Dihydrotestosterone influenced numbers of healthy follicles and follicular amounts of LH receptor mRNA during the follicular phase of the estrous cycle in gilts

H Cárdenas, E Jiménez and W F Pope

Department of Animal Sciences, The Ohio State University, 2027 Coffey Road, Columbus, Ohio 43210, USA

Correspondence should be addressed to H Cárdenas; Email: cardenas.21@osu.edu

Abstract

The present experiments were conducted to determine androgenic effects on numbers, health, and amounts of gonadotropin receptor mRNA in late developing follicles of gilts. Gilts ($n=5$ per group) received daily injections of one of the following treatments on days 13–16 or days 13–18 of the estrous cycle: corn oil, 5 α -dihydrotestosterone (DHT, 10 mg), flutamide (1.5 g, an androgen receptor inhibitor), DHT (10 mg) plus flutamide (1.5 g), testosterone (10 mg), and testosterone (10 mg) plus flutamide (1.5 g). Ovarian follicles ≥ 5 mm in diameter were evaluated on day 17 or 19, 24 h after receiving the last treatment dose. Follicles were classified as healthy (H), moderately atretic (MA), or very atretic (VA). Treatment with DHT increased ($P<0.05$) the numbers of H follicles relative to control gilts on days 17 and 19. DHT administration from days 13 to 16 diminished ($P<0.05$) the amounts of LH receptor (LHR) mRNA in H follicles from day 17 (relative amounts: 1.45 ± 0.33 and 2.72 ± 0.33 for DHT- and vehicle-treated gilts respectively). The effects of DHT on numbers of H follicles and LHR mRNA were not observed in gilts receiving DHT plus flutamide. Androgens did not influence numbers of MA, VA, and total follicles, or follicular estradiol-17 β concentrations and amounts of FSHR mRNA. Treating gilts with DHT during follicular recruitment and selection did not induce changes in the numbers of total follicles ≥ 5 mm, but rather increased the numbers of healthy follicles in this follicular population in association with decreased amounts of LHR mRNA.

Reproduction (2008) **135** 343–350

Introduction

Ovulation rate in pigs is variable and can be influenced by multiple factors (Cárdenas & Pope 2002b, Hunter *et al.* 2004, Knox 2005). Critical processes that define the number of ovulatory follicles are thought to occur during follicular recruitment and selection (Foxcroft & Hunter 1985). Follicular recruitment is the process of forming a pool of follicles that are small and medium in size occurring approximately from days 13 through 16 of the estrous cycle (day 0=onset of estrus) in pigs. The majority of recruited follicles subsequently become atretic while those that remain are chosen to complete their development through ovulation by a process called follicular selection (Foxcroft & Hunter 1985).

Among the factors that influence follicular development and ovulation rate, follicle-stimulating hormone (FSH) is important because it stimulates granulosa cell differentiation, steroidogenic function, and survival (Richards 1994, Chun *et al.* 1996, Markström *et al.* 2002) and follicular recruitment (Driancourt 2001, Hunter *et al.* 2004). Luteinizing hormone (LH) also appears important during late follicular development in pigs. Evaluations of follicular growth, serum concentrations of gonadotropins,

and amounts of gonadotropin receptors suggested that ovarian follicles of pigs were highly dependent on LH for developing from ~ 5 mm in diameter onward (Lucy *et al.* 2001). Like FSH and LH, any factor that influences follicular processes such as cell proliferation, oocyte development, atresia, and responses to ovulatory stimuli might be able to modulate ovulation rate. These factors are numerous and among them are the steroid hormones, progesterone, estrogens, and androgens.

Androgens have been demonstrated to influence early follicular development in nonhuman primates (Weil *et al.* 1999) and cattle (Yang & Fortune 2006). Female mice lacking a functional androgen receptor (AR) gene had fewer ovarian follicles and offspring during early reproductive life than the wild type, and became infertile later because of complete loss of follicles (Shiina *et al.* 2006). Previous research from our laboratory demonstrated that administration of testosterone (T) or 5 α -dihydrotestosterone (DHT) to gilts during the follicular phase of the estrous cycle increased ovulation rate (Cárdenas & Pope 1994, Cárdenas *et al.* 2002a). The effects of DHT suggested stimulation through the AR and possible exclusion of estrogenic actions. It is not known, however, what organ,

cell type, or molecular pathway is actually influenced by androgenic treatment. Whatever the mechanism, it is conceivable that the effects of androgens must be related, directly or indirectly, to at least one of the physiological processes that define ovulation rate; follicular recruitment and selection. In the present experiments, androgens were administered to gilts during follicular recruitment and selection (follicular phase of the estrous cycle) to determine androgenic effects on follicular numbers, health, and expression of the FSH receptor (FSHR) and LH receptor (LHR) genes.

Results

The total numbers of follicles (sum of healthy, H; moderately atretic, MA and very atretic, VA) ≥ 5 mm were not altered by the treatments and day of the estrous cycle (Table 1). The numbers of H follicles were influenced by treatment and day (no interaction was observed). DHT increased ($P < 0.05$) the mean number of H follicles on days 17 and 19. The effect of DHT on numbers of H follicles was blocked when administered to gilts simultaneously with flutamide (F). The numbers of H follicles in gilts receiving T, T+F, or F were not different from vehicle-treated gilts (Fig. 1 and Table 1). Treatments did not influence the numbers of VA and MA follicles or the proportions of H, MA, or VA follicles (Fig. 1 and Table 1). Regarding day effects, numbers and proportions of H follicles were less ($P < 0.05$) on day 17 than on day 19 of the estrous cycle, possibly because only follicles ≥ 5 mm were considered. Differently from H follicles, greater ($P < 0.05$) numbers and proportions of MA follicles were observed on day 17 than on day 19. However, the numbers of VA follicles did not differ between days (Table 1).

