Granulosa Cells are Refractory to FSH Action in Individuals with a Low Antral Follicle Count

Running title: Variation in Antral Follicle Count Negatively Impacts FSH Action

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Abstract. The reason ovarian function and fertility are diminished in women with a low antral follicle count (AFC), despite significant numbers of follicles remaining in ovaries, is unknown. The bovine model is unique to address this question because cattle and women with a low AFC exhibit similar phenotypic characteristics including a diminished ovarian reserve, reduced circulating concentrations of anti-Müllerian hormone (AMH) but heightened FSH secretion during reproductive cycles. Because women and cattle with a low AFC respond minimally to gonadotropin stimulation during IVF cycles or superovulation, granulosa cells in individuals with a low AFC are hypothesized to be refractory to FSH. The present study evaluates this hypothesis by testing whether capacity of granulosa cells to respond to FSH differs between cattle with low versus a high AFC. Granulosa cells from cattle with low (≤15 follicles ≥ 3 mm in diameter) or a high (>25 follicles) AFC were cultured with different doses of FSH. Treatments were evaluated by measurement of estradiol (E), progesterone (P) and AMH in media and abundance of mRNAs for aromatase (CYP19A1), AMH, FSH receptor (FSHR), and oxytocin (OXT). P and OXT mRNA are well established markers of granulosa cell luteinization. Although high doses of FSH induced granulosa cell luteinization, basal and FSH-induced increases in E and AMH production and expression of mRNAs for CYP19A1, FSHR and AMH in granulosa cells were much lower while P production and OXT mRNA expression were higher in non-luteinized and luteinized granulosa cells from the Low versus High AFC Group. Granulosa cells in cattle with a low AFC are refractory to FSH action, which could explain why ovarian function, responsiveness to gonadotropin stimulation and fertility are diminished in individuals with low versus a high AFC.
Introduction

Antral follicle count (AFC) is positively associated with responsiveness to gonadotropin stimulation during IVF cycles in women (Aflatoonian et al. 2009; Hsu et al. 2011). Thus, AFC is not only a useful index of potential responsiveness of women to gonadotropin stimulation, but the inherently high variation in AFC among women (Popovic-Todorovic et al. 2003; Kailasam et al. 2004) may also have a key, albeit unexamined role in regulation of ovarian responsiveness to gonadotropin stimulation.

Because of the high emotional (Verhaak et al. 2007) and financial (e.g., ~$15,771 per treatment cycle and ~$55,450 per live birth in the U.S. (Connolly et al. 2010)) expenditures associated with each unsuccessful IVF cycle (Connolly et al. 2010), better methods are needed to predict and improve responsiveness of patients to gonadotropin stimulation to enhance success of IVF cycles.

We have taken advantage of the bovine model to determine if a physiologically important link exists between the high variation in AFC and ovarian function for several reasons. Cattle are a single-ovulating species that have two or three well-characterized FSH-induced waves of growth and atresia of antral follicles during their reproductive cycles (Ginther et al. 1996) similar to women (Baerwald et al. 2003). Moreover, serial ovarian ultrasonography of cattle shows that peak number of antral follicles (> 3 mm in diameter) growing during each follicular wave, hereafter referred to as AFC, is very highly repeatable within individuals (0.85 to 0.95, 1 = perfect), despite the high variability among animals (Burns et al. 2005). For example, some cattle have as few as 8 follicles growing during each follicular wave of an estrous cycle while others have 56 (Burns et al. 2005; Ireland et al. 2007). Moreover, we have also demonstrated that the
high variation in AFC is positively associated with the total number of morphologically healthy follicles and oocytes in ovaries of cattle, and young adult cattle with a relatively low AFC (≤15 follicles) have an 80% smaller ovarian reserve than cattle with a higher AFC (>25 follicles) (Ireland et al. 2008). Thus, the bovine model is unique because individuals can be phenotyped reliably based on AFC and correspondingly size of the ovarian reserve (see review by (Ireland et al. 2011)).

