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Intrafollicular level of steroid hormones and the expression of androgen receptor in the equine ovary at puberty

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ABSTRACT

Steroidogenic activity in the equine ovary from birth to puberty has been poorly investigated. This study aimed to examine the capability of the ovarian follicles of prepubertal and pubertal fillies to produce steroid hormones and to evaluate the expression and cellular localization of androgen receptor (AR) in their ovaries. The ovaries of 6–18 month-old fillies were divided into two groups: prepubertal (PrP) – without preovulatory follicle (pF) and corpus luteum (CL), and ovulating/postpubertal (Ov/pB) – with pF and/or CL in at least one of the gonads. Adult mares (Me) were used as a control. The concentration of progesterone (P4), testosterone (T) and estradiol (E2) in follicular fluid (FF) was measured by radioimmunoassay. AR distribution was assessed by immunohistochemistry, while AR protein expression was examined by Western blot analysis. In the female groups, E2 concentration in FF of small follicles (<10 mm) was low and increased with the diameter of the follicle reaching the greatest value in pF (Ov/pB and Me group). In follicles (11–30 mm) of PrP fillies, the concentration of E2 was similar to that from Ov/pB fillies, but less than half ($P < 0.05$) than in Me follicles. In FF from all classes of follicles of Ov/pB fillies, the concentration of all steroids was similar to that in Me. AR immunolocalization, predominantly nuclear, was observed in all types of follicular cells (granulosa and theca cells) as well as in stroma and luteal cells. The pattern of staining was dependent on the follicle size and the group of females. In smaller antral follicles and in pF, the nuclear AR staining in granulosa cells was stronger than that found in follicles of 21–25 mm. In theca interna cells of pF, both nuclear and faint cytoplasmic reactions were seen. In luteal cells, AR labeling was noted in the nuclei and the cytoplasm: the strongest one in the early CL and almost negative in the late CL. AR protein expression in filly and mare ovarian tissues was confirmed by Western blot analysis and detected as a single band at approximately 110 kDa. In summary, the ovaries of fillies aged at least 6 months are capable of active steroidogenesis. ARs are present either in the cell nuclei or cytoplasm of all compartments of the equine ovary. AR expression in follicular and stroma cells may indicate the sensitivity of the filly ovarian tissue to androgens, the impact of androgens on folliculogenesis and the development of the equine ovary *via* a receptor-mediated pathway.

1. Introduction

Puberty is a complex biological process depending on sex, genetic background and environmental factors including the development of the reproductive tract, changes in body weight and activation of the hypothalamic-pituitary-gonadal axis [1–3]. Knowledge of the ability of the equine ovary to produce steroid hormones from birth to puberty is limited and based mostly on the assessment of the concentration of sex hormones in the peripheral blood serum [4–7]. It is worth mentioning that steroids are also produced by adrenal cortex, thus their presence in the blood does not directly reflect gonadal steroidogenic capacity. To date, studies on ovariectomized prepubertal fillies and mares have revealed that these animals exhibit estrous behavior despite of removal of the ovaries [4,8]. Thus sexual behavior was being induced either by non-ovarian estrogens originating from aromatization of adrenal androgens or directly by androgens itself [8]. The participation of androgens in the regulation of female sexual behavior was also reported in earlier studies on mares [9], pigs [10] and

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ovariectomized sheep [11]. Previously we have found the presence of aromatase in antral follicles of 6 month-old fillies, which provides evidence that equine ovaries are able to produce estrogens long before the first ovulation [12]. However, there is no data considering androgens production and action within the ovary at this developmental stage.

The influence of androgens on ovarian function and female fertility in mammalian species, including mares, is complex and still requires elucidation. As a substrate for aromatase, androgens are converted to estrogens and thus indirectly control folliculogenesis and female reproduction [13,14]. They can also directly (hormonal action) target ovarian follicles and modulate their development *via* specific androgen receptors (AR) expressed on ovarian cells [13–16]. Currently there is no data describing the presence of AR in equine ovaries or the steroidogenic activity of filly ovaries, including the evaluation of the concentration of steroid hormones in follicular fluid (FF). Progesterone (P4) and androgens produced by theca interna cells are precursors for estrogens, synthesized in the granulosa cells [16]. Thus the presence of aromatase in filly ovaries [12] not only indicates their ability to secrete estradiol, but also allows to assume that the filly ovaries are capable of active steroidogenesis and the production of all sex steroids. Therefore, the aim of the present study was: i) to compare the capability of the ovaries of prepubertal and pubertal fillies to produce steroid hormones, based on the concentration of progesterone, testosterone (T) and estradiol (E2) in FF, and ii) to evaluate the expression and cellular localization of AR protein in the ovaries of fillies and adult mares.

2. Materials and methods

2.1. Animals

The study was conducted on ovaries obtained from 6 to 18 month-old fillies and adult mares aged >5 years at a local abattoir. The age of fillies was estimated by examining their teeth just before slaughter. Ovaries were collected promptly after slaughter, submerged individually in phosphate-buffered saline (pH 7.4; Polfa, Lublin, Poland) and transported at 4 °C to the laboratory.

