The follicular phase in pigs: Follicle populations, circulating hormones, follicle factors and oocytes

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ABSTRACT: The predominant pattern of follicle development in pigs is characterized by continuous activation, slow growth to the antral stage, and rapid growth to 4 to 5 mm followed by atresia. The only time that this pattern is broken is when a small portion of the follicle population is selected for ovulation. The mechanisms that regulate the selection of ovulatory follicles are not well understood. However, the ovulatory cohort shifts from FSH to LH dependence at the expense of the nonovulatory follicles as indicated by the following: 1) decreased secretion of FSH, and 2) decreased expression of the FSH receptor and increased expression of the LH receptor. The selection of ovulatory follicles may be dependent on the interaction of members of the intraovarian IGF system to maintain a high level of IGF-I bioavailability. The maintenance of a proliferating population of antral follicles is critically dependent on circulating FSH. A naturally or experimentally induced increase in circulating FSH levels results in an increase in antral follicles; conversely, decreased secretion of FSH is followed by a decrease in the number and in health status of antral follicles. Gonadotropin treatment with eCG or PG600 triggers selection of ovulatory follicles, and although these treatments do not increase litter size, they are beneficial for treatment of anestrus and, in conjunction with hCG or GnRH analogs, provide better control of the time of ovulation. The use of porcine FSH has not increased ovulation rate or improved oocyte developmental competence. To improve reproductive efficiency in the future, research should be directed toward obtaining more knowledge about genetic and physiological regulation of ovulatory follicle selection and the effect of follicle development on oocyte developmental competence.

Key Words: Follicle, Follicle Stimulating Hormone, Oocyte, Ovulation, Pigs

Introduction

Antrum formation is the stage of development in which follicles become dependent on the secretion of pituitary gonadotropins (Fortune, 1994; Burns and Matzuk, 2002). After activation, follicles grow slowly to the antral follicle stage and then undergo a relatively short burst of cell proliferation (Hirshfield, 1991; Morbeck et al., 1992; Fortune, 1994). Most antral follicles die, but once during each estrous cycle, a small portion of the population is selected for ovulation. Based on estimated growth rates in colchicine-arrested prepubertal gilts, once activated, a follicle requires 84 d to reach the antral stage (Morbeck et al., 1992). Growth of newly formed antral follicles (400 μm to 3 mm) was calculated to require 2 wk, and when follicles grew to ovulatory size, five additional days were required. From their estimate of antral follicle growth rate, Morbeck et al. (1992) hypothesized that the follicles undergoing antrum formation at the beginning of the cycle reach 3 mm in diameter on d 14 to 16 of the estrous cycle and constitute the population from which the ovulatory follicles are selected. A better understanding of factors and molecular mechanisms of folliculogenesis could enhance our ability to produce developmentally competent oocytes either in vivo or in vitro.

Estrous Cycle: Follicle Population and Endocrine Changes

The populations of small (1 to 2 mm) and medium (3 to 5 mm) follicles essentially disappear during the follicular phase of the cycle as the ovulatory follicles mature (Foxcroft and Hunter, 1985; Guthrie et al., 1995). The depleted follicle populations are replenished after ovulation with small follicles restored to 35 to 40 per animal by d 2 and medium follicles restored to

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approximately 40 per gilt by d 8 of the cycle. During the first 5 d of the cycle, 95% of the small and medium follicles are healthy (nonatretic) and steroidogenically active (Guthrie et al., 1995; Garrett and Guthrie, 1997). However, by d 7, the level of atresia increased to 50%, and steroidogenesis and granulosa cell proliferative activity were declining. Hirshfield (1991) suggested that as growth continues, the follicle wall reaches a certain thickness, which becomes limiting with respect to gas and nutrient exchange in the membrana granulosa. At this point, cell proliferation slows and cells begin to die. Perhaps porcine follicles grown after ovulation have reached their limit of growth as d 7 approaches and are beginning to die. This process of growth and death is a continuous process; only those follicles positioned in the window of opportunity opened by progestin withdrawal escape this fate and undergo preovulatory maturation. Whether groups of antral follicles grow to medium size in a few successive waves beginning after ovulation or grow more or less continuously throughout the luteal phase is unknown. The FSH secretory pattern during the 41 h before recovery of follicles for analysis on different days during the luteal phase was not significantly correlated with follicle size distributions, atresia status, or intrafollicular steroid hormone concentrations (Guthrie and Cooper, 1996).

