PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia biologica (Kraków), vol. 60 (2012), No 1-2
doi:10.3409/fb60_1-2.27-33

Prenatal Exposure to Antiandrogen Flutamide Affects Androgen Receptor (AR) Expression in Postnatal Ovarian Development in Pig*

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Accepted October 5, 2011

DURLEJ M., WIECIECH I., WĘGRZYN P., SŁOMCZYŃSKA M. 2012. Prenatal exposure to antiandrogen flutamide affects androgen receptor (AR) expression in postnatal ovarian development in pig. Folia biologica (Kraków) **60**: 27-33.

The following study was undertaken to localize androgen receptors (ARs) in various structures of the porcine ovary after prenatal exposure to antiandrogen flutamide. *In utero* treatment by antiandrogens may have adverse effects on reproductive function in immature and adult animals. Flutamide was injected into pregnant swines between days 20 and 28 (GD20) or 80 to 88 (GD80) of gestation. The ovaries were collected from treated animals and from control ones (non-treated) at two different points of development: from immature and adult pigs. Immunoexpression of AR was determined for preantral and antral follicles and for stroma cells. Immunostaining showed that AR expression in immature animals was unaffected in the primary follicles, while in the preantral and antral follicles the AR level fluctuated depending on day of treatment as well as on analyzed tissue. In adult animals, the immunoexpression of AR slightly decreased in antral follicles independently on the day of flutamide treatment. Therefore, AR expression in postnatal life may be affected by *in utero* exposure to antiandrogen flutamide.

Key words: Androgen receptor, flutamide, ovary, pig.

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The ovary has two important roles which depends on a succession of multiple integrated processes in female reproduction (LENIE & SMITZ 2009). During the process of folliculogenesis the ovary produces fertilizable gametes and is an important site of steroid production that directly participates in folliculogenesis through endogenously expressed receptors and indirectly affects ovarian cyclicity through regulation of the neuroendocrine system. In mammals the ovary is controlled by endocrine and neural factors (WALTERS *et al.* 2008). Ovarian steroid hormones produced locally also regulate folliculogenesis through modulation of gonadotropin and growth factor activities. During antral follicle growth, theca cells predominantly secrete aromatizable androgens which accumulate within follicular fluid. Granulosa cells of all mammalian species examined to date express the AR and direct, AR-mediated activity has been implicated in the control of folliculogenesis through mechanisms that involve both FSH (WANG *et al.* 2001) and IGF1 (HICKEY *et al.* 2004, 2005).

Fully grown oocytes are arrested in the diploten stage of the first meiotic prophase, and the maturation of meiosis is triggered *in vivo* by a hormonal stimulus (FAN & SUN 2004). Androgens were shown to be involved in promoting oocyte maturation in mouse (JAMNONGJIT & HAMMES 2006), and could potentially trigger porcine oocyte meiotic resumption, which is mediated by intra-oocyte AR (LI *et al.* 2008). Hormones such as testosterone and dihydrotestosterone influences cell growth and differentiation by regulating specific target gene expression after binding to the androgen receptor (AR), a member of the steroid receptor superfamily (LAMB *et al.* 2001). Antiandrogens flutamide or bicalutamide work by inhibiting endogenous androgens from activating AR. Fluta-

*Supported by the Ministry of Science and Higher Education Grant no. N N303 017737 and partly by K/ZDS/001952. M. DURLEJ is a scholar of the Foundation for Polish Science 2011.

mide is a non-steroidal, pure anti-androgenic compound which binds AR and blocks androgen action (TEVELL *et al.* 2006).

A previous study revealed the presence of AR in the porcine fetal ovaries at different days of prenatal life. The study reported by BUREK *et al.* (2007) revealed that androgens may play a regulatory role in developing fetal ovary, as they are present in stroma cells surrounding and supporting the egg nests. The days of pregnancy selected for the experiment are critical for ovarian development. Day 20 is close to the time of implantation and the ovary is not differentiated yet, while at day 80 the formation of primordial follicles occurs. It is well known from our earlier papers, that exposure to flutamide during fetal development may result in defective expression of certain proteins (FSHR, Cx43) during postnatal life (DURLEJ *et al.* 2011a; 2011b). As those genes are androgen regulated, it could be assumed that differences in their expression level might be a result of changes in AR protein level/activity caused by flutamide treatment. However, the question remains whether in postnatal life the changes of expression of AR could be observed. Hence, the aim of this study was to localize AR protein immunologically in ovarian follicles of immature and adult pigs following prenatal flutamide exposure.

