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Effect of flutamide on folliculogenesis in the fetal porcine ovary – Regulation by Kit ligand/c-Kit and IGF1/IGF1R systems

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ABSTRACT

In pigs, primordial to primary follicle transition occur in the late pregnancy. The interactions between Kit ligand (KL) and its receptor (c-Kit), as well as insulin-like growth factor 1 (IGF1) and cognate receptor (IGF1R) are crucial for the primordial follicle activation. It is well established that hormonal disruption induces abnormalities in the developing reproductive system. Hence, this study investigated the influence of antiandrogen, flutamide, on genes involved in the primordial to primary follicle transition. Pregnant gilts were injected with flutamide (50 mg/kg bw, seven times, every day) or corn oil (control groups) starting on gestation days 83 (GD90) or 101 (GD108). Fetal ovaries were excised on days 90 and 108 of gestation. The proportion of primordial and primary follicles was determined, and immunohistochemistry for c-Kit and IGF1R was conducted. To assess KL, c-Kit, IGF1 and IGF1R mRNA expression real-time PCR was performed. Ovaries from both GD90 and GD108 animals exhibited a greater proportion of primordial to primary follicles when compared to respective control groups. C-Kit and IGF1R were immunolocalized in the oocytes of primordial and primary follicles. Both c-Kit mRNA and protein levels and KL mRNA expression were diminished in GD90 group. IGF1R expression decreased at mRNA and protein levels, whereas IGF1 mRNA expression was increased in GD90 and GD108 groups. In summary, our findings may indicate that the interactions between KL and c-Kit as well as IGF1 and IGF1R are relevant to the initiation of follicular transition from primordial into primary follicles and can be affected by AR signaling.

1. Introduction

Animals during fetal and neonatal life are susceptible to environmental chemicals which may affect the reproductive health (Fowler et al., 2012). In many mammals, including pigs, folliculogenesis begins already during fetal development and continues throughout adulthood. The formation of porcine ovarian follicles starts on day 56 of gestation and the assembly of primordial follicles and their subsequent transition to the primary stage occurs in late gestational period (Bielanska-Osuchowska, 2006). Androgens acting via androgen receptors (ARs) play an important role in the ovarian development and functions, as well as may regulate the expression of ovarian growth factors during different stages of follicle growth (Cárdenas and Pope, 1997; Drummond, 2006; Hickey et al., 2005; Walters et al., 2008). The presence of ARs was shown in the porcine fetal ovary at different stages of gestation (Burek et al., 2007), indicating the possible sites of androgen action. Moreover, cytochrome P450c17 was found in oocyte nests

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and oocytes of primary follicles, suggesting local androgen production during early development of porcine fetal ovaries (Knapczyk-Stwora et al., 2011). Studies using either androgen excess (Xita and Tsatsoulis, 2006) or deficiency (Durlej et al., 2011a, 2012; Grzesiak et al., 2012) as a model both showed that disturbed androgen action led to reprogramming of the trajectory of ovarian development, manifested by altered follicular development and reproductive function in adult life.

Primordial follicle assembly represents the most important event in establishing maximal reproductive potency of an adult ovary (Skinner, 2005). Follicular transition from primordial to primary stage is governed by a wide range of growth factors and cytokines (Fortune, 2003). The role of Kit ligand (KL, stem cell factor) and its tyrosine kinase receptor (c-Kit) was demonstrated in oocyte growth and follicular development (Driancourt et al., 2000; Merkwitz et al., 2011; Parrott and Skinner, 1999). Studies on mice have established that KL represents a direct downstream target of androgen signaling in a regulatory cascade controlling folliculogenesis (Shiina et al., 2006).

The other crucial receptor–ligand pair involved in the regulation of follicle growth from primordial stage is insulin-like growth factor (IGF1) and its cognate receptor IGF1R (Qu et al., 2000). Androgen treatment resulted in the increase of IGF1 and IGF1R mRNAs in the oocytes of primordial follicles in the primate ovary, suggesting that androgen induced activation of oocyte IGF1 signaling may trigger primordial follicle activation (Vendola et al., 1999b).