The high circulating progesterone concentrations established during the first week of the estrous cycle persist until approximately d 12 to 14 of the cycle (Guthrie and Bolt, 1983, 1990; Knox et al., 2003), when progesterone secretion decreases to mark the onset of luteolysis. The development of large follicles and the diminished numbers of small and medium follicles during the follicular phase are accompanied by a dramatic decline in circulating levels of FSH (Guthrie and Bolt, 1983, 1990; Knox et al., 2003) starting approximately 1.5 to 2 d after the initiation of the decline in circulating progesterone. During the transition from the luteal to the follicular phase, circulating levels of LH seem to shift from a luteal-phase pulsatile mode to a follicular-phase mode characterized by a decrease in the incidence of pulsatile secretion and in the mean concentration until the preovulatory surge (Guthrie and Bolt, 1990; Flowers et al., 1991; Guthrie et al., 1993, 1997). The first indication of follicle maturation in the pig, increased ovarian secretion of estradiol-17β into the ovarian vein, occurs in conjunction with decreasing progesterone in the absence of any significant change in plasma LH secretion pattern or concentration (Flowers et al., 1991). If LH secretion does not increase during the early follicular phase, then follicle maturation may result from a change in the balance between progestin and gonadotropin action at the ovarian level. The notion of progesterone playing a direct role in suppressing follicle development is supported by the suppression of large follicles (Guthrie et al., 1995) and steroidogenesis, including estradiol-17β, in healthy follicles during the luteal phase of the estrous cycle (Guthrie and Cooper, 1996), and by the fact that progesterone added to cultured granulosa cells of prepubertal gilts (Chan and Tan, 1986) antagonizes the stimulatory effect of gonadotropins on estrogen production. The subsequent decrease in circulating levels of FSH and LH during the follicular phase is postulated to be the result of inhibin and estradiol negative feedback at the anterior pituitary and hypothalamus, respectively (Guthrie et al., 1995).

**Figure 1.** The shift from FSH to LH dependence during the follicular phase of the estrous cycle. Panel A shows the decreasing plasma FSH concentration between d 1 and 5 (modified from Guthrie et al., 1993). Panel B shows decreasing expression of FSH receptor (FSHR) mRNA and increasing expression LH receptor (LHR) and aromatase (P450arom) mRNA (modified from Liu et al., 2000). Means for P450arom, FSHR, and LHR with no common superscript letter differ ($P < 0.05$).

**Gene Expression During Follicular Phase**

For the purpose of discussion, the follicular phase of cyclic gilts and weaned sows will be divided into different days or stages as illustrated in Figure 1. The first stage, d 1, represents the late luteal phase or prefollicular phase stage of development corresponding to 24 h
after the last feeding of altrenogest (d 0) or 24 h after weaning. Days 3 and 5 represent progressive stages of preovulatory maturation before the preovulatory LH surge, and d 7 represents terminal differentiation, 24 to 36 h after the onset of the preovulatory LH surge.

A major event associated with the selection of ovulatory follicles is the shift in follicle dependence from FSH to LH. Two physiological events result in a reduction in the bioavailability of FSH to preovulatory follicles. Circulating levels of FSH decrease by four- to fivefold between d 1 and 5 of the follicular phase after cessation of altrenogest administration (Guthrie et al., 1993) or following luteolysis (Guthrie and Bolt, 1983; Knox et al., 2003), as illustrated in Figure 1A. In addition, FSH-receptor (FSHR) mRNA expression in granulosa cells of small and medium healthy follicles also decreases between d 1 and 5 (Figure 1B) to very low levels (as measured by northern and RNase protection analysis) within hours of the expected preovulatory LH surge (Liu et al., 1998, 2000). The shift to LH dependence is indicated by increased expression of LH receptor (LHR) mRNA in granulosa and theca interna cells between d 1 and 3, and is greatest in granulosa and theca interna cells of large follicles, especially in granulosa cells on d 5 (Figure 1B). The changes in FSHR and LHR expression are positively associated with the function of FSHR and LHR during preovulatory follicle maturation. The loss of FSH response and the increase in LH response in porcine granulosa cells during preovulatory maturation in terms of adenosine cyclic 3′,5′-phosphate production in vitro and the relative number of FSH and LH binding sites (reviewed by Ainsworth et al., 1990) are in general agreement with the expression pattern of the FSHR and LHR mRNA in vivo. In addition, the shift to LH dependence may begin much earlier in the estrus cycle of the pig. Histological examination of follicles in hypophysectomized gilts, intact gonadotropin-releasing hormone inhibitor treated gilts, and intact control gilts indicated that development of healthy follicles 1.1 to 2 mm in diameter was FSH-dependent, whereas those >2 mm in diameter were dependent on LH pulsatile secretion (Driancourt et al., 1995).

