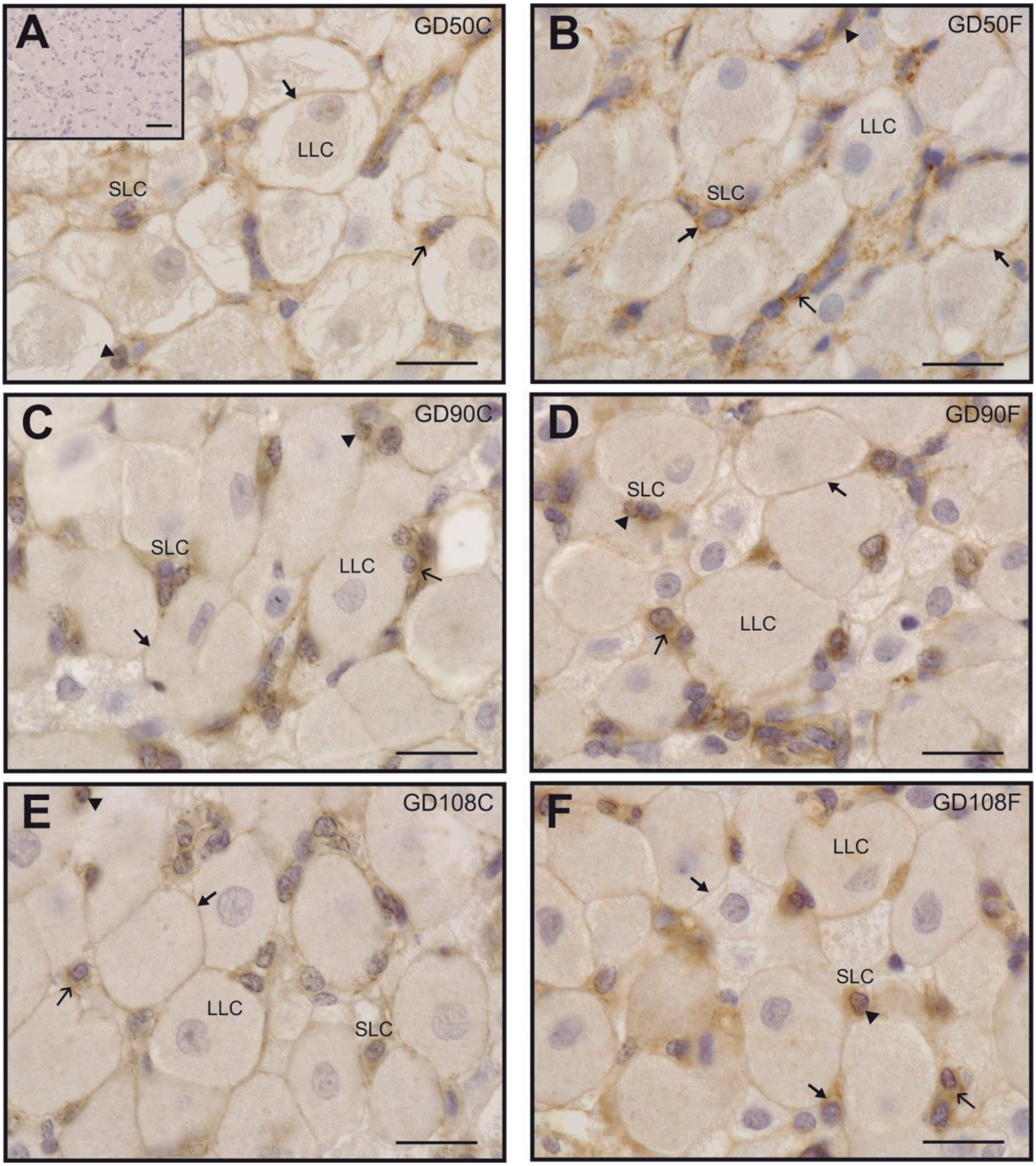