Material and Methods

Animals and tissue collection

All procedures were performed in accordance with the Polish legal requirements, under the license given by the National Commission of Bioethics at the Jagiellonian University, Kraków, Poland (No. 4/2008).

The study involved six pregnant crossbred pigs (Large White x Polish Landrace). They were divided into three groups. The first group served as a control, gilts of the second group were treated with flutamide (2-methyl-N-[4-nitro-3'-(trifluoromethyl)-phenyl]propamide) (Sigma-AldrichChemical Co., St. Louis, MO, USA) between gestational days 20-28 (GD20), and gilts of the third group between days 80-88 (GD80). Flutamide (50 mg/kg body weight) was dissolved in corn oil and was given as subcutaneous injections at every second day of gestation. Female offspring from control (n=3) and flutamide-treated pigs (n=3 for GD20 and GD80) were maintained until immaturity and ovarian samples were collected from three-month-old pigs. Simultaneously, part of the animals from each group (n=3 for control, GD20 and GD80) were bred until sexual maturity and ovarian tissues were obtained from nine-month-old ones (mature). Ovariectomy was performed under general anesthesia. Gestational days were chosen on the basis of literature data (FOSTER & HARRIS 2005) and our previous studies (BUREK *et al.* 2007; KNAPCZYK *et al.* 2008). Control animals were exposed only to corn oil.

AR immunolocalization

The ovarian samples were fixed in 10% buffered formalin overnight. Then, samples were dehydratated in an increasing gradient of ethanol and equilibrated with xylene prior to embedding in paraplast (Monoject Scientific Devision of Sherwood Medical, St. Lousis, MO, USA). Paraplast sections were cut by microtome (5 μm), mounted on slides, deparaffinized in xylene and rehydrated through a series of alcohol dilutions. Sections were subjected to a microwave antigen retrieval technique by incubation in 0.01 M citrate buffer (pH 6.0) in a microwave oven at full power for 3 x 4 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in TBS (Tris buffered saline, pH 7.4) for 20 min. After the treatment, the sections were left for 30 min in the buffer for cooling. To prevent nonspecific reactions, sections were incubated with 5% normal goat serum (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature. Thereafter, tissue sections were incubated overnight at 4°C with the primary rabbit polyclonal antibody anti-AR (1:2000, SantaCruz). Negative control sections were incubated with non-immune goat serum instead of androgen receptor antibody. After washing in TBS buffer for amplification, of the primary antibody reaction the secondary goat biotinylated antibody (1:300, Vector Lab.) was placed for 1.5 h and ABC Complex/HPR (avidin-biotinylated horseradishperoxidase complex; 1:100; Vector Lab.) was used.

The reaction product was visualized using TBS containing 0.01% H_2O_2 , 0.05% diaminobenzidine (DAB; Sigma Aldrich) and 0.07% imidazole. Finally, slides were dehydrated and mounted on DPX (Fluka Chemie GmbH, Buchs).

AR immunostaining evaluation and statistical analysis

For specific AR immunostaining analysis photomicrographs were taken using a Nikon Eclipse E200 microscope attached to Coolpix 5400 digital camera system and then quantified by a videodensitometric analysis using ImageJ software (National Institutes of Health, Bethesda, MD, USA). At least eight different sections from each investigated animal were examined. For the intensity of immunoreaction in stroma cells, five randomly chosen microscopic fields from each section were