The nature of follicle growth also changes during preovulatory maturation. Cell proliferation slows in healthy follicles as they became estrogen active (>100 ng/mL of estradiol) and increase in size >5 mm (Fricke et al., 1996; Garrett and Guthrie, 1997). The selected follicles continue to increase in size, but growth is primarily due to an increase in follicle diameter through accumulation of fluid. Granulosa and theca cell proliferation continue at a rate just sufficient to maintain the thickness of the follicle wall until proliferation is completely arrested after the preovulatory LH surge (Fricke et al., 1996).

The intrafollicular concentration of estradiol-17β is a well-established marker for preovulatory maturation and is closely correlated with expression of aromatase (P450arom) mRNA (Guthrie et al., 1994) and protein (Garrett and Guthrie, 1997). The expression of P450arom (Figure 1B) is maximal in large follicles on d 5 and decreases on d 7. Many genes that may play a role in preovulatory maturation are expressed in the ovarian follicle in a pattern similar to that of P450arom; inhibin α, inhibin/activin β(A) (Guthrie et al., 1992; Li et al., 1997), P450τ (Guthrie et al., 1994; Liu et al., 2000), LHR (Liu et al., 2000), IGF-I (Samaras et al., 1993), and IGF-II (Liu et al., 2000) increase to a maximum on d 5 in large follicles and then decrease by varying degrees between d 5 and 7. The increase in expression of steroidogenic enzymes is associated with an increase in LHR mRNA in both granulosa and theca interna cells. Experiments in LHR knock-out (KO) mice have shown that the expression of these enzymes and of the ovulatory process are dependent on the action of the LHR (Burns and Matzuk, 2002).

Role of IGF System in Selection of Ovulatory Follicles

Insulin-like growth factors and low-molecular-weight IGFBP are considered as stimulators and inhibitors, respectively, of follicle growth and maturation (see reviews of Hammond et al., 1993; Mazerbourg et al., 2003). The IGF system comprises two ligands, IGF-I and IGF-II; two receptors, the type I receptor (IGFR) and the type II/mannose-6-phosphate receptor, and six IGFBP, which bind IFG-I and IGF-II with high affinity. The low-molecular-weight IGFBP (ranging from 24 to 35 kDa) include IGFBP-1, -2, -4, -5, and -6, which are present in variable proportions in the follicular fluid of different species.

Ovarian IGF-I plays a critical role in folliculogenesis after the early antral stage (Mazerbourg et al., 2003). The KO of IGF-I in the mouse exhibit normal activation of primordial follicles and produces normal numbers of granulosa cells in preantral follicles (Baker et al., 1996). However, growth of antral follicles and ovulation do not occur even with gonadotropin replacement therapy. In contrast, growth of follicles and ovulation in IGF-I KO mice can be restored after 2 wk of exogenous IGF-I replacement (Zhou et al., 1997). The interaction of IGF-I and FSH in the development of antral follicles is quite complex. Expression of FSHR mRNA is depressed in the IGF-I KO, whereas full expression of IGF-I mRNA was found in FSHR KO (Zhou et al., 1997). These results indicate the hierarchy of control between these two genes, such that IGF-I serves to permit and augment the physiological responses of granulosa cells to FSH by induction of the expression of the FSHR. The FSHR is required for expression of other genes important for antral follicle growth, such as P450arom (Zhou et al., 1997), inhibin α and β subunits, and LHR (Burns and Matzuk, 2002).