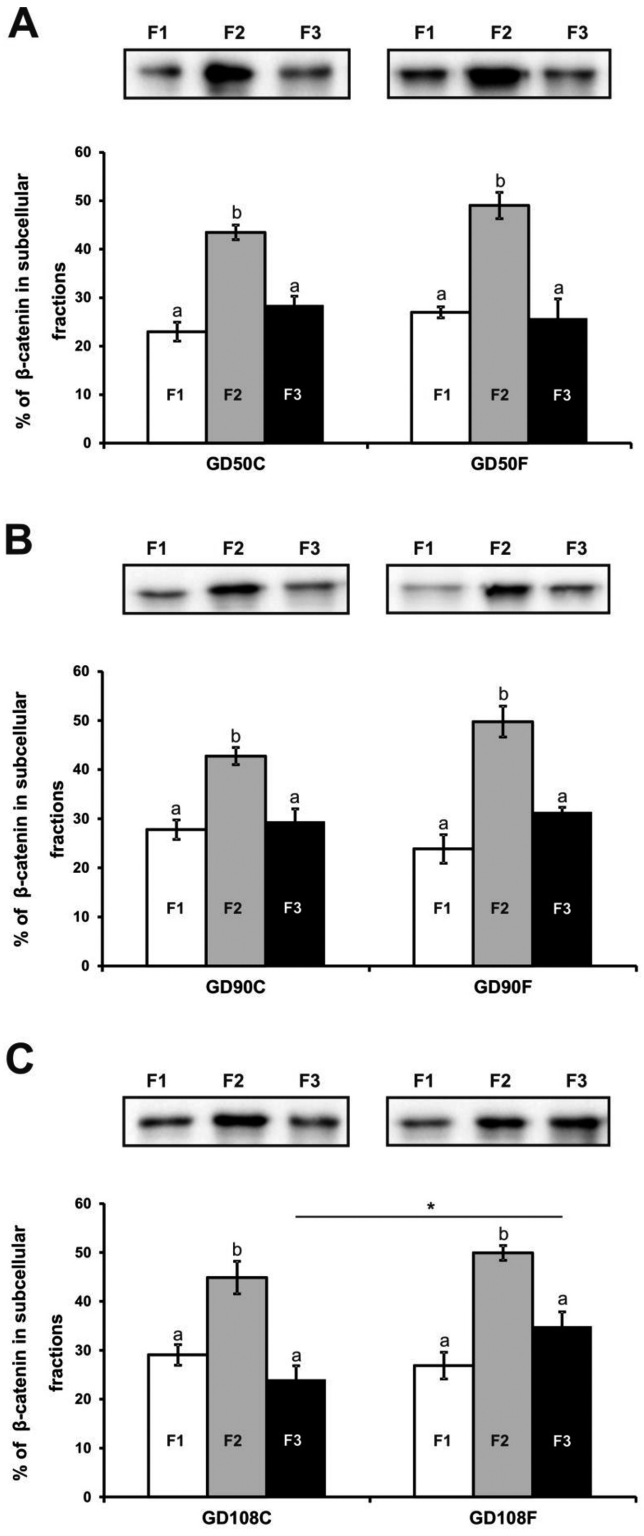