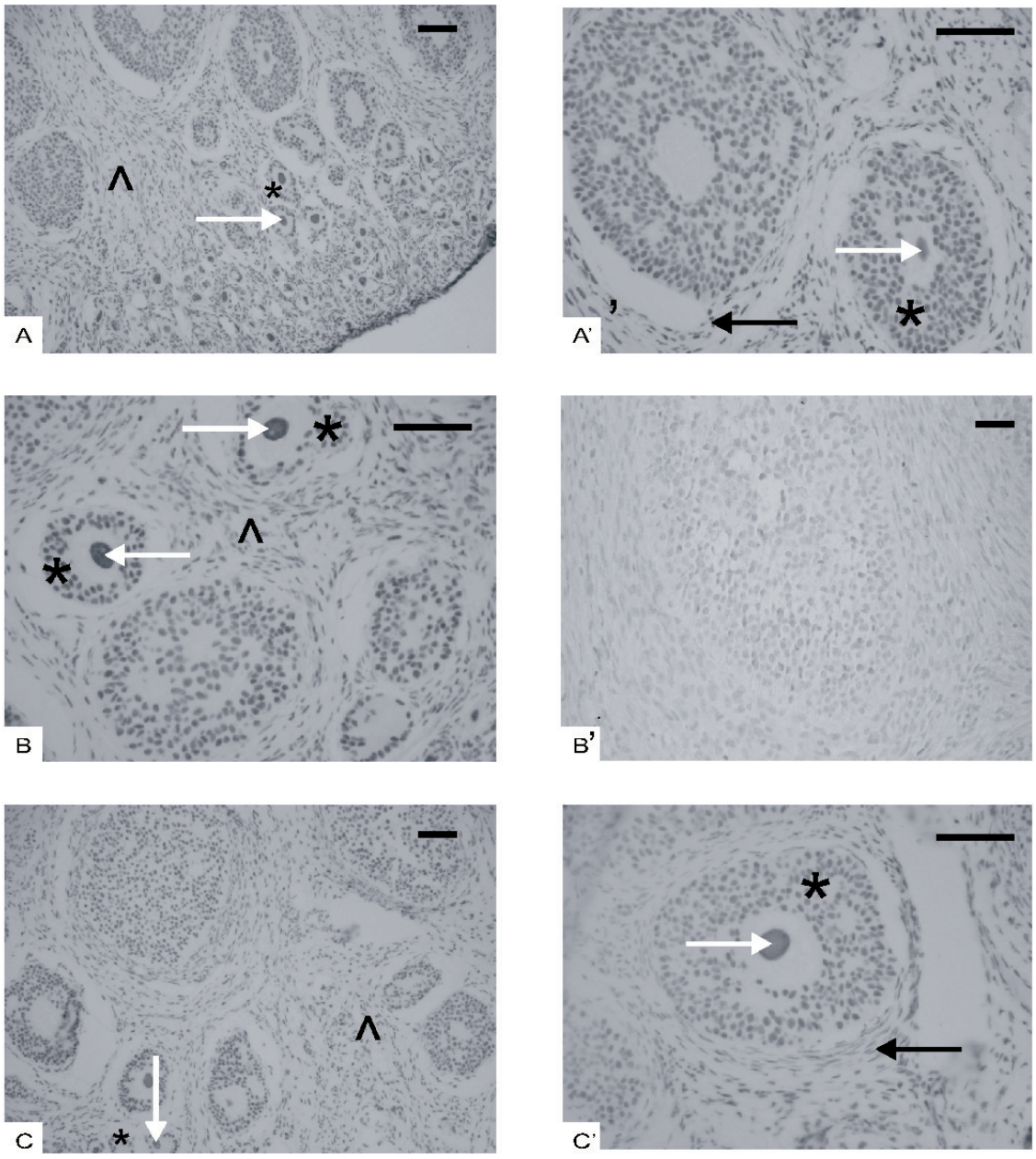

Fig. 1. Immunohistochemical localization of androgen receptor (AR) in primary (one layer of cuboidal granulosa cells) and preantral (more than one layer of granulosa cells) follicles of immature porcine ovaries following exposure to flutamide: A, A' – control group; B – flutamide treatment on gestational days 20-28; C, C' – flutamide treatment on gestational days 80-88. Positive reaction was observed in the nuclei of all primary and preantral follicle components: oocytes (white arrows), granulosa cells (*), theca cells (black arrows) and in the nuclei of stroma cells (^). B' – negative control, scale bar = 100 μm.

measured. Primary, preantral and antral follicles, all observed in the sections, were quantified. Measurements were repeated twice by two independent observers. The intensity of the immunohistochemical reaction was expressed as relative optical density (ROD) of diaminobenzidine brown reaction products (SMOLEN 1990). Statistical analysis was performed using the STATISTICA 5.1 program (StatSoft Imc, Tulsa, OK, USA). ANOVA test was used to analyze statistical differences and Tukey's test was used to determined differences between groups. Statistical significances were set at $P<0.01$.