In the pig, the hierarchy of importance for IGF-I and the FSHR is uncertain because transcripts for IGF-I and FSHR are present even in the primary follicles of mature gilts (Yuan et al., 1996). Abundant IGF-I mRNA is present in granulosa cells as well as theca externa
Granulosa cell expression of mRNA of IGF-I and type 1 IGF receptor (IGFR) in panel A and IGFBP-2 and -4 in panel B. Means for IGF-I and IGFBP-2 that do not have a superscript letter in common differ \( (P < 0.05) \) \( \text{modified from Samaras et al., 1993; Liu et al., 2000.} \) Cells during the follicular phase (Liu et al., 2000). The amount of IGF-I mRNA in the follicle walls of individual follicles increased \( (P < 0.001) \) approximately 4.5-fold between d 1 and 5 (Figure 2A) and is highly correlated with growth (diameter) and estradiol-17\( \beta \) production \( (\log \text{estradiol-17}\beta \text{concentration}) ; r > 0.7; P < 0.01 \) (Samaras et al., 1993). Results of other experiments also indicated abundant IGF-I expression, but no effect of follicular stage (follicle size or day), (Liu et al., 2000).

As an estimate of IGF-I translational output, follicular fluid IGF did not change significantly between d 1 and 5 (Liu et al., 2000). Granulosa cells express IGFR mRNA in a constitutive pattern, with no differences \( (P > 0.10) \) among different stages of development, size, or day (Figure 2A).

Although the levels of follicular IGF-I protein may or may not change significantly during the follicular phase, its biological availability may be closely regulated by the presence of IGFBP in follicular tissue. Granulosa and theca interna cells expressed IGFBP-2 mRNA (Liu et al., 2000), but expression of IGFBP-2 mRNA is greater in granulosa cells compared with theca cells \( (P < 0.05) \). A decrease in IGFBP-2 mRNA expression in the tissue of preovulatory follicles between d 1 and 5 (Figure 2B) supports the notion that the bioavailability of IGF-I increases in the maturing ovulatory follicles (Samaras et al., 1993; Liu et al., 2000.) Expression of IGFBP-4 mRNA seems to be less important as a regulatory factor as its mRNA is present at a much lower level than IGFBP-2 mRNA and did not differ \( (P > 0.10) \) among different stages of development (Figure 2B).

The major IGFBP in porcine follicular fluid as measured by ligand blotting were IGFBP-3 (43 to 40 kDa) and IGFBP-2 (34-kDa), (Besnard et al., 1997; Liu et al., 2000). Follicular fluid IGFBP-3 was greater \( (P < 0.01) \) on d 3 and 5 compared with d 1 and seemed to be associated with preovulatory maturation. The amount of IGFBP-2 in follicular fluid was greater \( (P < 0.01) \) on d 1 and 3 than on d 5, and is in agreement with the notion of an increase in IGF-I bioavailability. The IGFBP can inhibit IGF action by sequestration at the site of action because the affinity of IGFBP for IGF is similar to the affinity of the IGFR (Mazerbourg et al., 2003). Further, the affinity of IGFBP-3, -4, and -5 for IGF are decreased when they are proteolyzed. A physiological role for IGFBP in the survival and continued growth of ovulatory follicles is also indicated by a decrease in levels of intrafollicular IGFBP-2 and IGFBP-4. For example, the reduction of intrafollicular IGFBP-2 in the pig is likely due to a combination of decreased transcriptional/translational activity of the gene in follicular tissue as discussed above and to an increase in IGFBP-2 proteolytic degradation found in growing ovulatory follicles (Mazerbourg et al., 2003). In healthy small follicles corresponding to d 1, IGFBP-2 degradation activity is low, with a 30% loss of IGFBP-2 during a 20 h incubation assay (Besnard et al., 1997). However, by d 4 in large follicles, IGFBP-2 degradation activity increased to 80% (Figure 3). Degradation of endogenous IGFBP-2 was lower in atretic vs. healthy follicles (Figure 3). The end result is that during the terminal stages of ovulatory follicle growth, follicular fluids contain low to undetectable levels of native IGFBP-2 as assessed by Western ligand blotting in comparison with the corresponding serum. In contrast, much higher levels of 23- and 12-kDa proteolytic fragments were found by immunoblotting in bovine and porcine preovulatory fol-
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**Figure 3.** Effect of follicle size class on the percentage of endogenous follicular fluid IGFBP-2 degraded during 20 h of incubation. Means for healthy follicles that do not have a superscript letter in common differ \((P < 0.05)\), and means for atretic follicles with an asterisk differ \((P < 0.05)\) from those for healthy follicles of same size class (modified from Besnard et al., 1997).