Fig. 2. Imunohistochemical localization of β-catenin in corpora lutea obtained from control **(A, C, E)** and flutamide-treated **(B, D, F)** gilts on days 50 (GD50), 90 (GD90), and 108 (GD108) of gestation. In both control and flutamide-treated groups, positive β-catenin staining was localized in the membranes (arrows) of LLC and SLC. Moreover, β-catenin was also found in the cytoplasm (open arrows) and nuclei (arrowheads) of SLC. Control sections in which the primary antibody was replaced by non-immune rabbit IgG did not exhibit any positive staining **(A inset)**. GD, gestational day; C, control; F, flutamide; LLC, large luteal cells; SLC, small luteal cells. Scale bars: 50 μm.



formation of β-catenin/E-cadherin complexes, coimmunoprecipitation of β-catenin and E-cadherin in luteal tissue extracts obtained from CLs of control and flutamide-treated gilts on days 50, 90, and 108 of gestation was conducted using anti-β-catenin antibody (Fig. 4). In all analyzed control and flutamide-exposed samples, the presence of β-catenin protein in β-catenin directed immunoprecipitates was confirmed (Fig. 4A upper blots). Likewise, E-cadherin was found in all control and flutamide-treated groups (Fig. 4A lower blots) that indicates its coprecipitation with β-catenin. As shown in Fig. 4B, the complexes between β-catenin and E-cadherin were significantly more abundant in the GD90F ($p<0.05$) and GD108F ($p<0.01$) groups than in the respective controls. Moreover, the absence of both analyzed proteins in supernatants obtained during immunoprecipitation indicates the propriety of the experimental procedure (Fig. 4A).

Effect of flutamide on total E-cadherin mRNA and protein expression and E-cadherin phosphorylation

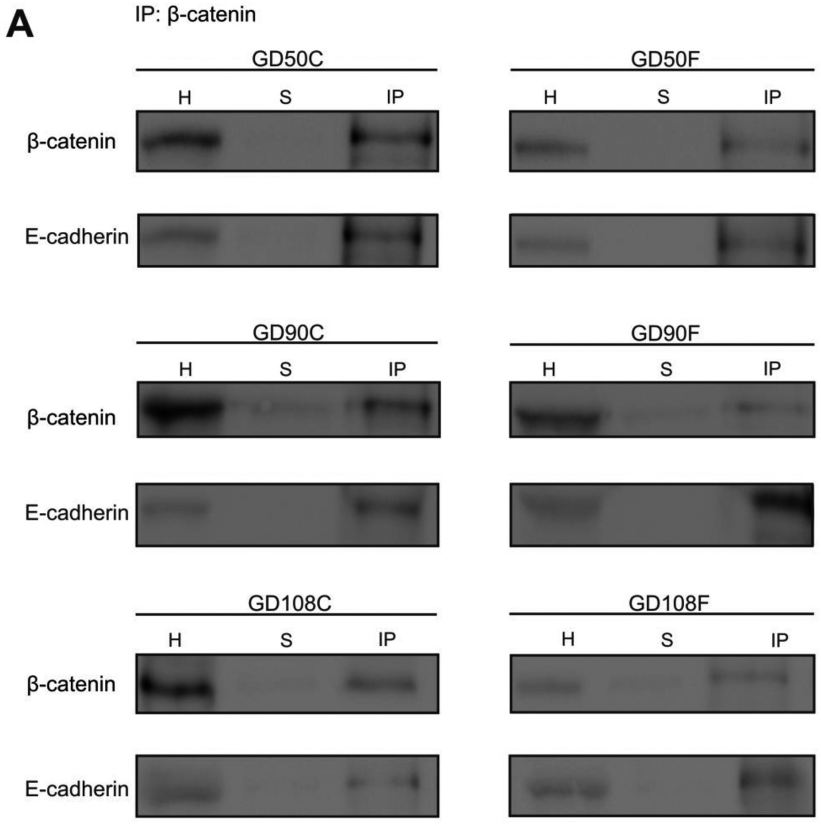
E-cadherin mRNA expression was significantly elevated in the GD50F ($p<0.05$), GD90F ($p<0.01$), and GD108F ($p<0.01$) groups (Fig. 5A), which corresponded with the increased E-cadherin protein level following flutamide administration on each examined day of gestation ($p<0.05$) (Fig. 5B$^{\#\#}$). Both E-cadherin mRNA (Fig. 5A) and protein (Fig. 5B$^{\#\#}$) expressions were at the same level within pregnancy progression.