Androgens and/or F did not influence the diameter of ovarian follicles classified as H, MA, or VA (Table 2). As expected, based on the progression of late follicular growth, H follicles were smaller ($P < 0.01$) on day 17 than on day 19 of the estrous cycle. MA and VA follicles were also smaller ($P < 0.05$) on day 17 than on day 19 (Table 2). Treating gilts with androgens and/or F did not influence the concentrations of estradiol-17 β (E_2) in follicular fluid. Concentrations of E_2 in follicular fluid of H follicles increased ($P < 0.001$) from days 17 to 19 of the estrous cycle. Unlike H follicles, MA follicles obtained on days 17 and 19 did not differ in concentrations of E_2 in follicular fluid, and a decrease ($P < 0.05$) from days 17 to 19 was observed in VA follicles (Table 3).

Androgens or F, alone or in combination, did not influence the relative amounts of FSHR mRNA in H follicles (Table 4). Follicles obtained on day 19 had less ($P < 0.01$) FSHR mRNA than those obtained on day 17. Regarding LHR, administration of DHT on days 13 to 16 decreased ($P < 0.05$) the mRNA amounts of this receptor in H follicles on day 17. Likewise, F decreased the amounts of LHR mRNA in similar follicles on day 17. The treatments DHT+F, T+F, and T did not alter the amounts of LHR mRNA on day 17 of the estrous cycle. No treatment effects were detected on LHR mRNA on day 19 (Table 4).

Discussion

Since enhancement of ovulation rate in pigs by administration of androgens was observed (Cárdenas & Pope 1994), an important research objective has been to elucidate the mechanisms mediating this effect.

Table 1 Mean numbers and percentages of healthy (H) moderately atretic (MA) and very atretic (VA) ovarian follicles ≥ 5 mm on days 17 and 19 of the estrous cycle in gilts treated with 5 α -dihydrotestosterone (DHT), testosterone (T), and/or flutamide (F)^a.

Treatment ^b	Day	H ^c	MA	VA	Total	H (%) ^d	MA (%)	VA (%)
Control	17	14.0	9.2	3.8	27.0	51.7	34.7	13.6
DHT		19.4	8.6	2.0	30.0	65.7	27.5	6.8
DHT+F		13.2	6.6	4.4	24.2	54.3	28.8	16.9
F		10.0	5.2	2.0	17.2	67.2	25.0	7.8
T		17.4	5.6	3.0	26.0	67.7	21.1	11.2
T+F		15.6	6.2	3.8	25.6	64.3	21.3	14.5
Control	19	16.4	3.2	2.0	21.6	74.9	14.8	10.3
DHT		19.8	1.0	2.2	23.0	86.2	4.4	9.4
DHT+F		16.8	1.2	2.0	20.0	84.0	6.1	10.0
F		14.2	5.6	3.6	23.4	60.7	24.3	15.0
T		19.8	4.4	5.8	30.0	67.1	15.3	17.6
T+F		19.4	2.4	4.0	25.8	77.3	7.8	14.9
Day mean	17	14.9 $\pm$ 0.8 ^e	6.9 $\pm$ 0.7 ^e	3.2 $\pm$ 0.5	25.0 $\pm$ 1.3	61.8 $\pm$ 2.7 ^e	26.4 $\pm$ 2.1 ^e	11.8 $\pm$ 1.5
	19	17.7 $\pm$ 0.8 ^f	3.0 $\pm$ 0.7 ^f	3.3 $\pm$ 0.5	24.0 $\pm$ 1.3	75.0 $\pm$ 2.7 ^f	12.1 $\pm$ 2.1 ^f	12.9 $\pm$ 1.5

^aNo day-treatment interactions were observed on any variable. Treatments altered (main effect, $P < 0.01$) numbers of H follicles but did not influence numbers of total, MA, or VA follicles. For clarity, means for treatment main effects (across days) are presented separately in Fig. 1. ^bTreatments were given daily on days 13 to 16 or days 13 to 18, and follicles were evaluated on days 17 or 19 respectively. ^cS.E.M. associated with the means of numbers for treatment $\times$ day combinations; H=2.0, MA=1.8, VA=1.1, Total follicles=3.1, $n=5$. ^dS.E.M. associated with the means of percentages for treatment $\times$ day combinations; H=6.6, MA=6.1, VA=3.7, $n=5$. ^{e,f}Means for days across treatments within a column having different superscripts differ ($P < 0.05$).

Figure 1 Mean numbers of healthy (H), moderately atretic (MA), and very atretic (VA) ovarian follicles ≥ 5 mm collected on days 17 and 19 of the estrous cycle in gilts treated with 5 α -dihydrotestosterone (DHT), testosterone (T), and/or flutamide (F). Treatments were given on days 13 to 16 or days 13 to 18 of the estrous cycle. Follicles were collected 24 h after the last injection. Values represent means of treatments irrespective of days (treatment main effect). Means of H follicles having different superscripts differ ($P < 0.05$). Treatments did not influence numbers of MA and VA follicles.

