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Original article

Interrelationships between ovarian follicles grown in culture and possible mediators

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ABSTRACT

Dominance or cooperation between ovarian follicles can determine the number of ovulations and fecundity, but interrelationships between follicles in mono- and poly-ovulatory species and their mechanisms are poorly understood. The goals of this work were to determine the existence and compare the character of mutual influence of cultured ovarian follicles from a mono-ovulatory species (cow) with established follicular dominance with those from a poly-ovulatory species (pig), in which interrelationship between follicles remain unknown, and to examine the role of ovarian cell proliferation, the insulin-like growth factor I (IGF-I)- oxytocin (OT) system, and steroid hormones in mediating interrelationships among ovarian follicles. Bovine and porcine ovarian follicles were isolated and cultured alone and in pairs, and the percentage of growing follicles was calculated. Porcine follicles were cultured alone and in pairs after addition of exogenous OT and IGF-I (100 ng mL^{-1}) or inactivation of endogenous OT and IGF-I by antisera against these hormones (1%). Proliferation of porcine follicular cells was assessed by SDS PAGE-Western immunoblotting, the release of IGF-I, progesterone, androstenedione and estradiol by cultured porcine ovarian follicles was analyzed by RIA/EIA. Overall, our observations suggest (1) competition/dominance (mutual suppression of growth) in bovine ovarian follicles, (2) cooperation (mutual support of growth) in porcine ovarian follicles, (3) that this mutual growth of porcine ovarian follicles was caused by the promotion of cell proliferation, (4) that this mechanism was probably not involved in bovine follicular dominance, (5) that communication between both porcine and bovine follicles affects their secretory activity, and (6) that both follicular dominance in cows and cooperation of follicles in pigs can be mediated by either down- or up-regulation of the IGF-I-OT system, which in turn affects follicular steroidogenesis and promotes follicular cell proliferation and follicular growth.

1. Introduction

Interrelationships between follicles within the ovary can determine both individual and species fecundity. In mono-ovulatory species (e.g., cow, sheep, goat, buffalo, and horse) usually only one dominant follicle grows and develops until ovulation and can result in the generation of offspring, whereas development of subordinate follicles is suppressed by this follicle, and they cease growth and development, and undergo atresia via apoptosis [1–3]. In poly-ovulatory species (e.g., rodents), several follicles are developed and ovulated during one cycle, and both follicular dominance and cooperation have been observed. Large murine follicles can reversibly induce apoptosis and inhibit growth of co-cultured smaller follicles [4,5]. Conversely, the co-culture of ovarian follicles of similar size as that of rats [6] and mice [4] can produce unknown peptide hormone(s), which can promote growth of neighboring follicles. The interrelationships between ovarian follicles in other poly-ovulatory species than rodents (e.g., pigs) have not yet been studied.

The intra- and extracellular mechanisms of inter-ovarian communication remain virtually unknown. Results of *in vivo* studies have shown that dominant follicles in mono-ovulatory

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species can suppress the growth of subordinate follicles via the promotion of their atresia via apoptosis [1,2], but it remains unknown whether follicular dominance can be mediated with changes in ovarian cell proliferation as well. One study failed to demonstrate follicular dominance *in vitro* [7]. It is unknown whether mutual support of follicles in poly-ovulatory species is caused by follicular atresia via apoptosis or proliferation.

The hormonal mediators of inter-follicular interrelationships have also been insufficiently studied. Local ovarian hormones appear to affect follicular interrelationships more than ovarian cycle-dependent changes in pituitary or exogenous follicle stimulating hormone (FSH) [5,7,8]. Possible candidates for hormonal mediators of follicular selection and dominance are insulin-like growth factor I (IGF-I) and IGF-binding proteins [2,8,9], which, in close collaboration with oxytocin (OT) [10,11], can control ovarian follicular growth, development, and selection. OT-IGF-I axis which can mutually stimulate both OT and IGF-I output can promote growth of ovarian follicles both *in vivo* [2,8,9,11] and *in vitro* [10,12,13]. OT-IGF-I axis promoted porcine ovarian follicular cell proliferation (accumulation of promoter and marker of proliferation, proliferating cell nuclear antigene, PCNA [14]) and growth of isolated porcine ovarian follicles [10], as well as the release of ovarian steroid and peptide hormones-regulators of ovarian folliculogenesis in different mammalian species [10,11]. Nevertheless, it remains unknown whether the OT-IGF-I axis can mediate inter-follicular communication and related follicular cell proliferation in both poly- and mono-ovulatory species. Therefore, the interrelationships between follicles in poly-ovulatory species, as well as the mediators of such interrelationship in both poly- and mono-ovulatory species require further elucidation.