Immunoneutralization and immunoprecipitation studies have shown the protease that degrades IGFBP-4, IGFBP-2, and IGFBP-5 in ovine, porcine, bovine, and equine ovulatory follicles is the pregnancy-associated plasma protein-A (PAPP-A), previously identified in human fibroblasts and osteoblasts (Mazerbourg et al., 2003). In support of the physiological role of PAPP-A in the pig, expression of PAPP-A mRNA is lower in atretic and small healthy follicles than in ovulatory follicles (correlation between exogenous IGFBP-4 degradation and PAPP-A mRNA abundance, \(r = 0.55, P = 0.0016\)) (Mazerbourg et al., 2001). Those follicles containing high levels PAPP-A mRNA have greater capacity to degrade exogenous IGFBP-4.

**Experimental Manipulation of Follicle Development**

A series of experiments will be reviewed to illustrate the role of FSH and eCG in maintaining a population of ovarian follicles from which the ovulatory follicles are selected and to determine whether exogenous FSH might be an alternative method to stimulate follicle growth and increase recruitment or selection of ovulatory follicles.

**FSH Administration During an Artificial Luteal Phase**

Cyclic gilts were administered altrenogest to maintain an artificial luteal phase and were treated for 3 d at 8-h intervals with four treatment combinations to decrease or increase circulating levels of FSH: 1) saline and charcoal-stripped porcine serum as a control, 2) saline and charcoal stripped porcine follicular fluid (pFF) as a source of inhibin, 3) USDA porcine FSH (pFSH) and serum, and 4) pFSH and pFF (Guthrie et al., 1988). This FSH preparation was considered to be free of LH activity, with <1% contamination based on ascorbic acid depletion assay and a very low level of LH displacement in a luteal tissue LHR assay (Guthrie et al., 1990). The results of this experiment are summarized in Figure 4A and B. The number of small and medium follicles in the saline + serum control gilts, 53 and 31 per gilt, respectively, were similar to the numbers that would be found during the mid-luteal phase.
of the cycle. Administration of saline + pFF caused a decrease \( (P < 0.05) \) in circulating levels of FSH compared with gilts injected with saline-serum and decreased \( (P < 0.05) \) the number of medium follicles compared with saline + serum to 0.8 per animal. Injections of FSH + serum increased circulating levels of FSH compared with saline + serum, and increased \( (P < 0.05) \) the number of medium follicles to 57 per animal without a significant effect on the number of small follicles. The combination of pFSH + pFF also increased circulating FSH levels and increased \( (P < 0.05) \) the number of medium follicles compared with saline + pFF, in effect, restoring the population to a distribution similar to that in the control gilts. In another experiment, within 48 h after initiation of pFF injections, follicles were atretic by morphological criterion being opaque in appearance and containing cellular debris (Guthrie et al., 1987). From these experiments, we concluded that changes in circulating FSH over a 3-d interval were closely coupled to changes in follicular growth during the same period of time. The surprising result was that the pFSH treatment did not result in development of large, estrogenic follicles or increased secretion of estradiol-17\( \beta \). In contrast, a single injection of eCG induced growth of large, estradiol-17\( \beta \)-secreting follicles during an artificial luteal phase induced by altrenogest, and a subsequent injection of hCG was capable of ovulating these follicles (Guthrie and Bolt, 1985).