Conceivably, androgen treatment during the follicular phase might increase the ovulation rate by stimulating incorporation of more follicles into the recruited pool and/or enhancement of follicular health resulting in the decreased losses due to atresia. As follicular recruitment in pigs has been estimated to occur up to day 16, which is then followed by follicular selection (Foxcroft & Hunter 1985), treatments were given to gilts for 4 or 6 days, beginning on day 13 of the estrous cycle to examine effects of androgens related to these processes. Evaluations of the total numbers of follicles (healthy plus atretic) ≥ 5 mm in diameter, soon after the estimated completion of recruitment (day 17), provided no apparent indication that androgens induced incorporation of more follicles into the recruited pool.

Overall effects of androgens on follicular health were assessed by initially categorizing follicles according to health status and then comparing their numbers and proportions. DHT increased the numbers of H follicles

by ~ 5 on day 17 and 3 on day 19 (13% average increase across days). This effect was absent in gilts treated with DHT+F, which would be consistent with effects mediated by the AR. An increase in numbers of H follicles without a change in total numbers of follicles might be expected to occur concurrently with a decrease in numbers of atretic follicles. This decrease was observed only in terms of absolute values (no statistical significance) of numbers and proportions of MA and VA follicles in DHT- versus vehicle-treated gilts (Fig. 1 and Table 1). The numbers of H follicles in gilts treated with T were similar to those receiving DHT but compared with control gilts the difference only approached significance ($P = 0.09$). Responses of different magnitude to DHT and T (also observed on LHR mRNA, Table 4) might be explained by the differences in biological activity between these two androgens (Grino *et al.* 1990) or possible interactions of androgenic and estrogenic effects elicited by T due to its conversion into estrogens.

Table 2 Mean diameter (mm) of healthy (H), moderately atretic (MA), and very atretic (VA) ovarian follicles ≥ 5 mm on days 17 and 19 of the estrous cycle in gilts treated with 5 α -dihydrotestosterone (DHT), testosterone (T), and/or flutamide (F)^a.

Treatment ^b	Day	H	MA	VA	All follicles
Control	17	6.7	6.0	5.9	6.4
DHT	17	6.0	5.4	5.3	5.8
DHT+F	17	5.6	5.4	5.2	5.5
F	17	5.5	5.4	5.1	5.4
T	17	6.0	5.6	5.2	5.8
T+F	17	6.4	5.6	5.2	6.1
Control	19	7.7	6.6	5.4	7.3
DHT	19	7.8	5.8	5.6	7.5
DHT+F	19	8.6	7.8	6.2	8.2
F	19	8.5	7.6	6.3	8.0
T	19	8.4	7.0	5.9	7.8
T+F	19	7.1	6.4	5.3	6.7
Day mean	17	6.1 $\pm$ 0.2 ^c	5.6 $\pm$ 0.2 ^c	5.3 $\pm$ 0.2 ^c	5.8 $\pm$ 0.2 ^c
	19	8.0 $\pm$ 0.2 ^d	6.9 $\pm$ 0.3 ^d	5.8 $\pm$ 0.2 ^d	7.6 $\pm$ 0.2 ^d

^aFollicular diameter was not influenced by treatment or treatment-day interactions but it was influenced by day. For simplicity, means for treatment across days are not presented. S.E.M. associated with the means of treatment $\times$ day combinations; H=0.5, MA=0.6, VA=0.4, all follicles=0.4.

^bTreatments were given daily on days 13 to 16 or days 13 to 18 and follicles were evaluated on days 17 or 19 respectively, $n=5$ gilts per group.

^{c,d}Means for days across treatments within a column having different superscripts differ ($P < 0.05$).

Table 3 Means ($\pm$ S.E.M.) of estradiol-17 β concentrations (ng/ml) in follicular fluid of ovarian follicles ≥ 5 mm collected on days 17 and 19 of the estrous cycle from gilts treated with 5 α -dihydrotestosterone (DHT), testosterone (T), and/or flutamide (F)^a.

Treatment ^b	Day	H	MA	VA
Control	17	162.5 $\pm$ 12.9	63.1 $\pm$ 14.4	30.9 $\pm$ 7.4
DHT		196.4 $\pm$ 10.4	112.7 $\pm$ 17.7	29.8 $\pm$ 11.0
DHT+F		134.4 $\pm$ 13.6	55.1 $\pm$ 17.0	44.3 $\pm$ 7.6
F		128.1 $\pm$ 16.4	87.5 $\pm$ 27.3	19.4 $\pm$ 12
T		223.9 $\pm$ 11.3	95.3 $\pm$ 20.0	20.5 $\pm$ 10.5
T+F		212.8 $\pm$ 11.6	28.1 $\pm$ 27.6	42.7 $\pm$ 8.8
Control	19	345.8 $\pm$ 11.9	170.4 $\pm$ 30.0	7.4 $\pm$ 11.5
DHT		305.1 $\pm$ 10.3	100.6 $\pm$ 42.5	43.8 $\pm$ 11.5
DHT+F		420.1 $\pm$ 11.2	113.5 $\pm$ 42.5	16.8 $\pm$ 9.7
F		303.7 $\pm$ 15.5	69.8 $\pm$ 27.3	10.9 $\pm$ 9.3
T		325.3 $\pm$ 10.4	77.0 $\pm$ 22.1	10.6 $\pm$ 6.4
T+F		383.7 $\pm$ 10.5	77.7 $\pm$ 35.7	9.5 $\pm$ 8.6
Day mean	17	176.4 $\pm$ 5.2 ^c	73.6 $\pm$ 8.7	31.3 $\pm$ 4.0 ^c
	19	347.3 $\pm$ 4.8 ^d	101.5 $\pm$ 14.0	16.5 $\pm$ 4.0 ^d

^aH, healthy; MA, moderately atretic; VA, very atretic. No effects of treatment or day-treatment interactions were observed. Day effects were observed on H and VA follicles. For simplicity, means for treatment across days are not presented. ^bDaily treatments were administration on days 13 to 16 or days 13 to 18 follicles were collected on days 17 or 19, 24 h after the last injection, $n=5$ gilts per group. ^{c,d}Means for days across treatments within a column having different superscripts differ ($P<0.05$).