Results

In ovaries of immature pigs, AR immunoexpression was examined in primary (one layer of cuboidal granulosa cells), preantral (more than one layer

of granulosa cells) and antral follicles. In ovaries of adult animals AR immunostaining was analyzed in antral follicles. Immunolocalization of AR was observed in all investigated animals. However, the intensity of reaction differed in the examined groups.

Immature animals

In the primary follicles no differences in AR expression were observed in flutamide treated animals (Fig. 1). The intensity of staining was unaffected by flutamide treatment in experimental groups in oocytes and granulosa cells (Fig. 4A). At this stage of follicle development it was difficult to analyze theca cells which were not well differentiated.

Immunohistochemistry demonstrated that AR was present in preantral follicles in GD20, GD80 and control groups (Fig. 1). The intensity of immunostaining increased in oocytes from GD20 ($P<0.01$), and in theca cells from GD80, while the intensity of staining decreased in granulosa cells from GD80 group (Fig. 4B).

In the antral follicles AR protein was decreased in granulosa, theca and stroma cells in the GD80 experimental group while no statistically significant differences were demonstrated in GD20 animals (Figs 2 & 4C).

Adult animals

In large antral follicles AR protein was observed in granulosa and theca cells (Fig. 3). However, the intensity of staining decreased in flutamide treated pigs in comparison to control animals (Fig. 4D).

Quantitative evaluation of the intensity of the immunohistochemical reaction in all ovarian compartments (Figs 4A-D) was expressed as relative optical density (ROD). Significant differences were detected between oocyte, granulosa and stroma cells of flutamide treated animals GD20 in comparison to the control group and GD80 ($P<0.01$).

Discussion

The current study is the first which demonstrated changes in the expression of AR in the ovaries of pigs treated with flutamide during fetal life.

AR is a ligand activated nuclear transcription factor which regulates the functions of many organs. In the embryo, androgens stimulate male differentiation and determine morphogenesis of the genital tract. The genomic effect of androgens is mediated via nuclear ARs and androgens have the ability to up- and down-regulate their own receptor. The study by BUREK *et al.* (2007) shows that in the developing fetal ovary androgen receptor immunoreaction is limited predominantly to stroma cells surrounding and suppoting the egg nests. Immunolabeling was observed in developing follicles in pregranulosa cells (day 90 p.c.) independent of their origin from surface epithelial cells or intraovarian rete. Granulosa cells of ovarian preantral follicles collected from 1 day-old neonatal piglets are AR-positive (BUREK *et al*. 2007). However, the lack of AR in granulosa cells of preantral follicles was observed in the ovaries of neonatal rats (our unpublished observation).

Previous research revealed that exposure to flutamide during gestation led to alteration in AR mRNA levels at least in preantral and large antral follicles of adult pigs (DURLEJ *et al.* 2011b). However, there was no influence of flutamide exposure on Cx43 expression in the gonads of neonatal (DURLEJ *et al.* 2011c) and prepubertal pigs (KOPERA *et al.* 2010).

Prenatal testosterone deficiency (as a result of antiandrogen action) results in postnatal changes in ovarian morphology and function. As shown here, exposure to flutamide *in utero* results in changes of AR protein level in animals in neonatal life as well as in immature and adult pigs. Observed changes are not very dramatic, and in some tissues the increase of immunostaining level can be observed while in other compartments, a decrease was documented. There is no doubt that exposure to flutamide during fetal life can affect AR expression in fetal life as well as in different developmental points after delivery. Although differences are statistically relevant, they fluctuate during development. Flutamide has been shown to block effectively the action of both T and DHT by competing at the level of the androgen receptor whereas it has no effect on T or DHT biosynthesis (IMPERATO-MCGINLEY *et al.* 1992). Flutamide promotes AR translocation to the nucleus and binding to DNA but no transcription is observed (TRUSS *et al.* 1994). Single cell analysis of live cells during hormone addition shed light on the rapid changes in AR function that result in nuclear translocation (MARCELLI *et al.* 2006).

