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DO PROGESTERONE, IGF-I, IGFBP-3 AND IGFBP-4 RELATE TO SEXUAL MATURATION?

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ABSTRACT

Hormones and binding proteins can regulate reproduction, but their involvement in sexual maturation remains to be elucidated. This study describes possible hormonal regulators of female sexual maturation. For this purpose, the release of steroid hormone progesterone (P_4), insulin-like growth factor I (IGF-I) and IGF-binding proteins (IGFBP-3, IGFBP-4) were shown in this study. Sexual maturation in gilts was found to be associated with a significant increase in the release of P_4, IGF-I and IGFBP-3 *in vitro*. Furthermore, sexual maturation was associated with significant increase in the expression of IGFBP-3 but not in IGFBP-4. The present data obtained from *in vitro* study indicate that sexual maturation in females is influenced by puberty-related changes in porcine ovarian signalling substances: increase in P_4, IGF-I, IGFBP-3 but not IGFBP-4. It suggests that these signalling molecules could be potential regulators of porcine sexual maturation.

Keywords: Sexual maturation, porcine granulosa cells, progesterone, insulin-like growth factor I and IGF-binding proteins

INTRODUCTION

Sexual maturation is associated with ovarian follicular growth and differentiation (**Onagbesan *et al.*, 2009; Palma *et al.*, 2012**). These processes are governed by hormones, growth factors and their binding proteins (**Kolesarova *et al.*, 2008; Sirotkin, 2013**). There is indirect evidence for involvement of several candidate signalling substances in control of sexual maturation and/or related ovarian follicle development. Steroid hormone progesterone (P_4) is essential for normal ovarian cycles (**Arnhold *et al.*, 2009; Hagan *et al.*, 2009**) and contributes to regulation of ovarian follicular development and remodelling (**Astiz, 2013; Mahajan, 2008**). Progesterone produced by porcine ovarian granulosa cells (**Duda *et al.*, 2012; Kolesarova *et al.*, 2009b, 2010**) and the *corpus luteum* (**Gregoraszczuk, 1992,1997; Mahajan, 2008; Shah and Nagarajan, 2013**) is a local paracrine or autocrine promoter of ovarian cell luteinization (**Gregoraszczuk, 1994**). In cyclic animals, when the early follicular growth is initiated, a high amount of P_4 is secreted by secondary, tertiary and luteinized ovarian follicles and active corpora lutea, into the peripheral blood (**Mahajan, 2008**).
Insulin-like growth factor I (IGF-I) is known to stimulate ovarian follicular growth (**Lucy, 2008**) and development (**Carter *et al.*, 2006**) by promoting granulosa cell proliferation, follicular antrum formation (**Mao *et al.*, 2004**), hyperplasia of ovarian surface epithelium (**King *et al.*, 2013**), releasing ovarian hormones (**Kolesarova *et al.*, 2008**) and decreasing ovarian cell apoptosis (**Mao *et al.*, 2004**). IGF-I has been found to be produced by porcine (**Kolesarova *et al.*, 2008, 2009b, 2010**), chicken (**Sirotkin *et al.*, 2006**) and human (**Karamouti *et al.*, 2008**) ovarian cells. The effects of IGF-I on the ovary may be modified by the local production of IGF binding proteins (IGFBPs) (**Sandhu *et al.*, 2002; Yi *et al.*, 2001**). In the ovary, IGFBP-3 appears to neutralize the actions of IGF-I (**Bicsak *et al.*, 1990,1991; Ui *et al.*, 1989**). IGFBP-3 not bound to IGF also affects cells via mechanisms involving binding to specific cell surface receptors and/or transport into the cell (**Xi *et al.*, 2007**). IGFBP-4 modulates autocrine/paracrine action of IGF in both follicular growth and differentiation in the porcine ovary (**Zhou *et al.*, 1996**). The secretion of IGFBP-4 is higher in immature granulosa cells as compared to mature porcine ovarian follicles (**Grimes *et al.*, 1994**).
The general aim of the *in-vitro* experiments with porcine ovarian granulosa cells was to identify possible hormonal regulators of female sexual maturation. For this purpose, these signaling molecules were evaluated in granulosa cells collected from sexually mature and immature gilts.