Treatments did not induce changes in follicular size (diameter) within the categories of H, MA, and VA follicles. Therefore, any treatment effect including that of DHT on numbers of H follicles was not related to changes in follicular size. Associations between follicular size and ovulation rate have been described

Table 4 Relative amounts (mean $\pm$ S.E.M.) of follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) mRNAs in tissues of healthy ovarian follicles ≥ 5 mm in diameter on days 17 and 19 of the estrous cycle in gilts treated with 5 α -dihydrotestosterone (DHT), testosterone (T), and/or flutamide (F)^a.

Treatment ^b	Day	FSHR	LHR
Control	17	1.10 $\pm$ 0.15	2.72 $\pm$ 0.33 ^c
DHT		1.04 $\pm$ 0.15	1.45 $\pm$ 0.33 ^{d,e}
DHT+F		1.12 $\pm$ 0.15	2.36 $\pm$ 0.33 ^{c,e}
F		1.29 $\pm$ 0.15	1.72 $\pm$ 0.33 ^{d,e}
T		1.15 $\pm$ 0.15	2.80 $\pm$ 0.33 ^c
T+F		1.29 $\pm$ 0.15	2.50 $\pm$ 0.33 ^c
Control	19	1.02 $\pm$ 0.15	3.57 $\pm$ 0.29
DHT		0.79 $\pm$ 0.15	3.29 $\pm$ 0.18
DHT+F		0.78 $\pm$ 0.15	3.11 $\pm$ 0.84
F		0.54 $\pm$ 0.15	2.50 $\pm$ 0.83
T		0.71 $\pm$ 0.15	1.22 $\pm$ 0.60
T+F		0.76 $\pm$ 0.15	2.58 $\pm$ 0.82
Day mean	17	1.17 $\pm$ 0.06 ^c	2.26 $\pm$ 0.15
	19	0.77 $\pm$ 0.06 ^d	2.71 $\pm$ 0.27

^aRelative amounts are ratios to ribosomal protein L19. For simplicity, means for treatment across days are not presented. FSHR mRNA was influenced ($P<0.05$) only by day of the estrous cycle. Treatments affected ($P<0.05$) LHR mRNA on day 17 but not on day 19. ^bDaily treatments were given on days 13 to 16 or days 13 to 18, and follicles were collected on days 17 or 19, 24 h after the last injection, $n=5$ gilts per group. ^{c,d,e}Means for days across treatments for FSHR, or for treatments within day 17 for LHR, having different superscripts differ ($P<0.05$).

in pigs. For instance, smaller size of day 19 preovulatory follicles and greater ovulation rate were observed in Meishan sows when compared with Large-White hybrid sows (Miller *et al.* 1998). Smaller size of preovulatory follicles was interpreted as relatively fast follicular differentiation in preparation for ovulation (Hunter 2004). The difference in average diameter of H follicles between days 17 and 19 was ~ 2 mm, which agrees with previous estimations of growth during late follicular development in the pig (1.1 mm/day, Dailey *et al.* 1976). Although the average diameter of MA follicles on day 19 was greater than on day 17, the difference between days (1.3 mm) was not as large as for H follicles. This might indicate that MA follicles on day 17 can continue to grow but at a slower rate than H follicles or that atresia started in a proportion of follicles after day 17. Unlike H and MA follicles, VA follicles from days 17 and 19 differed only slightly in average size.

In the present study, follicular fluid concentrations of E₂ in follicles from the different categories of health were not influenced by treatments. Noteworthy is that the concentrations of E₂ were measured 24 h after the last treatment and that E₂ concentrations could have changed before 24 h and then returned to normal. Indeed, the previous research described a transient increase in serum concentrations of E₂ after administration of 1 mg T on day 17 of the estrous cycle in gilts (Cárdenas & Pope 1994). Consequently, it might be assumed that enhancement of ovulation rate by androgens probably occurs independently of factors influencing the synthesis of E₂ by ovarian follicles in a substantial and extended manner. Other researchers did not observe significant changes in plasma E₂ concentrations in gilts having increased ovulation rate as a result of genetic selection (Mariscal *et al.* 1998).

Results on follicular numbers and health presented above suggest that androgen treatment increases the ovulation rate in gilts possibly by enhancing health among follicles of the recruited pool resulting in increased follicular selection. Results of a previous experiment in which DHT increased ovulation rate when administered during the post-recruitment period (day 17 to estrus) also suggested enhancement of follicular selection as a possible mechanism (Cárdenas *et al.* 2002a). Follicular health is regulated by interactions among multiple physiological processes that stimulate cell proliferation and survival or induce apoptotic cell death (Tsafiri & Braw 1984). Apoptosis-inducing factors that have been identified in pig granulosa cells include tumor necrosis factor (TNF)- α , Fas ligand (TNF receptor superfamily member 6 or APO1), TNF related apoptosis-inducing ligand (also known as apoptosis-2, APO2), and APO3 (Manabe *et al.* 2004). Whether androgens influenced the actions of one of these factors, other components of the complex intracellular apoptotic pathways, or apoptosis inhibitory mechanisms (survival factors), is not known.