Several genes are known to be involved in embryonic gonad development and sexual differentiation in large mammals such as the pig. Some of them may be regulated by androgens (MCCOARD *et al.* 2001). In order to regulate transcription, nuclear receptors must exist as a part of large multiprotein complexes. AR-dependent transcriptional activity is modulated by co-regulators. Multiple factors are considered to regulate the expression of several steroid hormone receptors in some cells, emphasizing the importance of balance of physiological androgen and estrogen for the differentiation of many organs during embryogenesis

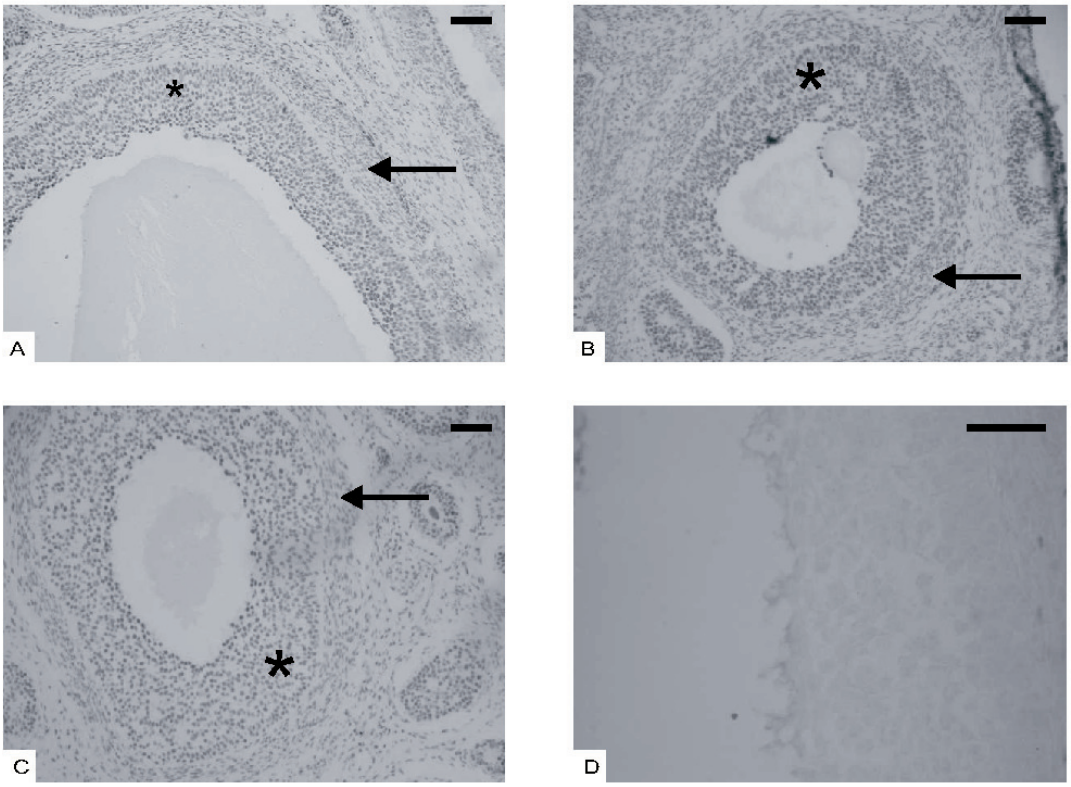

Fig. 2. Immunohistochemical localization of androgen receptor (AR) in antral follicles of immature porcine ovaries following exposure to flutamide: A – control group; B – flutamide treatment on gestational days 20-28; C – flutamide treatment on gestational days 80-88.
Positive reaction was observed in the nuclei of granulosa cells (*) and theca cells (black arrows) of antral follicles. D – negative control, scale bar = 100 μm.