The first goal of our study was to determine the existence and compare the character of mutual influence of cultured ovarian follicles of a mono-ovulatory species (cow) with established follicular dominance of a poly-ovulatory species (pig), in which interrelationships between follicles remain unknown. The second goal was to examine the role of ovarian cell proliferation and the oxytocin-IGF-I system in mediating the interrelationships among porcine ovarian follicles. To accomplish our goals, isolated similar-sized bovine and porcine ovarian follicles were cultured alone and in pairs, whereas the percentage of growing follicles in each group was calculated. In addition, porcine follicles were cultured alone and in pairs after the addition of exogenous OT and IGF-I, or inactivation of endogenous OT and IGF-I by antisera against these hormones. In both bovine and porcine follicular cell proliferation (accumulation of proliferating cell nuclear antigen, PCNA), and release of some key ovarian hormones (IGF-I, progesterone, androstenedione, and estradiol) were analyzed.

2. Materials and methods

2.1. Isolation, culture, and processing of ovarian follicles

The ovaries were collected from non-cycling Slovakian white gilts, 180 days of age (weight 85–100 kg) and from cows 2–4 years old (weight 600–700 kg) at follicular stage of the estrous cycle without visible reproductive abnormalities, killed at a local slaughterhouse in accordance with the corresponding European and Slovak regulations. Ovarian follicles (2.5–3.5-mm diameter, representing the majority of visible growing but not ovulating follicles) were collected, processed, and cultured in a DME/F-12 1:1 mixture (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma), and 1% antibiotic-antimycotic solution (Sigma), as it was validated and described previously [10,15], although in these experiments only the whole, but not dissected follicles were cultured.

Randomly selected whole ovarian follicles were cultured individually or in pairs for 10 d in the medium described above. Bovine follicles were cultured without additions, whereas porcine follicles were cultured with and without recombinant IGF-I (100 ng mL^{-1} medium; Calbiochem, Lucerne, Switzerland) or synthetic OT (100 ng mL^{-1}; Sigma), sheep antiserum against OT (1%; kindly provided by Prof. A.P.F. Flint, University of Nottingham, Sutton Bonington, U.K.) or rabbit antiserum against human IGF-I (1%; kindly provided by Dr. A.F. Parlow, National Hormone & Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA, USA). This concentration of antisera binds 85–100% of OT or IGF-I produced by follicular cells during culture. Previous studies [10,15] demonstrated that hormones and antisera at these concentrations have maximal effects on both the secretory activity and the size of cultured porcine follicles by the 10th day of culture. The hormones and antisera were of biological grade. They were dissolved in the incubation medium immediately before the experiments. Control tissues were cultured in medium without additions of hormones or antisera. The blank control was represented by medium cultured without follicular tissue. Before and after culture, follicles were weighed and their diameters were measured using a micrometer to $\pm$ 0.1 mm. Culture medium and follicular tissue were frozen at $-18\,^{\circ}C$ until radioimmunoassay/enzymatic immunoassay (RIA/EIA) and SDS PAGE-Western blotting, respectively. The number and viability of cells within the follicles were not determined, although PCNA was detected by western blotting of follicular lysates after culture (see below).

2.2. Immunoassay

Concentrations of progesterone, androstenedione, estradiol, and IGF-I were determined in 25–100 μL of incubation medium by RIAs or EIA. Progesterone and androstenedione concentrations were determined using RIA and EIA kits from DSL (Webster, TX, USA). Estradiol was assayed using an RIA kit (BioChem Immuno Systems Italia S.P.A., Rome, Italy), and IGF-I was extracted from the samples and concentrations determined using RIA as described previously [16] using rhIGF-I from Sigma as standard and anti-IGF-I antiserum (dilution 1:10.000) provided by Dr. A.F. Parlow (National Hormone and Pituitary Program, Torrance, USA). All procedures followed the manufacturer's instructions. All assays were validated for our culture medium. The characteristics of these assays are presented in Table 1.

2.3. Western blotting

The separation of PCNA was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, [17]). Subsequent visualization was by western immunoblotting by using mouse monoclonal antibodies against human PCNA (36 kDa protein; Santa Cruz, Santa Cruz, CA, USA, dilution 1:500 which binds either human, mouse, rat, chicken porcine or bovine PCNA), secondary HRP-conjugated anti-mouse rabbit IgG antibodies (DAKO, Carpinteria, CA, USA; dilution 1:1000), ECL detection reagents, and ECL hyper-film (Amersham International) according to the manufacturer's instructions and quantified by fraction densitometry normalized for the housekeeping protein fraction (see below). The primary antibody against PCNA is specific for proliferating mouse, rat, human, insect, yeast, porcine, and bovine cells at G1 and S phases of the cell cycle. Incubation medium without cells, or samples processed in the absence of the primary antibody, were used as negative controls. As housekeeping protein, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and the corresponding antibody (BD Trans Lab, dilution 1:500) were used (not shown). The molecular weights of fractions were evaluated using a molecular weight calibration set (18, 24, 45, and 67 kDa;