In a recent experiment, DHT and T administered to gilts during the follicular phase of the estrous cycle increased serum concentrations of FSH (Jiménez *et al.* 2006). Considering the effects of FSH on follicular development (see Introduction), an increase in its concentrations could mediate, at least in part, an increase in ovulation rate that is supported by enhancement of follicular health. However, other regulators of follicular development, whether or not related to FSH, could also be affected by DHT. Two important receptors were examined in Experiment 2, FSHR and LHR. No differences were observed among treatments in relative amounts of FSHR mRNA in H follicles collected on day 17 or 19. These results were somewhat unexpected because of a previous report of increased amounts of FSHR mRNA in pre-ovulatory follicles from DHT-treated gilts (Cárdenas *et al.* 2002a). In this previous experiment, gilts were treated with DHT longer (day 13 to first day of estrus) and follicles were more developed at the time of evaluation (first day of estrus) than in the present experiment. Duration of treatment and advancement of follicular development might be important for the effects of DHT on FSHR mRNA because FSHR is actively regulated and its mRNA amounts remarkably decrease during the final days of follicular development in pigs (Lucy *et al.* 2001, Cárdenas *et al.* 2002a, present experiment). Although the influence of DHT on FSHR appears variable in our pig model, other investigators have observed positive effects of androgens on FSHR mRNA in monkey granulosa cells *in vivo* (Weil *et al.* 1999) and bovine granulosa cells *in vitro* (Luo & Wiltbank 2006).

Contrary to FSHR, which was unaltered by the treatments, amounts of LHR mRNA were ~50% less in H follicles collected on day 17 from DHT-treated gilts compared with those receiving vehicle. Consistent with androgenic actions mediated by the AR, the inhibitory effect on LHR mRNA did not occur when gilts received DHT + F. This is a novel observation on regulation of LHR mRNA in ovarian follicles by administration of androgens. It is not known whether the effect of DHT was directly exerted on transcription of the LHR gene or indirectly by influencing other regulators. It is likely that DHT might have exerted its effects indirectly because no androgen response element has been reported in the LHR promoter. Some factors influencing LHR in ovarian follicles have been identified. LHR mRNA was enhanced synergistically by FSH and E₂ in granulosa cells of hypophysectomized rats (Segaloff *et al.* 1990). Likewise, FSH increased LHR mRNA in pig cumulus cells (Okazaki *et al.* 2003). In addition, the LHR gene can be regulated by repression. For example, orphan receptors and histone deacetylase (Zhang & Dufau 2000, 2003) and an upstream initiator-like element (Youn *et al.* 2005) have been determined to inhibit expression of the LHR gene.

The temporary nature of the effect of DHT on LHR mRNA (the effect was observed on day 17 but not on day 19) indicates that other factor(s) might have influenced

LHR mRNA as follicles approached final maturation, overcoming the effects observed on day 17. The factor(s) is as yet undetermined, but based on the results of Segaloff *et al.* (1990, see previous paragraph), FSH (increased after DHT treatment, Jiménez *et al.* 2006), and E₂ (increased twofold from day 17 to 19 in follicular fluid, Table 3), might be candidate modulators of the LHR mRNA pattern observed in DHT-treated gilts.

Treatment of gilts with F alone also decreased the amounts of LHR mRNA. In rats, F treatment produced a significant decrease in testicular LH receptors (Marchetti & Labrie 1988) but had no effects on ovarian LH receptors (Luthy *et al.* 1987). It is expected that inhibition of AR by F would block, at least partially, the actions of endogenous androgens. If this was the case, then androgens might be necessary to maintain normal amounts of LHR mRNA in pig ovarian follicles. The reasons by which both inhibition of endogenous androgen signaling (F treatment) and increased androgen stimulation (DHT treatment) produced inhibitory effects on LHR mRNA are not clear. A similar change in the type of action of androgens on follicular development, from stimulatory to inhibitory, and apparently in direct association with the magnitude of AR stimulation has been observed in Rhesus monkeys (Zelevnik *et al.* 2004).

Lesser amounts of LHR mRNA might presumably result in decreased LHR protein synthesis and subsequent inhibition of LH actions in ovarian follicles. An important function of LH is the stimulation of androgen synthesis (pigs, Evans *et al.* 1981). Decreased amounts of androgens might result in decreased synthesis of E₂. In Experiment 1, concentrations of E₂ in follicular fluid were not affected by DHT treatment. In a previous experiment, plasma concentrations of E₂, T, and androstenedione in gilts were not altered by DHT treatment (authors' observations, unpublished). These results suggest that androgen synthesis might not have been affected by a possible decrease in LH stimulation.

In conclusion, androgenic stimulation through the AR during the follicular phase of the estrous cycle enhanced the numbers of healthy follicles among a numerically unaltered pool of total recruited follicles. How a simultaneous decrease in amounts of LHR mRNA might be related to this increase in healthy follicles is interesting but at present obscure.

Materials and Methods

Animals and treatments

Gilts (cross of domestic European breeds) near puberty were exposed to boars and observed for the signs of estrus twice a day (~0800 and 1700 h). To reduce possible influences of the variability in estrous cycle duration, only gilts that exhibited two consecutive estrous cycles of 19.5–21.0 days (day 0 = first day of estrus) were included in the experiments. The mean ($\pm$ S.D.) estrous cycle duration for these gilts was 20.2 $\pm$ 0.5 days.