Western blot analysis was conducted to assess the effect of flutamide on E-cadherin phosphorylation in CLs obtained from control and flutamide-exposed gilts on days 50, 90, and 108 of pregnancy (Fig. 5B). Using an antibody which detects E-cadherin phosphorylated at serine 838/840, we have observed increased level of phosphorylated E-cadherin normalized to total E-cadherin in the GD90F and GD108F groups ($p<0.05$), when compared with their controls (Fig. 5B$^{\#}$). E-cadherin phosphorylation level was unchanged within pregnancy progression among control and flutamide-treated groups (Fig. 5B$^{\#}$).

Fig. 3. Western blot analysis of β-catenin expression in subcellular fractions: cytoplasmic (F1), membranous (F2), and nuclear (F3) of luteal tissue extracts obtained from corpora lutea of control and flutamide-treated gilts on days 50 (GD50; **A**), 90 (GD90; **B**), and 108 (GD108; **C**) of gestation. Results are expressed as the percentage of β-catenin protein within each subcellular fraction relative to total β-catenin content (set as 100%) in the control or flutamide-exposed groups. Asterisk indicates significant difference between control and flutamide-treated groups on 108 gestational day (Mann-Whitney U-test; *$p<0.05$). Different letter superscripts indicate differences among control groups and among flutamide-treated groups (Kruskal-Wallis test; $p<0.05$). Each value represents mean of relative β-catenin content (%) ± SEM. GD, gestational day; C, control; F, flutamide.

Discussion

Recently it has been demonstrated that β-catenin, apart from its involvement in cell adhesion, might also act as a transcriptional factor and regulate luteal progesterone synthesis (Roy et al., 2009). The present study showed decreased β-catenin mRNA and protein expression following flutamide administration in the porcine CL during late pregnancy (GD90F and GD108F). Furthermore, we observed stronger interactions between β-catenin and E-cadherin in those animals due to enhanced E-cadherin phosphorylation. Therefore, the results suggest the presence of compensatory mechanism to prevent the disruption of



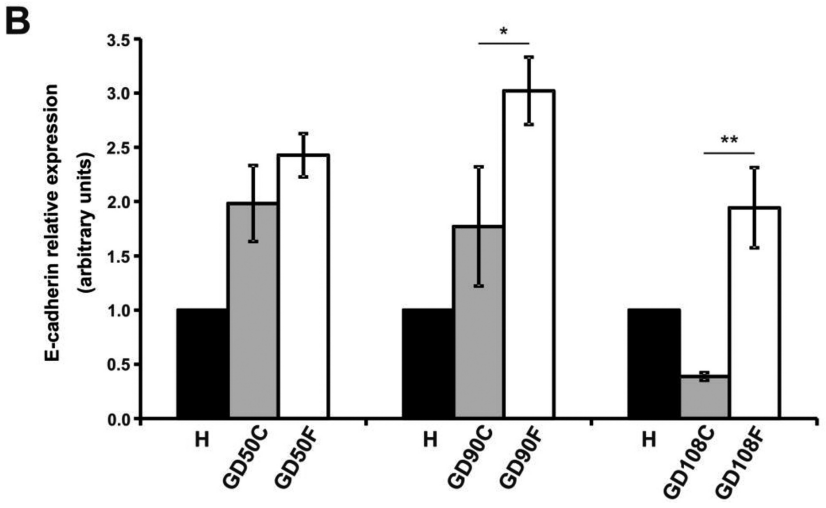


Fig. 4. Coimmunoprecipitation of β-catenin and E-cadherin in luteal tissue extracts obtained from corpora lutea of control and flutamide-treated gilts on days 50 (GD50), 90 (GD90), and 108 (GD108) of gestation. **A.** Upper blots represent immunoprecipitated samples, wich were blotted for β-catenin as a control of precipitation efficiency. Lower blots represent the same samples blotted for E-cadherin detection. No positive signal was found in supernatants obtained during immunoprecipitation (negative control). **B.** E-cadherin bands from the control and flutamide-treated groups were densitometrically quantified and normalized to their corresponding E-cadherin bands in the whole homogenates. Only one bar representative for whole homogenates of control and flutamide-treated groups was shown (set as 1 respectively). Each value represents the mean ± SEM. *p<0.05, **p<0.01 (Mann-Whitney U-test). GD, gestational day; C, control; F, flutamide; H, whole luteal tissue homogenates; S, supernatants obtained during immunoprecipitation; IP, immunoprecipitates.

cell-cell communication within the porcine CL of pregnancy.