**Gonadotropin Administration to Prepubertal Gilts**

The results of the experiments described in the previous section indicated that whereas pFSH treatment induced growth of medium size follicles, it did not induce development of estrogen active large follicles during an altrenogest artificial luteal phase. To further characterize the effect of pFSH in a different experimental model, free of a possible progestin antagonism, an experiment was conducted in prepubertal gilts. Crossbred gilts at 160 d of age were injected 1) once with saline, 2) nine times at 8-h intervals with USDA pFSH; or 3) once with 15 IU/kg BW of eCG (Guthrie et al., 1990). Plasma was collected during the 72-h treatment period and ovaries were collected at slaughter 72 h after the first injection. The saline-injected gilts contained essentially no large follicles, and the numbers of small and medium follicles were 104 and eight per gilt, respectively. Injections of gonadotropins caused radical shifts in the distribution of follicles in these size classes (Figure 5A). Compared with saline, the 3-d exposure of ovaries to FSH treatment increased \( (P < 0.05) \) the number of small follicles to 152 per gilt, but had no significant effect on the number of medium or large follicles. In contrast to pFSH, eCG increased development of large follicles and decreased \( (P < 0.05) \) the total number and the number of small follicles compared with saline-injected gilts. The pFSH treatment increased \( (P < 0.05) \) plasma FSH concentration, whereas eCG decreased \( (P < 0.05) \) FSH secretion (Figure 5B). Secretion of estradiol-17\( \beta \) increased \( (P < 0.05) \) only following eCG treatment (Figure 5B), and aromatase activity was induced only in granulosa cells isolated from the follicles of the eCG-treated gilts. The common themes emerging from this work were as follows: 1) FSH plays an important role in the maintenance of a proliferating population of small or medium follicles in sexually mature and prepubertal swine, 2) selection of ovulatory follicles (spontaneous or gonadotropin induced) is accompanied by a decrease in FSH secretion, and 3) pFSH treatment did not induce the growth of large follicles or estradiol production.

Recently, results were published that described the acute effects of eCG on follicle size distribution and granulosa cell apoptosis in luteal phase gilts (Liu et al., 2003). Injection of eCG (1,000 IU) on d 11 of the cycle caused a dramatic, transient increase \( (P < 0.05) \) in the number of small and medium follicles on d 12 followed by a gradual decline to pretreatment numbers on d 13 (Figure 6A). The percentage of apoptotic granulosa cells in small and medium follicles was transiently decreased \( (P < 0.05) \) from 20 to 21% on d 11 to 8 to 10% 24 h later and then increased to d 11 levels at 96 h after injection (Figure 6B). The increased number of healthy follicles 24 h after injection may have been triggered by the FSH-like activity known to reside in eCG (Guthrie et al., 1990). The decrease in small and medium follicles and increased granulosa cell apoptosis after d 12 could be a result of 1) decreased secretion of FSH that follows the injection of eCG, 2) the potential decay of follicle stimulating activity in the eCG molecule itself, and 3) the potential atretogenic effect of the LH activity in eCG. The comparison of pFSH and eCG effects on follicular development indicates that the LH activity of eCG or the relatively long half life of eCG might explain the differences in biological effects relative to pFSH in maturation of preovulatory follicles.

**Follicular Phase FSH Treatment**

Treatment regimens such as eCG or PG600 have provided benefit for treatment of anestrus and, in conjunction with hCG or GnRH analogs, provide better control of the time of ovulation and have increased ovulation rate; however, litter size has not been increased. Reproductive efficiency might still be improved with new protocols and management tools to provide for more precise or reliable scheduling of estrus and ovulation.

An experiment was performed to compare the effects of pFSH and eCG on follicle development in terms of estradiol-17\( \beta \) secretion, ovulation rate, and fertilization rate (Guthrie et al., 1997). Postpuberal gilts were synchronized using altrenogest and assigned to one of four treatments: 1) one injection of saline (control) on d 1, 2) twice daily injections of Super Ov (Ausa Int., Tyler, TX) at a dose of 2.8 NIH FSH-S1 IU of pFSH/100 kg BW on d 1, 2, and 3, and 3) Super Ov at a dose of 4.6 NIH FSH-S1 IU of pFSH/100 kg BW on d 1, 2, and 3, or 4) one injection of 1,300 IU of eCG on d 1. The gilts in treatments 2 through 4 were artificially inseminated,
Figure 5. Effects of injections of porcine FSH (pFSH) and eCG at 8-h intervals on the number of 1- to 3- and 4- to 6-mm follicles (A) and on the linear regression of plasma FSH and estradiol concentrations on time (B) 72 h after the first injection in prepubertal gilts. Means for the number of 1- to 3-mm follicles and the linear regression coefficients for FSH and estradiol that do not have a superscript letter in common differ ($P < 0.05$; modified from Guthrie et al., 1990).