At the time, treatments were given gilts ranged from 120 to 130 kg. Gilts ($n=5$ per treatment) received daily (at ~0800 h) injections (i.m.) of the corresponding treatment on days 13 to 16 (estimated period of follicular recruitment) or days 13 to 18 (follicular recruitment and selection). Treatments were as follows: corn oil (vehicle), DHT (10 mg), flutamide (F, 1.5 g, nonsteroidal AR inhibitor), DHT (10 mg) plus F (1.5 g), T (10 mg), and T (10 mg) plus F (1.5 g). Hormones and F were obtained from Sigma. Gilts were ovariectomized by mid-ventral laparotomy on day 17 or 19, 24 h after receiving their last treatment. Two experimental protocols having the same treatments and days of ovarian evaluations were conducted. All the experimental procedures involving animals were approved by the University Animal Care and Use Committee.

Experiment 1

The objectives were to determine the effects of androgens on numbers of follicles, follicular health, and concentrations of estradiol-17 β (E_2) in follicular fluid on days 17 and 19 of the estrous cycle. All evaluations were performed on follicles ≥ 5 mm in diameter. It was determined that among follicles ≥ 2 mm on day 16 (end of recruitment) most were ~4 mm or larger (Grant *et al.* 1989). We considered that follicles ≥ 4 mm represented the majority of the recruited pool and that they would be ≥ 5 mm by day 17. The group of follicles ≥ 5 mm evaluated on day 19 would include those selected for ovulation and most that were initially recruited but had become atretic.

Immediately after ovariectomy, follicles were dissected individually or in groups of two to six if clustered together. Diagrams were drawn for groups of follicles to ensure follicle identification. A total of 1469 follicles were collected. Follicular fluid (10–50 μ l depending on the size of the follicle) was obtained from each follicle using a tuberculin syringe. Follicular fluid was transferred to a 1.5 ml tube and stored at -20°C until E_2 concentrations were determined. Follicles were then fixed in 4% paraformaldehyde for 20 h at 4°C and embedded in paraffin (Paraplast Plus; Fisher Scientific, Florence, KY, USA). Tissue sections (7 μ m) were cut and attached to positively charged slides (Superfrost Plus, Fisher Scientific) for subsequent staining and morphological assessment of follicular health.

Follicular health

Tissue sections (two to three per follicle) were stained using the Mason Trichrome procedure, which included sequential staining with hematoxylin, acid fuchsin, ponceau de xylinide, and fast green (Humason 1972). Sections were examined using a light microscope (Nikon Labophot) at magnifications up to $400\times$. Follicular health was determined based on morphological integrity of the membrana granulosa, presence of granulosa cells with pyknotic nuclei, and observation of cells and/or apoptotic bodies in the follicular antrum (Maxson *et al.* 1985, Cárdenas & Pope 1994, Clark *et al.* 2004). Pyknotic nuclei appeared intensely stained, frequently compacted, and sometimes crescent in shape. Apoptotic bodies resulting from cell disintegration following apoptotic death were round or irregularly shaped structures, intensely stained, and smaller than healthy nuclei. Healthy granulosa and theca cells had light-blue or red-

blue nuclei with darker dots in the nucleoplasm and were abundant in the membranes having good morphological integrity.

Follicles were classified as H, MA, or VA. Examples of follicles within these classifications are depicted in Fig. 2. H follicles had sound membrana granulosa with none or only few isolated cells with pyknotic nuclei. MA follicles had numerous granulosa cells with pyknotic nuclei detached from the membrana granulosa. Additionally, apoptotic bodies were observed within the clusters of detached cells. Granulosas of MA follicles had partial integrity (at least three layers) but their antral border appeared rough due to cell detachment. VA follicles had nearly all their granulosa cells with pyknotic nuclei, and granulosas were completely or mostly (≤ 2 cell layers remaining) disintegrated. Consequently, in VA follicles most granulosa cells and apoptotic bodies were observed dispersed, individually or in small clusters, within the follicular antrum. Evaluations of follicular health were performed using a person unaware of treatment allocation of gilts.

RIA of E_2

Concentrations of E_2 in follicular fluid were measured using a single-antibody RIA procedure routinely employed in our laboratory for measuring E_2 in serum or plasma (Anderson *et al.* 1996). This assay was modified by increasing the range of standard concentrations (2–2000 pg/ml) and using samples

Figure 2 Sections of representative healthy (H), moderately atretic (MA), and very atretic (VA) ovarian follicles collected during the follicular phase of the estrous cycle in gilts treated with androgens and/or flutamide (F). Different degrees of morphological integrity of the granulosas (G) were important criteria for follicular classification. T, theca interna. Right bottom segment represents 100 μ m.

directly (without extraction of lipids). Displacement curves of serially diluted samples were parallel to the standard curve. Samples were diluted 1/250 or 1/500 in PBS containing 0.1% gelatin before analysis. Inter-assay coefficients of variation (CVs) of pools of follicular fluid having high or low concentrations of E_2 were 12.3 and 11.9% respectively. The average CV of duplicate determinations per sample was 10.2%. The sensitivity was estimated to be 2 pg/ml.

Experiment 2

The objective of this experiment was to evaluate the influence of androgens on amounts of the FSHR and LHR mRNAs in the tissues of H follicles. After ovariectomy, six to eight healthy follicles (pink walls and abundant capillary bed) measuring 5–6 mm on day 17, or 7–8 mm on day 19, were quickly dissected. Follicles were punctured with a needle, gently squeezed to eliminate follicular fluid, pooled, and then frozen in liquid nitrogen. Samples were frozen no later than 15 min after ovariectomy. Follicular tissues were stored at -80°C until isolation of total RNA.