MATERIALS AND METHODS

Animals

Healthy gilts of Slovakian White breed were reared under standard conditions at the Experimental Station of the Slovak University of Agriculture in Nitra, Slovakia. Conditions of their care and handling corresponded to the instructions of the European Commission (EC) no. 178/2002 and related EC documents and as approved by local ethics committee. Animals (n=35) were assigned at slaughter into two groups: sexually immature (n=18) and animals of the same age having reached sexual maturity (n=17) according to visual characteristics of ovaries (presence of follicles larger than 5 mm).

Preparation, culture and processing of granulosa cells

Ovaries were transported to the laboratory at 4ºC and washed in sterile physiological solution. Ovaries from immature and mature gilts were processed separately. Follicular fluid was aspirated from 3-5 mm follicles, granulosa cells were isolated by centrifugation for 10 min at 200xg followed by washing in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium) and resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittaker™) and 1% antibotic-antimycotic solution (Sigma, St. Louis, Mo, USA) at a final concentration of 10^6 cells/mL of medium. Portions of the cell suspension were dispensed to 24-welled culture plates (Nunc™, Roskilde, Denmark, 1 ml/well; for RIA) or Lab-Tek 16-welled chamber slides (Nunc Inc., International, Naperville, USA, 100 µl/well; for immunocytochemistry). Both the plate wells and chamber slides were incubated at 37.5ºC and 5% CO_2 in humidified air until a 75 % confluent monolayer was formed (5-7 days), at which point the medium was replaced with fresh medium. Further culture was performed in 300 µl medium in 16-welled chamber slide cells or 1 ml of culture plate. After 2 days of culture the media from wells were removed, wells from chamber slides were washed in ice-cold PBS (ph 7.5). Cells were fixed for 1 h at room temperature in 4% paraformaldehyde, dehydrated in alcohols (70, 80, 96%; 10 min each) and stored in 96% alcohol at -4ºC to await immunocytochemical analysis. Media from plate wells were aspirated and kept at -70 ºC to await RIA.

Immunocytochemistry

Immunocytochemistry was used to detect IGFBP-3, IGFBP-4 in granulosa cells plated on chamber slides. Primary mouse monoclonal antibodies to each petide IGFBP-3, IGFBP-4 (cross-reacting with corresponding rat, human, porcine and chicken substances; all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used as directed by the manufacturer at a dilution of 1:100. Visualisation of the primary antibody binding sites was done with a secondary rabbit polyclonal antibody against mouse IGs, labelled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:500) and diaminobenzidine (DAB) reagent (Roche Diagnostics Corporation, IN, USA, 10%). The presence of each peptide was determined by light microscopy. To verify these data, in some selected cases primary antibodies were visualised by secondary rabbit or goat monoclonal antibodies against mouse IGs labelled with FITC (Sevac, Prague, Czech Republic) and fluorescent microscopy. Negative control was presented by stained cells omitting primary antibody. During microscopic inspection, the percentage of cells containing visible antigen was determined.

Immunoassay

Levels of progesterone and IGFBP-3 were determined in duplicate in 20-100 µl samples by radioimmunoassay (RIA). Progesterone and IGFBP-3 were evaluated after ethanol extraction using RIA kits from DSL (Webster, USA) according to manufacturer's instructions while IGF-I was assayed as described previously. All RIA were validated for use in samples of culture medium. RIA assay for P_4: the antiserum cross-reacted was <0.001%, the sensitivity was 0.12 ng/mL. RIA assay for IGFBP-3: the antiserum cross-reacted was <0.03%, the sensitivity was 0.5 ng/mL. RIA assay for IGF-I: Inter- and intra-assay coefficients of variation did not exceed 10% and 16%, respectively. The sensitivity of the assay as determined by the dilution method was 0.3 ng/mL.