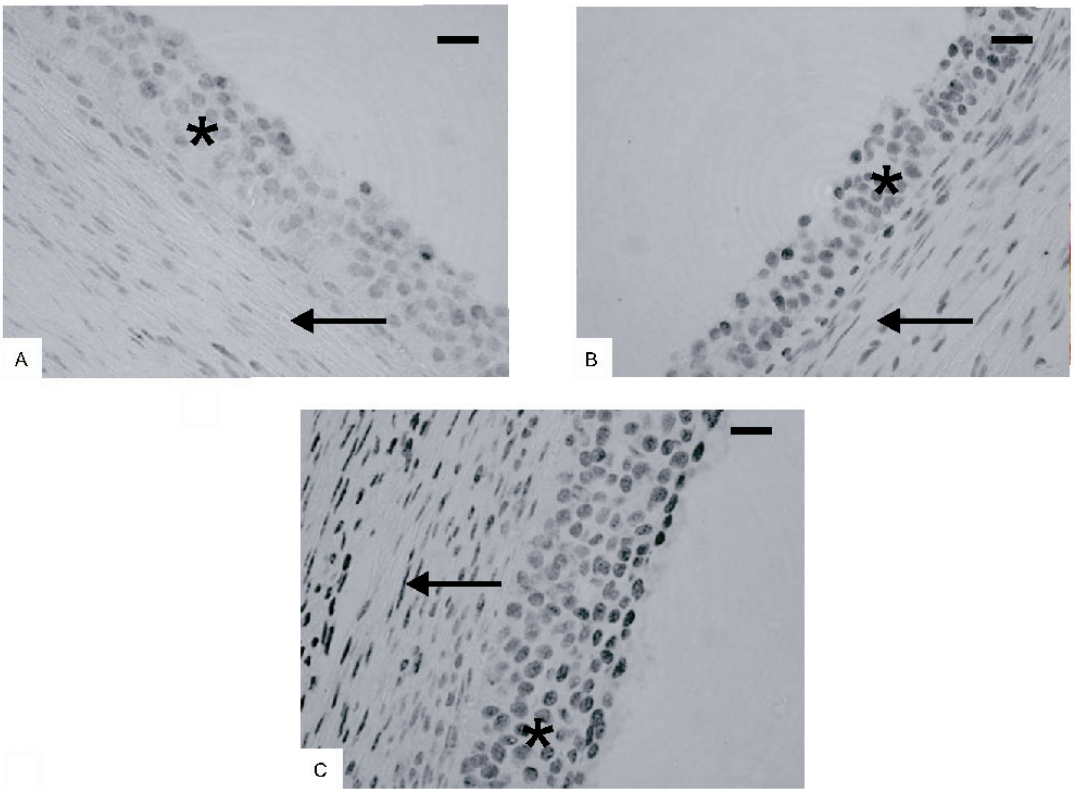

Fig. 3. Immunohistochemical localization of androgen receptor (AR) in antral follicles of mature porcine ovaries following exposure to flutamide: A – control group; B – flutamide treatment on gestational days 20-28; C – flutamide treatment on gestational days 80-88.
Positive reaction was observed in the nuclei of granulosa cells (*) and theca cells (black arrows) of antral follicles, scale bar = 100 μm.