Measurement of mRNAs

Total cellular RNA was isolated using TRI reagent as described by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). RNA integrity was verified by estimating the 28s to 18s rRNA ratio following agarose gel electrophoresis. RNA purity and concentration were evaluated by u.v. spectroscopy.

Relative amounts of FSHR and LHR mRNAs were determined using SYBR Green-based real-time reverse transcription PCR. RT (2 μg total RNA per 100 μl reaction) was performed using a kit and random hexamers as primers (TaqMan RT Reagents; Applied Biosystems, Foster City, CA, USA). Aliquots of the RT reactions, SYBR Green PCR master mix (Applied Biosystems), and specific primers (300 nM) were used for PCR amplification of the first-strand cDNAs for LHR, FSHR, and ribosomal protein L19 (endogenous control used as normalizer). Volume of PCRs was 25 μl and reactions were run in triplicate for each gene. The forward and reverse primers (5'–3' sequences) and size (bps) of products for FSHR, LHR, and L19 were: TCGAGGCAA-ATGTGTTCTCC, AAGGTTCTGGAAGGCATCAG, 101; TCTCC-CTATCAAAGTAATCC, GTTCTGGATCAGTATTTTCAG, 147; TACTGCCAATGCTCGAATGC, ACATGTGGCGGTCAATCTTC, 110 respectively.

PCR mixtures were incubated for 10 min at 95°C and then 40 cycles at 95°C for 15 s (DNA denaturing) and 60°C for 1 min (annealing/extension). Default incubation parameters for generation of dissociation curves were included at the end of each run. Reactions in which reverse transcriptase was replaced by water were used as negative controls. All assays were performed in 96-well plates using an ABI Prism 7700 SDS real-time PCR machine. Amounts of RNA for the target and normalizer genes were estimated by the relative standard curve method using the ABI 7500 System SDS software. cDNA for standards (1 to 1×10^{-4} relative concentrations) was synthesized using a pool of total RNA from six experimental samples. Authenticity of the PCR products was determined by sequencing. No significant differences in relative amounts of

the normalizer (L19) among treatments were detected. Single products were present in all the reactions as indicated by the dissociation curves, which was verified in some samples by agarose gel electrophoresis. Results of the amounts of FSHR and LHR mRNAs are presented as ratios to the amounts of the normalizer (L19) in the same sample.

Statistical analysis

Data were analyzed by ANOVA using general linear models of the SAS (SAS Institute, Cary, NC, USA) or SPSS (SPSS Inc., Chicago, IL, USA) software. Analyses for completely randomized two-way factorial designs were applied to follicular numbers, proportions, diameters, and also to the amounts of mRNA. Analysis of LHR mRNA was performed independently for each day because heterogeneity of variance could not be eliminated. Amounts of LHR mRNA on day 17 were analyzed by ANOVA as described above, but data for day 19 were analyzed using a nonparametric test (Kruskal–Wallis). In this case, means and standard errors of original data are presented. Repeated measurements analysis was used for the concentrations of E_2 . Means were compared using least significant difference tests. Statistical effects and differences among means were considered significant when $P < 0.05$.

Acknowledgements

This project was supported by National Research Initiative Competitive Grant no. 2003-35203-13489 from the USDA Cooperative State Research, Education, and Extension Service. Salary and research support were also provided by State and Federal funds appropriated to The Ohio Agricultural Research and Development Center and The Ohio State University. Manuscript no. 28/07AS. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Anderson LH, McDowell CM & Day ML 1996 Progesterone-induced puberty and secretion of luteinizing hormone in heifers. *Biology of Reproduction* **54** 1025–1031.
- Cárdenas H & Pope WF 1994 Administration of testosterone during the follicular phase increased the number of corpora lutea in gilts. *Journal of Animal Science* **72** 2930–2935.
- Cárdenas H & Pope WF 2002a Androgen receptor and follicle-stimulating hormone receptor in the pig ovary during the follicular phase of the estrous cycle. *Molecular Reproduction and Development* **62** 92–98.
- Cárdenas H & Pope WF 2002b Control of ovulation rate in swine. *Journal of Animal Science* **80** (Supplement 1) E36–E46.
- Cárdenas H, Herrick JR & Pope WF 2002 Increased ovulation rate in gilts treated with dihydrotestosterone. *Reproduction* **123** 527–533.
- Chun S-Y, Eisenhauer KM, Minami S, Billig H, Perlas E & Hsueh AJW 1996 Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor. *Endocrinology* **137** 1447–1456.
- Clark LJ, Irving-Rodgers HF, Dharmarajan AM & Rodgers RJ 2004 Theca interna: the other site of bovine follicular atresia. *Biology of Reproduction* **71** 1071–1078.
- Dailey RA, Clark JR, Staigmiller RB, First NL, Chapman AB & Casida LE 1976 Growth of new follicles following electrocautery in four genetic groups of swine. *Journal of Animal Science* **43** 175–183.