Statistics

Each experimental group was represented by four culture wells with granulosa cells. Assays of hormonal substances in incubation medium were performed in duplicate. The data presented concerning the effects of each substance are means of values obtained in three separate experiments performed on separate days using separate ovaries. The values of blank controls were subtracted from the values determined by RIA in cell-conditioned medium to exclude any non-specific background (less than 13% of total values). The rates of hormone secretion were calculated per 10^6 cells per day. The proportion of cells containing each analysed substance was calculated following immunocytochemical analysis by counting at least 1000 cells per chamber slide well. Firstly, the data obtained in each experiment were processed by ANOVA. Thereafter, significant differences between the immature groups and mature gilts were evaluated by paired t-test or chi-square (χ^2) test by using statistical software Sigma Plot 9.0 (Jandel, Corte Madera, USA). Differences from controls (P<0.05) were considered as significant.

RESULTS

Release of progesterone by ovarian granulosa cells was significantly higher (p<0.05) in sexually mature gilts (5.3±0.1 ng/mL vs 4.3±0.3 ng/mL) in comparison to sexually immature animals (Fig. 1). Release of IGF-I also followed the same pattern (7.8±0.6 ng/mL vs 6.2±0.2 ng/mL) (Fig. 1). Percentage of ovarian granulosa cells expressing IGFBP-3 was also significantly higher (p<0.05) in sexually mature gilts (53.1±0.4%) than the immature animals (36.5±2.3%) (Fig. 2). Although the IGFBP-4 expression by granulosa cells did not change significantly with sexual maturity (41.9±02.3% in mature gilts vs. 37.2±3.1% in immature ones) (Fig. 2).

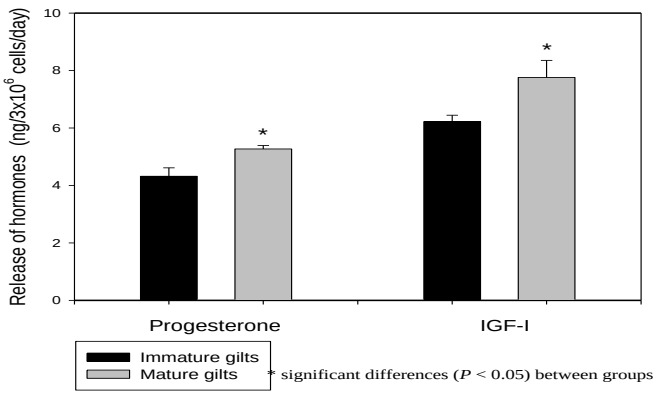

Figure 1 Release of progesterone and IGF-I by ovarian granulosa cells of sexually immature and mature gilts. Values are means ± SD, *significant difference ($P < 0.05$) between corresponding groups of sexually immature (n=18) and mature (n=17) gilts were evaluated by paired t-test and chi-square (χ^2) test.

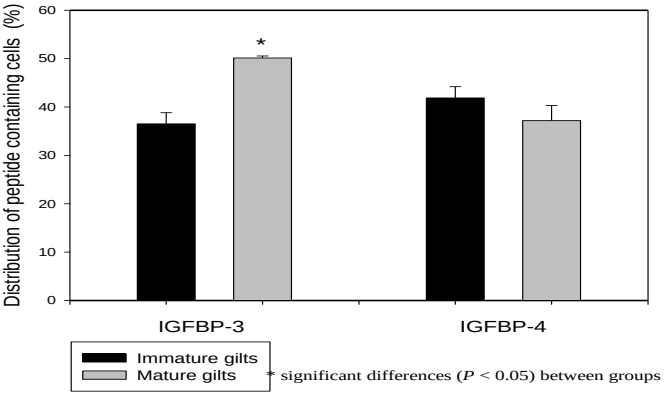

Figure 2 Expression of IGFBP-3 and IGFBP-4 in ovarian granulosa cells of sexually immature and mature gilts. Values are means ± SD, *significant difference ($P < 0.05$) between corresponding groups of sexually immature (n=18) and mature (n=17) gilts were evaluated by paired t-test and chi-square (χ^2) test.

DISCUSSION

Do progesterone, IGF-I, IGFBP-3 and IGFBP-4 relate to sexual maturation?

The previous observation in primates, rats, cattle (**Prunier and Louveau, 1997)** and pig (**Kolesarova *et al.*, 2010; Kolesarova *et al.*, 2008**) make it clear that sexual maturation was associated with the increase in blood concentrations of IGF-I. *In vivo* results concerning IGF-I levels in blood plasma in the study of **Kolesarova *et al.* (2008)** were also confirmed by *in vitro* results from IGF-I release by cultured ovarian granulosa cells indicating the sexual maturation-dependent increase in gilts . We report that increase in IGF-I release *in vitro* in gilts was associated with sexual maturation, and that therefore IGF-I may be involved in control of this process.