There is a direct link between β-catenin and androgen signaling. Protein of β-catenin selectively binds to the ligand binding domain of AR, but not to other steroid hormone receptors. Noteworthy, β-catenin augmented the ligand-dependent activity of AR in prostate cancer cells via this specific interaction (Yang et al., 2002). To assess changes in mRNA and protein levels of β-catenin in CLs of pregnant gilts exposed to flutamide, we used real-time PCR and Western blot analyses, respectively. Down-regulation of β-catenin mRNA was observed in all examined groups, whereas decreased β-catenin protein level was found only during late pregnancy (GD90F and GD108F). In agreement with the present findings, recent studies demonstrated flutamide-induced down-regulation of β-catenin expression in testes (Hejmej et al., 2012) and epididymis (Gorowska et al., 2014) of adult boars, as well as in porcine fetal gonads (Knapczyk-Stwora et al., 2013). Overall, these observations underscore the regulation of β-catenin by androgens. The further question should be addressed whether altered β-catenin expression might be directly involved in diminished progesterone production by the same CLs as stated previously (Grzesiak et al., 2014).

Apart from transcript and protein levels, a cellular distribution of β-catenin importantly determines its functions. The current research revealed for the first time the presence of β-catenin in the porcine CL of pregnancy, predominantly in submembranous region of luteal cells in both control and flutamide-treated gilts. These findings are consistent with the results on primates (Kahan-Dawood et al., 1996a,b) and rodents (Sundfeldt et al., 2000) and support the idea about β-catenin involvement in cell adhesion. Noteworthy, we have also observed cytoplasmic and nuclear β-catenin localization in small luteal cells suggesting that this protein might have transcriptional activity in the porcine CL during pregnancy.

Since β-catenin was immunolocalized in different cell compartments, the further aim of the study was to demonstrate the detailed cellular distribution of β-catenin within porcine CL of pregnancy following subcellular fractionation. In both control and flutamide-