Eggs were recovered surgically 48 h after an injection of hCG, and ovarian structures (corpora lutea and unovulated follicles) were recorded. An average of 18 corpora lutea were recorded for the saline, control gilts. Injection of eCG increased ($P < 0.05$) the number of corpora lutea to 43 per gilt compared with controls. Neither the low nor high dose of pFSH had a significant effect on ovulation rate compared with the saline-injected gilts. The high-dose pFSH seemed to stimulate more follicle growth than low-dose FSH or saline, but only 38% of the follicles ovulated. A greater proportion of the saline- (100%) and eCG-treated gilts (87%) were detected to be in estrus compared with 53.2% for the FSH-treated gilts ($P < 0.001$). The fertilization rate among the eggs recovered did not differ between the FSH and eCG treatment groups. Analysis of circulating hormone levels showed that plasma FSH increased between d 1 and 4 in the FSH-injected gilts and decreased in saline- and eCG-injected gilts. Plasma estradiol increased between d 1 and 4 in all experimental groups, but the increase in the eCG-injected gilts was greater ($P < 0.05$) than that for the saline- and FSH-injected gilts. Plasma estradiol profiles did not differ among saline- and FSH-injected gilts. Administration of the high dose of pFSH during the follicular phase stimulated growth of large, estrogenic follicles, but circulating levels of estradiol-$17\beta$ did not reflect that increased follicle growth.
Figure 6. Effects of a single injection of eCG on d 11 of the estrous cycle on the number of <3 mm, 3 to 5 mm, and >5 mm follicles (A) and percentage of apoptotic granulosa cells (GC) in <3 mm, 3 to 5 mm, and >5 mm follicles (B). Means for the number of <3 mm and 3- to 5-mm follicles that do not have a superscript letter in common differ (P < 0.05; modified from Liu et al., 2003).

Oocyte Developmental Competence

The rationale for the production of so many germ cells during early fetal life and their subsequent loss in pigs and other species is unknown. The importance of apoptosis and follicular atresia may be that after antrum formation, follicles may have a finite life span (Hirshfield, 1991). Generally 50% of antral follicles on the ovarian surface are atretic. The meiotic status of porcine oocytes is very heterogeneous, 70% of oocytes in 3 to 5 mm diameter follicles have initiated germinal vesicle breakdown (GVBD) in prepubertal (Funahshi and Day, 1997; Grupen et al., 1997; my unpublished observations) and postpubertal gilts (Brüssow et al., 1996; Guthrie and Garrett, 2000). The incidence of GVBD did not differ significantly between healthy and atretic follicles before the start of ovulatory follicle maturation. However, follicles containing oocytes undergoing GVBD may be eliminated during the follicular phase because oocytes examined immediately before the preovulatory LH surge were predominantly in meiotic arrest (Hunter and Polge, 1966; Motlik and Fulka, 1976). Therefore, atresia may be physiologically important to eliminate oocytes that are degenerate or have escaped meiotic arrest.

The oocytes used for in vitro maturation/fertilization (IVM/IVF) are predominantly from prepubertal gilts. Typically, the IVM period is 40 to 46 h, and it is possible that a subpopulation of oocytes has reached metaphase II by as much as 20 h earlier than the rest. Three approaches have been used to reduce meiotic heterogeneity before the induction of the maturation process: 1) administration of gonadotropins to gilts 72 h before oocyte collection, 2) preincubation of oocytes without gonadotropins, and 3) exposure of oocytes to dibutyryl cyclic adenosine 3’,5’-monophosphate or hypoxanthine to induce synchronization (Funahshi and Day, 1997). These meiotic synchronization protocols would imply that the GVBD is reversible; however, this reversal in meiotic status requires experimental confirmation.

Developmental competence of IVM/IVF oocytes has been investigated following different gonadotropin treatments of prepubertal gilts (Bolamba et al., 1996). The following treatments were assigned to five groups of six prepubertal gilts each: 1) one injection of saline, 2) two injections of 8 mg of pFSH (Schering) at 12-h intervals, 3) 16 mg of pFSH at 12-h intervals, 4) one injection of 1,000 IU of eCG, and 5) one injection of PG600. Gilts were killed to count follicles and recover oocytes for IVM/IVF 72 h after the first injection for each treatment. Additional gilts were treated and killed at 48 h. At 48 h, two significant changes in the follicle population were found. The number of large follicles in the eCG and PG600 groups was already significantly greater than those in the saline group, and the level of atresia in all follicles was 54% for the saline and FSH treatment groups compared with 87% for the eCG and PG600 groups.