In our previous study (**Kolesarova *et al.*, 2008**), we also noted lower (p<0.05) levels of IGFBP-3 in blood plasma and granulosa cells of sexually immature gilts in comparison to mature animals. These results confirm our previous *in vivo* study (**Kolesarova *et al.*, 2010**). Plasma 43-39 kDa IGFBP levels were found to increase whereas plasma 34 kDa IGFBP decreased with age (p<0.01) (**Prunier and Louveau, 1997**).

Expression of IGFBP-4 in granulosa cells did not change with sexual maturity (**Kolesarova *et al.*, 2008**). **Grimes *et al.* (1994)** reported that the secretion of IGFBP-4 was higher in granulosa cells from immature porcine ovarian follicles (**Grimes *et al.*, 1994**). Low molecular weight IGFBPs, especially IGFBP-4, was the highest in small immature follicles that are predominantly atretic in pigs (**Howard *et al.*, 1991; Mondschein *et al.*, 1991; Ryan, 1981**). Our observations, together with previous reports (**Liu *et al.*, 1993; Sirotkin *et al.*, 2001**) suggest that IGFBPs could be important regulators of follicular growth and differentiation.

Possible interrelationships between studied substances

Certain changes observed in our investigations could be primary; others could be secondary, i.e. mediated by upstream regulators. For example, changes in P_4 release may be due to changes in IGF-I output. At least a positive relationship between P_4 and IGF-I concentrations in porcine blood (**Langendijk *et al.*, 2008**) and the ability of IGF-I to activate porcine ovarian steroid hormone release (**Sirotkin *et al.*, 2004**) has been reported. The opposite action of steroids on IGF-I is less probable because previous study showed that gonad steroids are not involved or play only a minor role in the control of IGF-I and IGFBP plasma levels during pubertal development in gilts (**Prunier and Louveau, 1997**). Previous authors showed stimulatory action of IGF-I on granulosa cell steroidogenesis which increased with follicular development, whereas its mitogenic action on granulosa cells decreased with follicular phase progression (**Kolodziejczyk *et al.*, 2003**). Interrelationships between IGF-I and IGFBPs are well known. A strong positive correlation between IGF-I and IGFBP-3 concentration was apparent with increasing age of the animals suggesting functional interrelations between the substances during sexual maturation (**Lee *et al.*, 2002**). Cooperation between IGF-I and IGFBP-4 in control of porcine ovarian folliculogenesis, follicular selection and luteinization was outlined earlier (**Grimes *et al.*, 1994**), wherein increased expression of both IGF-I and IGFBP-4 mRNAs during follicular selection and luteinisation was reported. Furthermore, it was observed that the action of IGFBP-4 on the ovary can be mediated by modulation (**Zhou *et al.*, 1996**) or stimulation (**Sirotkin *et al.*, 2001**) of IGF-I release and/or by inhibition of P_4 output (**Sirotkin *et al.*, 2001**). Therefore, ovarian follicular growth, selection, luteinization and related increase in progesterone release during porcine sexual maturation can be regulated by members of ovarian IGF-I/IGFBP system. In our experiments, the puberty-related changes in IGF-I, IGFBP-3 and P_4, but not in IGFBP4 were observed (**Kolesarova *et al.*, 2008**).

CONCLUSION

The present data obtained from *in vitro* study indicate that sexual maturation in females is influenced by puberty-related changes in porcine ovarian signaling substances: increase in P_4, IGF-I, IGFBP-3 but not IGFBP-4. It suggests that these signaling molecules could be potential regulators of porcine sexual maturation. Therefore, it may be suggested that porcine sexual maturation can be regulated by IGF-I-IGFBP3-P_4, but not by IGF-I-IGFBP4-P_4 system. Although the puberty-related changes don't provide direct evidence of the involvement and physiological role of these signaling molecules in control of sexual maturation, our study enables to identify extracellular signaling substances, which could be potential candidates for induction of porcine puberty and sexual maturation.

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