Chemicals with androgenic/antiandrogenic properties are able to interfere with ARs and regulate the endocrine system (Uzumcu and Zachow, 2007). It was demonstrated that androgen deficiency during fetal life (an antiandrogen flutamide was administered to the pregnant sows) resulted in delayed primordial follicle formation (Knapczyk-Stwora et al., 2013). The aim of this study was to examine whether administration of an antiandrogen flutamide during late pregnancy may influence primordial to primary follicle transition in developing ovaries of fetal pigs. To meet this goal, the expression KL/c-Kit and IGF1/IGF1R systems, which are crucial for the primordial follicle activation, were examined using real-time PCR and immunohistochemistry.

2. Materials and methods

2.1. Animals

The experimental protocol was approved by the Local Ethics Committee at the Jagiellonian University (approval no. 122/2009) and all surgical procedures were performed by a veterinarian.

Fetal ovaries used in the present investigation derived from the same experimental animals that were examined in our previous study (Knapczyk-Stwora et al., 2013). Briefly, eight sexually mature crossbred gilts (Large White × Polish Landrace) of similar age and body weight (10–11 months; 100–120 kg) were kept at the same farm conditions with food and water *ad libitum*. Animals were checked daily for signs of the estrous behavior. After two consecutive estrous cycles, gilts were mated with a fertile boar at the onset of estrus and again 12 and 24 h later. The gestation day was estimated from the first mating day. Pregnant gilts were randomly allotted into two experimental groups: animals injected with flutamide (Sigma–Aldrich, St. Louis, MO, USA) between days 83 and 89 of gestation (GD90, $n = 2$) and animals injected with flutamide between days 101 and 107 of gestation (GD108, $n = 2$). Flutamide was suspended in corn oil and administered as subcutaneous injections daily for seven days at a dose of 50 mg/kg body weight. This dose level was chosen on the basis of the literature (Foster and Harris, 2005; Williams et al., 2001) and our previously published data (Durlej et al., 2011a,b), to antagonize androgen action without toxic effect in the sows and offspring. For each flutamide-exposed group, a respective control group was used ($n = 2$, per each gestation period) and control animals were treated with corn oil in a manner similar to the flutamide-treated pigs. The days chosen for in utero flutamide exposure are critical for ovarian development in pigs and include the period of primordial follicle formation (GD90) and its transition to primary follicles (GD108) (Bielanska-Osuchowska, 2006).

2.2. Tissue collection

Pregnant gilts were fasted for 12 h before surgery, but had free access to water. For premedication, the gilts received atropine (0.05 mg/kg bw i.m.; Biowet, Gorzow Wielkopolski, Poland) and azaperone (2 mg/kg bw i.m.; Stresnil, Janssen Pharmaceutica, Beerse, Belgium). Once the sow was sedated (20–30 min later), anesthesia was induced by injecting thiopental (10 mg/kg bw; SandozGmbH, Austria) into an ear vein, and a silastic catheter (o.d. 2.4 mm; i.d. 1.8 mm) was aseptically placed through the desensitized skin into the external jugular vein to continuous infusion of thiopental till deep anesthesia. The female fetuses were obtained from flutamide-treated and control pregnant gilts on GD90 ($n = 14$ and $n = 5$, respectively) or on GD108 ($n = 11$ and $n = 6$, respectively). Ovaries of each fetus were immediately excised: one ovary was fixed in Bouin's solution for immunohistochemical localization of c-Kit and IGF1R, while the contralateral ovary was snap frozen in liquid nitrogen for RNA isolation and real-time PCR analysis for c-Kit (*KIT*), KL (*KITLG*), IGF1R (*IGF1R*) and IGF1 (*IGF1*).

2.3. Assessment of primordial to primary follicles proportion

Ovarian primordial and primary follicles were counted in at least five sections (the middle cross section and four other sections) per ovary. Each slide was routinely stained with hematoxylin and eosin. Primordial follicle classification was based on the presence of a few follicular cells or a single layer of flattened follicular cells surrounding an individual oocyte. An oocyte surrounded by a single layer of cuboidal follicular cells was classified as a primary follicle (Bielanska-Osuchowska, 2006). Proportion of examined ovarian follicles was calculated as the percentage of each follicle group on the number of total follicles per section (Ding et al., 2010). The counting was performed in each field using a microscope at ×20 objective.