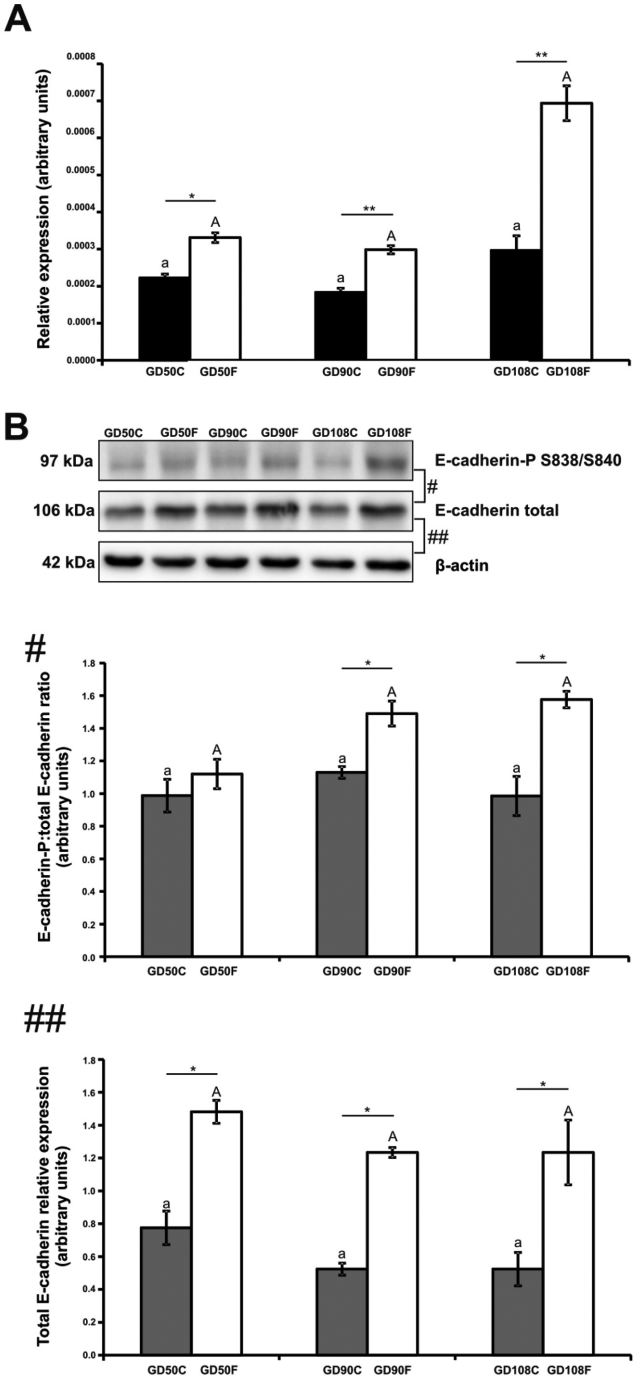


Fig. 5. Relative expression of E-cadherin mRNA, total and phosphorylated protein in corpora lutea obtained from control and flutamide-treated gilts on days 50 (GD50), 90 (GD90), and 108 (GD108) of gestation. **A.** The expression of E-cadherin mRNA was expressed as the ratio relative to *GAPDH*, and presented as $2^{-\Delta Ct}$ (solid bars, control groups; open bars, flutamide-treated groups). **B.** Representative blots of Western blot analysis and the expression of total and phosphorylated E-cadherin (S838/S840) proteins are shown. (B#) Semi-quantitative densitometric analysis of phosphorylated E-cadherin content was expressed as the ratio relative to total E-cadherin (solid bars, control groups; open bars, flutamide-treated groups). (B##) Total E-cadherin protein content was expressed as the ratio relative to β-actin (solid bars, control groups; open bars, flutamide-treated groups). Asterisks indicate significant differences between control and flutamide-treated groups for each gestational stage (Mann-Whitney U-test; *$p<0.05$, **$p<0.01$). Different letter superscripts indicate differences (Kruskal-Wallis test; $p<0.05$) among control groups (lowercase letters) and among flutamide-treated groups (uppercase letters). Each value represents the mean ± SEM. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; GD, gestational day; C, control; F, flutamide.

treated groups, the most abundant β-catenin expression was found in the membranous fraction similarly to immunohistochemical results described above. Interestingly, flutamide exposure resulted in a markedly increased β-catenin protein level within nuclear fraction on day 108 of gestation that suggests enhanced transcriptional activity of β-catenin. Recent studies implicated β-catenin as a cofactor regulating expression of hormone-dependent genes in steroidogenic tissues (Gummow et al., 2003; Parakh et al., 2006). Of particular importance, Roy et al. (2009) showed that LH-stimulated overexpression of β-catenin increased progesterone production in bovine luteal cells possibly via its action as a cotranscriptional factor for steroidogenic acute regulatory protein (StAR) gene. Notably, redistribution of β-catenin to the nucleus might not always be sufficient for the regulation of progesterone production because of a large number of factors and signaling pathways that should be activated for optimal steroidogenesis (Roy et al., 2009). In the present study, the increased nuclear localization of β-catenin appears not to be involved directly in luteal progesterone biosynthesis, which was reduced in the same CLs as shown previously (Grzesiak et al., 2014). Although we have demonstrated significantly decreased levels of total mRNA and protein for β-catenin in the GD90F and GD108F groups, establishing an unequivocal association with diminished progesterone concentration is problematic. Of physiological importance, an alternative explanation for nuclear β-catenin distribution would be related to pathological stages. Boerboom et al. (2005) showed nuclear β-catenin localization in spontaneously occurring human and equine granulosa cell tumors, suggesting that misregulation of the Wnt/β-catenin signaling pathway might be a predictive marker of cancer.