Administration of FSH-16 tended to increase the total number of follicles per gilt at 72 h, but it did not increase the number of large follicles compared with the saline control group (Figure 7). Treatment with eCG and PG600 decreased the total number of follicles compared with the saline-injected gilts and increased the number of large follicles compared with saline- and FSH-injected gilts. The FSH-16 gilts yielded greater (P < 0.05) numbers of good oocytes (uniform appearing cytoplasm and compact or expanding cumulus cell mass) than the saline, eCG, and PG600 groups (Figure 8). However, this apparent advantage was not maintained when oocytes representing these treatment groups were examined after IVM/IVF (Figure 9). The increased number of good oocytes recovered from pFSH-treated gilts was neutralized by the decreased oocyte developmental competence; the percentage of normal fertilized eggs recovered from eCG and PG600 gilts was
Figure 7. Effect of gonadotropin treatment on the number of small, medium, and large follicles in prepubertal gilts 72 h after the first injection. Treatments were a single injection of saline, injections of Schering porcine FSH at 12-h intervals (8 or 16 mg), a single injection of eCG, and a single injection of PG600. Means for the number of small, medium, and large follicles, and the percentage of apoptotic granulosa cells in small and medium follicles that do not have a superscript letter in common differ ($P < 0.05$; modified from Bolamba et al., 1996).

45 to 50% compared with 10 to 12% for pFSH-injected gilts.

Although exogenous gonadotropin treatments did not enhance apparent oocyte developmental competence in vitro, there are some changes in the experimental protocol that could improve the results. The ovarian response to Schering FSH preparations is unpredictable because they are known to contain LH contamination. Although two injections of Schering pFSH did not result in ovulations (Bolamba et al., 1996), additional injections did result in ovulation (Bolamba and Sirard, 2000). Presumably, each injection of Schering pFSH induced development of estrogen active follicles; these follicles may have induced their own ovulation by positive feedback on LH release or alternatively (or simultaneously), the LH activity in this particular FSH preparation itself elicited luteinization and perhaps ovulation. A FSH preparation with a lower LH content should be tested. Other modifications could include collection of oocytes sooner after initial gonadotropin treatment (24 or 48 h) and development of improved IVM protocols.

**Conclusions**

The purpose of preovulatory follicle maturation is to provide an environment that ensures delivery of a subpopulation of developmentally competent oocytes and capacitated sperm to the site of fertilization. Follicles selected for ovulation must be at the right place at the right time; a window of opportunity exists on d 14 to 16 of the estrous cycle or following an injection of eCG in prepubertal gilts. To improve reproductive efficiency in the future, research should be directed toward obtaining more knowledge about genetic and physiological regulation of ovulatory follicle selection and the effect of follicle development on oocyte developmental competence. The FSH treatment protocols were not found to be useful as assisted reproductive techniques in swine for the following reasons: 1) the failure to increase ovulation rate, 2) association with a low incidence of estrus compared with eCG treatment, and 3) low in vitro oocyte developmental competence compared with eCG.

**Implications**

Increasing ovulation rate and obtaining more precise control of the time of ovulation have been sought after...
goals for many years. Gonadotropin treatment with equine chorionic gonadotropin or prostaglandin 600 triggers selection of ovulatory follicles. Although these treatments do not increase litter size, they are beneficial for treatment of anestrus and, in conjunction with human chorionic gonadotropin and gonadotropin-releasing hormone analogs, provide better control of the time of ovulation. However, there is still room for more precise timing of ovulation, more reliable estrus detection, and improved oocyte developmental competence (better embryo survival). Follicle maturation itself has been investigated by cell culture and by analysis of tissues and blood plasma recovered at different stages of development. Treatments of a stimulatory and inhibitory nature have been applied in vitro and in vivo. These approaches have been valuable in generating a physiological database; however, there are still massive gaps in our understanding of recruitment and selection of follicles destined to ovulate in the pig. In the future, we should keep in mind some of the lessons of the mouse knockout models and begin using genomic and proteomic technologies to determine novel gene association and expression patterns controlling the selection of ovulatory follicles in swine. Such knowledge will be important in applying reproductive biotechnologies in swine.

**Literature Cited**


Follicular phase in pigs

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