- Driancourt MA** 2001 Regulation of ovarian follicular dynamics in farm animals, implications for manipulation of reproduction. *Theriogenology* **55** 1211–1239.
- Evans G, Dobias M, King GJ & Armstrong DT** 1981 Estrogen, androgen, and progesterone biosynthesis by theca and granulosa of preovulatory follicles in the pig. *Biology of Reproduction* **25** 673–682.
- Foxcroft GR & Hunter MG** 1985 Basic physiology of follicular maturation in the pig. *Journal of Reproduction and Fertility Supplement* **33** 1–19.
- Grant SA, Hunter MG & Foxcroft GR** 1989 Morphological and biochemical characteristics during ovarian follicular development in the pig. *Journal of Reproduction and Fertility* **86** 171–183.
- Grino PB, Griffin JE & Wilson JD** 1990 Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. *Endocrinology* **126** 1165–1172.
- Humason GL** 1972 *Animal Tissue Techniques*, 3rd edn. San Francisco: W H Freeman and Co.
- Hunter MG, Robinson RS, Mann GE & Webb R** 2004 Endocrine and paracrine control of follicular development and ovulation rate in farm species. *Animal Reproduction Science* **82–83** 461–477.
- Jiménez E, Cárdenas H & Pope WF** 2006 Administration of androgens increased serum concentrations of FSH in gilts. *Journal of Animal Science* **84** (Supplement 2) 129.
- Knox RV** 2005 Recruitment and selection of ovarian follicles for determination of ovulation rate in the pig. *Domestic Animal Endocrinology* **29** 385–397.
- Lucy MC, Liu J, Boyd CK & Bracken CJ** 2001 Ovarian follicular growth in sows. *Reproduction* **58** 31–45.
- Luo W & Wiltbank MC** 2006 Distinct regulation by steroids of messenger RNAs for FSHR and CYP19A1 in bovine granulosa cells. *Biology of Reproduction* **75** 217–225.
- Luthy I, Caron S, Bélanger A & Labrie F** 1987 Effects of flutamide, a pure antiandrogen, on endocrine parameters, in the adult female rat. *Gynecological Endocrinology* **1** 151–168.
- Manabe N, Goto Y, Matsuda-Minehata F, Inoue N, Maeda A, Sakamaki K & Minayo T** 2004 Regulation mechanism of selective atresia in porcine follicles: regulation of granulosa cell apoptosis during atresia. *Journal of Reproduction and Development* **50** 493–514.
- Marchetti B & Labrie F** 1988 Characteristics of flutamide action on prostatic and testicular functions in the rat. *Journal of Steroid Biochemistry* **29** 691–698.
- Mariscal DV, Bergfeld EG, Cupp AS, Kojima FN, Fike KE, Sanchez T, Wehrman ME, Johnson RK, Kittok RJ, Ford JJ *et al.*** 1998 Concentrations of gonadotropins, estradiol and progesterone in sows selected on an index of ovulation rate and embryo survival. *Animal Reproduction Science* **54** 31–43.
- Markström E, Svensson EC, Shao R, Svanberg B & Billig H** 2002 Survival factors regulating apoptosis-dependence on follicular differentiation. *Reproduction* **123** 23–30.
- Maxson WS, Haney AF & Schomberg DW** 1985 Steroidogenesis in porcine atretic follicles: loss of aromatase activity in isolated granulosa and theca. *Biology of Reproduction* **33** 495–501.
- Miller AT, Picton HM, Craigon J & Hunter MG** 1998 Follicle dynamics and aromatase activity in high-ovulating Meishan sows and in Large-White hybrid contemporaries. *Biology of Reproduction* **58** 1372–1378.
- Okazaki T, Nishibori M, Yamashita Y & Shimada M** 2003 LH reduces proliferative activity of cumulus cells and accelerates GVBD of porcine oocyte. *Molecular and Cellular Endocrinology* **209** 43–50.
- Richards JS** 1994 Hormonal control of gene expression in the ovary. *Endocrine Reviews* **15** 725–751.
- Segaloff DL, Wang HY & Richards JS** 1990 Hormonal regulation of luteinizing hormone/chorionic gonadotropin receptor mRNA in rat ovarian cells during follicular development and luteinization. *Molecular Endocrinology* **4** 1856–1865.
- Shiina H, Matsumoto T, Sato T, Igarashi K, Miyamoto J, Takemasa S, Sakari M, Takada I, Nakamura T, Metzger D *et al.*** 2006 Premature ovarian failure in androgen receptor-deficient mice. *PNAS* **103** 224–229.
- Tsafriri A & Braw RH** 1984 Experimental approaches to atresia in mammals. *Oxford Reviews of Reproductive Biology* **6** 226–265.
- Weil S, Vendola K, Zhou J & Bondy CA** 1999 Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. *Journal of Clinical Endocrinology and Metabolism* **84** 2951–2956.
- Yang MY & Fortune JE** 2006 Testosterone stimulates the primary to secondary follicle transition in bovine follicles *in vitro*. *Biology of Reproduction* **75** 924–932.
- Youn H, Koo Y, Ji I & Ji TH** 2005 An upstream initiator-like element suppresses transcription of the luteinizing hormone receptor gene. *Molecular Endocrinology* **19** 1318–1328.
- Zelevnik AJ, Little-Ihrig L & Ramasawamy S** 2004 Administration of dihydrotestosterone to Rhesus monkeys inhibits gonadotropin-stimulated ovarian steroidogenesis. *Journal of Clinical Endocrinology and Metabolism* **89** 860–866.
- Zhan Y & Dufau ML** 2000 Nuclear orphan receptors regulate transcription of the gene for the human luteinizing hormone receptor. *Journal of Biological Chemistry* **275** 2763–2770.
- Zhan Y & Dufau ML** 2003 Dual mechanisms of regulation of transcription of the luteinizing hormone receptor gene by nuclear orphan receptors and histone deacetylase complexes. *Journal of Steroid Biochemistry and Molecular Biology* **85** 401–414.

Received 21 September 2007

First decision 19 October 2007

Accepted 4 December 2007