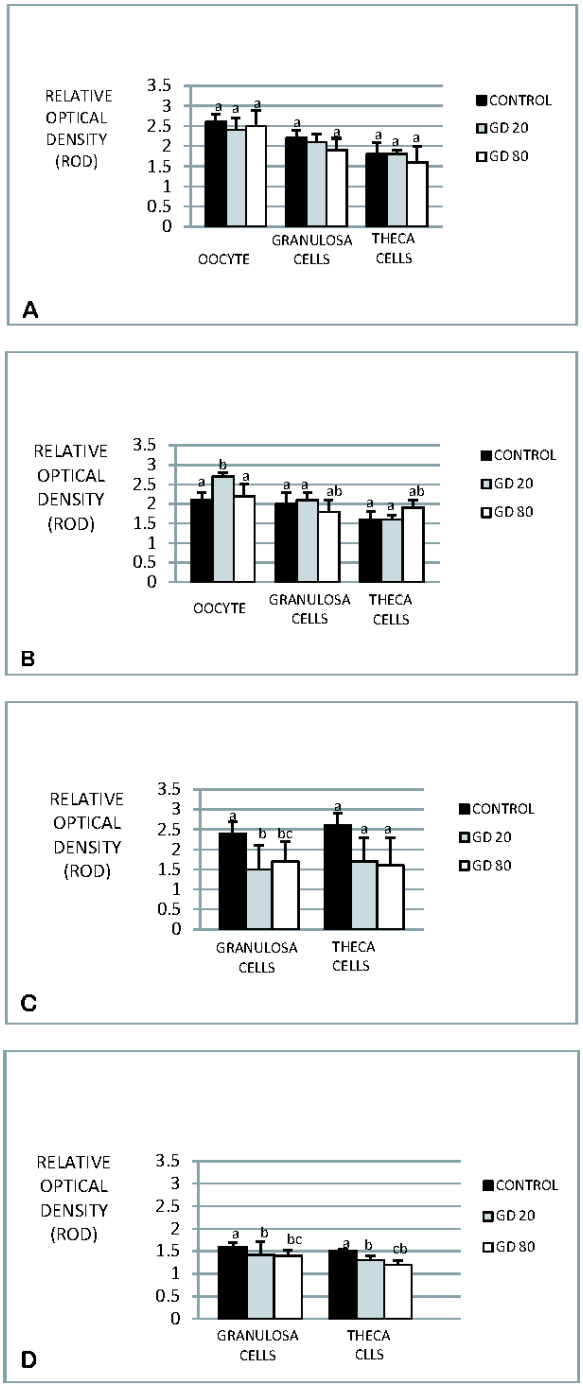

Fig. 4. The intensity of androgen receptor (AR) immunostaining expressed as relative optical density (ROD) in: primary follicles (A), preantral follicles (B) and antral follicles (C) of immature pig ovaries and antral follicles of adult porcine ovaries (D) exposed to flutamide at different days of gestation versus control. Values are means ± SD. Different letter superscripts indicate differences at P<0.01.

(HUGHES *et al.* 2001). Exposure to androgen excess *in utero* induces irreversible changes in gonadotropin secretion and results in disruption of reproductive endocrine and ovarian function in adulthood (FORSDIKE *et al.* 2007). An abnormality in early follicle development in ovaries with polycystic ovary syndrome may be the effect of androgens. Prenatal exposure to androgens not only caused abnormality in gonadotropin secretion, but also exerted a direct effect on the early stages of folliculogenesis which is supported by recent data from fetal sheep ovaries as well as from studies in other species. A reduced proportion of primordial and an increased proportion of growing follicles was the result of exposure to high concentrations of androgen (STECKLER *et al.* 2005). The increase in AR immunostaining in the ovarian stroma and granulosa of primordial and primary follicles of sheep fetus under testosterone (or DHT) treatment was documented. Therefore, AR is involved in the pathway of androgen action (ORTEGA *et al.* 2009). Studies using a culture system demonstrated that testosterone stimulates primary to secondary follicle transition via AR-dependent mechanism (YANG & FORTUNE 2006).

It is not clear how a decreased level of testosterone during the fetal life influence the level of AR in the adult ovary and how it is involved in its dysfunction. The epigenetic modification of key ovarian regulatory genes has been implicated (ORTEGA *et al.* 2009). Sex steroids have the ability to influence the methylation of DNA and AR acetylation (VOTTERO *et al.* 2006; XUE *et al.* 2007). Thus, the non-genomic action of androgens should also be taken into consideration. Further experiments showing the AR level in fetal ovaries of pigs treated *in utero* with flutamide are required.

In conclusion, our data show that androgens regulate their own receptor and the most visible effect is observed in adult animals. Exposure to antiandrogen during fetal life results in alteration of some androgen-regulated genes in adulthood.

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