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The impact of sex steroid agonists and antagonists on folliculogenesis in the neonatal porcine ovary via cell proliferation and apoptosis

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ABSTRACT

The objective of the study was to examine the effects of androgen and estrogen agonists or antagonists on the follicle formation, ovarian cell proliferation and apoptosis as well as plasma steroid concentration in neonatal pigs. Piglets were injected with testosterone propionate (TP, 20 mg/kg bw), flutamide (FLU, 50 mg/kg bw), 4-*tert*-octylphenol (OP, 100 mg/kg bw), ICI 182,780 (ICI, 400 μg/kg bw), methoxychlor (MXC, 100 mg/kg bw) or corn oil (CTR, controls) between postnatal Days 1 and 10 ($n = 4$/group). Heart blood was collected and ovaries were excised from the 11-day-old piglets. The lower percentage of oocytes within an egg nest and higher ovarian expression of active caspase 3 were found in TP (androgen excess) piglets compared to controls. FLU-induced androgen deficiency decreased the percentage of primordial follicles, increased that of early primary follicles and diminished ovarian cell proliferation. OP-induced estrogen action increased the percentage of primordial and developing follicles as well as cell proliferation. ICI-induced estrogen deficiency decreased the percentage of transitional follicles and ovarian cell proliferation, while increased the percentage of primordial follicles and the abundance of active caspase 3. Treatment with MXC, exhibiting estrogenic, antiestrogenic, and antiandrogenic activities, declined the percentage of developing follicles and cell proliferation. Moreover, the investigated compounds differentially affected plasma steroid level. In conclusion, the present study demonstrated clear effects of TP and FLU during the earliest stages of folliculogenesis in pigs (nest breakdown and follicle assembly), whereas OP and ICI influenced also the subsequent stages of follicle initial recruitment and growth. Therefore, the androgen and estrogen seems to be important for the follicle assembly and follicle growth in neonatal porcine ovaries.

1. Introduction

The establishment of the pool of primordial follicles during fetal and/or neonatal period is important for reproductive lifespan of females. In pigs, the breakdown of the oocyte nests leading to the formation of primordial follicles begins around prenatal day 56 [1], and is still observed during the neonatal period [2]. The oocyte nest breakdown involves hormone- and growth factor-dependent apoptosis of germ cells and migration of somatic cells [3,4]. Once formed, some primordial follicles are recruited into the primary follicle pool and the recruitment lasts until the ovarian reserve depletion [5]. Therefore, a disturbed formation of primordial follicles may lead to premature exhaustion of the primordial follicle reserve, resulting in primary ovarian insufficiency [6].

Several factors involved in oocyte-granulosa cell communication, including members of the transforming growth factor β (TGFβ) superfamily, have been shown to regulate early stages of folliculogenesis [7,8]. The transition of primordial follicles into primary follicles is coordinated mainly by oocyte-derived paracrine factors capable of promoting the proliferation and differentiation of surrounding somatic cells [9]. Steroid hormones also seem to be indispensable for the intraovarian regulation of folliculogenesis [9,10]. It was shown that estradiol inhibited the breakdown of oocyte nests and the primordial-to-primary follicle transition in

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neonatal rats [11]. Moreover, an exposure of neonatal mice to estrogenic compounds inhibited the oocyte nests breakdown and increased oocyte survival [12]. The results of our previous studies showed that neonatal exposure to antiandrogen flutamide influenced ovarian development and resulted in reproductive dysfunctions in adulthood [13,14]. In addition, we have recently found that androgen deficiency affected expression of several genes as well as delayed the formation of primordial follicles and their transition into the primary stage in porcine fetal ovaries [15,16]. Therefore, we hypothesized that sex steroid availability during the neonatal period is important for the assembly of follicles and their primordial-primary transition.

The usage of animal models that mimic androgen/estrogen deficiency or excess can help to elucidate the impact of steroid hormones on female reproductive processes. The steroid receptor agonists and antagonists, including environmental chemicals (endocrine-active chemicals, EACs) may be employed to study the significance of sex steroid hormones in neonates. The EACs may affect multiple signal transduction pathways by mimicking or blocking the action of endogenous steroids [17]. Therefore, in the current study we aimed to examine the effects of testosterone propionate (TP; a synthetic androgen), flutamide (FLU; a nonsteroidal antiandrogen), 4-*tert*-octylphenol (OP; compound with estrogenic activity), ICI 182,780 (ICI; a pure antiestrogen), and methoxychlor (MXC; compound with estrogenic, antiestrogenic and antiandrogenic properties) on folliculogenesis in the neonatal porcine ovary. To meet this goal, histological examination of the ovary as well as analysis of ovarian cell proliferation and apoptosis were performed in the ovaries of untreated and EACs-treated porcine neonates.