Steroids, including androgens, have been shown to modulate cell adhesion in a tissue-specific manner. It is relevant for the present study that activated AR represses the expression of E-cadherin (Liu et al., 2008). Nightingale et al. (2003) reported that dihydrotestosterone acting via AR downregulated E-cadherin expression in prostatic cancer cells. Herein, we suggest that flutamide binding to AR blocked its transcriptional activity and up-regulation of E-cadherin mRNA and protein were found in CLs in all examined days of pregnancy. Because β-catenin plays a role in retaining normal cell adhesion by interaction with E-cadherin, the next goal of this research was to examine whether flutamide treatment influenced formation of complexes between β-catenin and E-cadherin. From the immunoprecipitation data, the association between these two proteins was significantly greater in the CLs during late pregnancy and before parturition in pigs. The functional β-catenin/E-cadherin complexes are necessary for the stabilization of β-catenin at the membrane (Gottardi and Gumbiner, 2001). It was reported that overexpression of E-cadherin can result in withdrawal of β-catenin from the cytoplasm and nucleus that in turn antagonizes its transcriptional activity (Orsulic et al., 1999). Therefore, it seems possible that increased luteal E-cadherin expression and enhanced β-catenin binding following flutamide administration might reflect a yet unidentified adaptive response that compensates decreased β-catenin level and promotes intercellular adhesion within the CL of pregnancy in pigs.

Another important question is the molecular mechanism of augmented stabilization of β-catenin in complexes with E-cadherin. Cell adhesion is a dynamic process that requires unremitting interactions between cell adhesion molecules and their posttranslational modifications, such as phosphorylation and dephosphorylation (Bertocchi et al., 2012). Phosphorylation of E-cadherin is restricted to a short region in the cytoplasic domain, which is crucial for β-catenin and plakoglobin attachement. Thus, E-cadherin phosphorylation is a common mechanism by which adhesion occurrence is modulated (Stappert and Kemler, 1994). Indeed, Lickert et al. (2000) have shown the increased binding affinity towards β-catenin upon phosphorylation of E-cadherin by casein kinase II at serine residues in human fibroblasts. Moreover, the serines localized in the β-catenin-binding site of E-cadherin were confirmed to be conservative among cadherins, supporting the concept that these residues are important for β-catenin binding (Lickert et al., 2000). In the present study, the increased serine phosphorylation (S838/S840) within β-catenin-binding region of E-cadherin in the GD90F and GD108F groups were found. In turn, these results might explain the stronger binding of β-catenin to E-cadherin, marked by more abundant complexes between these proteins.

In conclusion, although our study showed decreased β-catenin expression in the porcine CL during late pregnancy and preparturient period, we are not able to link these results directly with the reduced progesterone secretion described recently (Grzesiak et al., 2014). The luteal β-catenin expression has changed within pregnancy progression. The highest protein level was found around parturition, which indicates extensive β-catenin contribution to luteal function during this period. Regardless of the day of pregnancy and flutamide treatment, β-catenin was mainly found in the membrane of luteal cells that suggests its involvement in cell adhesion. Herein, we propose androgen-mediated regulation of cell adhesion through E-cadherin in the porcine CL of pregnancy. This conclusion is further supported by the fact that increased E-cadherin expression and its stronger binding to β-catenin were observed in the CL in the GD90F and GD108F groups. Therefore, androgen signaling via AR appears to be crucial in the regulation of luteal cell adhesion by affecting the expression of cell adhesion molecules and their interactions. These findings might be relevant especially in relation to processes contributed to luteal regression (apoptosis or autophagy) that occurs during late pregnancy and prior to parturition.

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