Before follicle isolation, the ovaries were weighed and palpated in order to locate the follicles and make an initial evaluation of their size as well to detect the possible presence of corpus luteum (CL) (see *2.2.*). During isolation of the follicles special attention was given to the largest follicle, in particular preovulatory ones (pF: > 30 mm in diameter). After isolation of the follicles (see *2.2*), the remaining ovarian tissue was cut and checked again to see if a CL was present.

The presence of preovulatory follicle and/or CL was used as a criterion for allocating pairs of ovaries collected from fillies (and thus fillies) into groups. Group I – prepubertal (PrP) – consisted of ovaries without any of the above mentioned structures. Group II – ovulatory/post-pubertal (Ov/pB) – comprised ovaries with pF and/or CL present at least in one of the ovaries [17]. Ovarian tissues (for AR protein expression and localization) and FF (for steroids analysis) were assigned to one of the above groups taking into account the size of the follicle (see *2.2.*). The material obtained from the ovaries of adult mares (Me group) was used as control (mares ovaries were weighed and estimated as filly ovaries).

2.2. Follicular fluid collection and tissue fixation

The preovulatory follicle (if present) and 2 to 6 smaller randomly selected antral follicles were isolated from each ovary. If pF was not present, the largest follicle and 2 to 6 smaller randomly selected follicles were isolated as well. When the largest follicle could not be collected, 2–6 smaller antral follicles randomly selected were isolated.

Each isolated follicle was measured and allocated to a size group, according to diameter: small (5–10 mm; sF), medium (three classes: 11–15, 16–20 and 21–25 mm; mF1, mF2 and mF3, respectively), large (26–30 mm, lF) and preovulatory (pF; see *2.1).* The follicles were cut individually on a Petri dish (to obtain FF and/or fix follicular wall) or fixed without cutting. Collected FF was centrifuged (3000 × g; 10 min), frozen individually in liquid nitrogen and stored at −20 °C until radioimmunological analysis of steroid concentrations.

For AR protein detection, whole follicles or sections of follicular walls (left after FF collection), pieces of ovarian stroma and CLs were either fixed in 4% paraformaldehyde for immunohistochemistry or frozen in liquid nitrogen and stored at −80 °C for Western blot analysis. Before fixation, CLs were cut and classified according to the criteria described by Al-Zi'abi et al. [18] and Wesson and Ginther [19] as: I) early (eCL) – with a clearly visible huge red-brown clot inside forming the luteal structure of soft consistency with thicker pale edges; II) mature (mCL) – well-developed mushroom- or gourd-shaped with a thick consistency and pink-to-purple in color, or III) late (laCL) – smaller and harder than mCL, and yellow or cream in color.

2.3. Immunohistochemistry

After fixation ovarian tissues were dehydrated, embedded in paraplast[1] and cut into serial sections (6 μm). For immunohistochemical visualization of AR, polyclonal rabbit IgG against N-terminus of AR N-20 (Santa Cruz Biotechnology, Inc., USA) at 1:500 dilution was used as a primary antibody. Biotinylated goat anti-rabbit (1:300) IgG (Vector Laboratories, Inc, USA) was applied as a secondary antibody. The antibodies were diluted in Tris-buffered saline (TBS; 0.05 M Tris-HCl, pH 7.4) containing 0.1% (v/v) Tween 20[1] (TBST).

After routine deparaffinization and rehydration, sections were heated in a microwave oven in 0.01 M citrate buffer and then incubated in 0.5% (v/v) H_2O_2[1] in TBS. After washing in TBS, sections were incubated with 5% (v/v) normal goat serum (Vector Laboratories, Inc; USA) and then with a primary antibody overnight at 4 °C. Subsequently, the sections were incubated with a secondary antibody followed by the avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Inc; USA). Finally, the color reaction was developed in TBS supplemented with 0.07% (w/v) imidazole[1], 0.05% (w/v) diaminobenzidine (MP Biomedicals, Inc, USA) and 0.01% (v/v) H_2O_2[1]. For negative controls normal goat serum was used instead of the primary antibody.

2.4. Protein isolation and Western blot analysis

Equal amounts of frozen ovarian tissues (30 mg) were homogenized on ice in cold Tris/EDTA buffer (50 mM Tris, 1 mM EDTA, pH 7.5) containing 10 μL/mL Protease Inhibitor Cocktail[1], sonicated, and centrifuged at 10,000 × *g* for 20 min at 4 °C to remove non-homogenized tissue debris. Protein content was determined using the Bradford method [20]. Samples containing 20 μg protein were denatured for 5 min in Laemmli sample buffer (Bio-Rad Laboratories Inc., GmbH, Munich, Germany) and separated by SDS-PAGE under reducing conditions according to Laemmli [21], as previously described [22]. Separated proteins were electroblotted onto a PVDF membrane in transfer buffer (20 mM Tris, 150 mM glycine in 20% (v/v) methanol, pH 8.4). After overnight blocking in 5% (w/v) non-fat milk in TBST at 4 °C with shaking, the membranes were incubated with a primary anti-AR antibody (1:10000) for 1.5 h at room temperature. Subsequently, the membranes were