2. Materials and methods

2.1. Experiment design and tissue collection

In the present study, 24 female piglets (Large White × Polish Landrace) from different litters were randomly allocated into six groups. The animals were injected subcutaneously with: 1) TP (Sigma-Aldrich, St. Louis, MO, USA; 20 mg/kg body weight [bw]; $n = 4$), 2) FLU (Sigma-Aldrich; 50 mg/kg bw; $n = 4$), 3) OP (Sigma-Aldrich; 100 mg/kg bw; $n = 4$), 4) ICI (Sigma-Aldrich; 400 μg/kg bw; $n = 4$), 5) MXC (Sigma-Aldrich; 100 mg/kg bw; $n = 4$), and 6) vehicle only (corn oil) which served as a control group (CTR, untreated, $n = 4$). The effective doses were chosen on the basis of literature [18–21] and the results of our previous studies [15]. The animals were injected daily between postnatal Days 1 and 10. One day after the last injection, the ovaries were excised from all animals, and one gonad was fixed in Bouin's solution for routine histology (hematoxylin-eosin staining, H&E), immunohistochemistry and TUNEL analysis, while the contralateral ovary was snap frozen in liquid nitrogen for protein isolation. Blood samples were collected from the heart of each animal, placed in a heparinized test tube, centrifuged (2000 × g, 10 min, 4 °C) and stored (−20 °C). All surgical procedures were performed by a veterinarian. The animal protocols were conducted in accordance with the national guidelines and approved by the Local Ethics Committee at the Jagiellonian University in Krakow, Poland (approval number 150/2013, 122/2014, 187/2014 and 188/2014).

2.2. Histology of porcine ovaries

To investigate the development of ovarian follicles, ovaries were embedded in paraffin, sectioned (5 μm sections), and stained with H&E. The number of oocytes within an egg nest and the number of follicles at each developmental stage were counted in five separate sections (the central section and two lateral sections on each side of the ovary) per ovary. The examined ovarian sections did not overlap since they were separated by at least 20-μm distance. First, the total number of oocytes, regardless of their follicular origin, was counted across the entire section. Next, the number of follicles at various stages corresponding to those oocytes was also counted [24]. The examined oocytes and follicles were classified as: (i) naked oocytes within an egg nest (stage 0), (ii) primordial follicles - consisting of an oocyte partially or completely encapsulated by squamous pregranulosa cells (stage 1), (iii) early primary follicles, which initiated development and contained one or more cuboidal granulosa cells (stage 2), (iv) primary follicles - having a single layer of cuboidal granulosa cells surrounding the oocyte (stage 3) and (v) transitional follicles which were identified by 1–2 layer of columnar granulosa cells (stage 4) [25]. The proportion of an ovarian structure was expressed as the percentage of naked oocytes (stage 0) or follicles representing each developmental stage (stages 1, 2, 3 or 4) in relation to the number of total ovarian structures (naked oocytes plus all follicles) per section. The sections were coded and counted blindly by two independent investigators, and always the average of two readings was taken. All sections were examined at 200 × magnification under light microscopy (Nikon Eclipse Ni-U microscope) and photographed with a Nikon Digital DS-Fi1-U3 camera (Nikon, Tokyo, Japan) with corresponding software.

2.3. Immunohistochemistry

Immunohistochemistry was conducted routinely as previously described [15]. Blocking of nonspecific binding sites was performed with 10% (v/v) normal horse serum (Sigma-Aldrich) before incubation with mouse anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (MAB424R, Millipore, Temecula, CA, USA) at a dilution 1:200. After overnight incubation (4 °C) in a humidified chamber, the antigens were visualized using secondary biotinylated horse anti-mouse IgG (1:300; 1.5 h; RT; Vector Laboratories, Burlingame, CA, USA), avidin–biotin–peroxidase complex (1:100; 40 min; RT; StreptABComplex-HRP, Dako A/S, Glostrup, Denmark), and 3,3′-diaminobenzidine (DAB, Sigma–Aldrich) as a chromogen-staining substrate. Sections were counterstained with Mayer's hematoxylin. Negative controls were incubated with non-immune mouse IgG instead of primary antibody and processed as described above. Selected sections were photographed using a Nikon Eclipse Ni-U microscope and a Nikon Digital DS-Fi1-U3 camera (Nikon) with corresponding software. Next, the percentage of follicles (stages 1–4) with PCNA positive oocytes and percentage of developing follicles (stages 2–4) with PCNA positive granulosa cells were evaluated in five separate sections per ovary as described above. The former percentage denotes the number of PCNA-positive oocytes enclosed in follicles (stages 1–4) in relation to the total number of follicles per section; the latter percentage means the number of developing follicles (stages 2–4) with at least one PCNA-positive granulosa cell in relation to the total number of developing follicles. The oocytes and follicles were counted across the entire section under light microscopy using a 200 × magnification (Nikon Eclipse Ni-U microscope) and a cell counter. Sections were coded and counted blindly by two independent investigators and the average of two readings was taken.

2.4. TUNEL staining

Apoptotic cells were detected in ovarian sections using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Melbourne, Australia) following the manufacturer's protocol [15], and visualized with DAB. Sections were then counterstained with Hematoxylin QS. A minimum three randomly