Recent studies show that remarkable differences exist in pituitary-ovarian function between young adult nulliparous cattle with a consistently relatively low (≤15 follicle) versus a high (≥25 follicles) AFC (Ireland et al. 2011). For example, quantity of morphologically healthy oocytes and follicles (Ireland et al. 2008), circulating concentrations of anti-Müllerian hormone (AMH) (Ireland et al. 2008), progesterone (Jimenez-Krassel et al. 2009) and androgens (Mossa et al. 2010b), responsiveness to superovulation (Ireland et al. 2007), and number of transferable embryos and in vitro blastocyst development (Ireland et al. 2007) are much lower while circulating FSH (Burns et al. 2005; Ireland et al. 2007; Mossa et al. 2010b) concentrations are chronically higher in young adult cattle with low versus a high AFC. Fertility is also diminished in cows with low versus a higher AFC (Cushman et al. 2009; Mossa et al. 2010a). Many of these same phenotypic differences between young adult cattle with low versus a high AFC are also reported for older, less fertile versus younger women (Block 1953; Richardson et al. 1987; Miller 2001; Scheffer et al. 2003) or cattle (Erickson 1966; Cushman et al. 1999; Wolfenson et al. 2004; Malhi et al. 2005), young women with high versus normal circulating FSH concentrations (El-Toukhy et al. 2002), and young women born small versus normal size for gestational age (Ibanez et al. 2003). Consequently,
these similarities between cattle and women with low compared with a higher AFC strongly support the utility of the bovine model to identify and examine the potential mechanisms whereby the high variation in AFC may negatively impact ovarian function, gonadotropin responsiveness and fertility.

Because of the poor responsiveness of women (Melo et al. 2009; Hsu et al. 2011) and cattle (Ireland et al. 2007) with a low AFC to gonadotropin stimulation, the capacity of granulosa cells to respond to FSH is hypothesized to be diminished in individuals with a low compared with a higher AFC. To test this hypothesis, the bovine model was used in the present study to determine if FSH action on granulosa cells, as measured by alterations in a variety of biomarkers for FSH action (estradiol, progesterone, and mRNAs for aromatase, FSH receptor, AMH and oxytocin), differed between individuals with low versus a high AFC.

Materials and Methods

*Long-Term Serum-Free Bovine Granulosa Cell Culture*

Pairs of ovaries from individual non-pregnant Bos taurus beef and dairy cows (approximately 2 to 8 years old) were obtained at random stages of the estrous cycle (based on presence of a corpus luteum) from JBS Packing Company Inc. (Plainwell, MI) and classified into a high or low AFC group based on the total number of antral follicles ≥ 3 mm in diameter per pair of ovaries for each animal: High = ≥ 25 antral follicles; Low = ≤ 15 follicles. Each pair of ovaries was placed in ice-cold supplemented Dulbecco's phosphate buffered saline solution (DPBS) and transported to the laboratory.

Approximately 8 to 10, 3 to 5 mm follicles (without blood in follicular fluid) of unknown
health status (e.g, non-atretic or atretic) were dissected from each pair of ovaries in the Low and High AFC Groups, and each AFC Group contained follicles from 3 to 5 cows. Each follicle was washed with 75% ethanol for 3-5 seconds and placed into serum-free MEM-α culture media. The dissected follicles were punctured with a needle to drain follicular fluid and split with scissors. Granulosa cells were then removed from each follicle piece with a spatula and pooled into the Low or High AFC Group separately in a 15-ml centrifuge tube containing MEM-α culture media supplemented with sodium bicarbonate (10 mM), HEPES (20 mM), antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin), Fungizone-amphotericin B (0.625 µl/ml), nonessential amino acids (1.1 mM), bovine insulin (1 ng/ml), long R3-IGF-I (2 ng/ml), sodium selenite (4 ng/ml), apo-transferrin (5 µg/ml), and androstenedione (10⁻⁶ M). Each pool of granulosa cells from the Low or High AFC Group was washed with culture media three times and resuspended in 2 ml media. Cell number was estimated by a Coulter Counter Particle Z1 (Beckman Coulter, Inc., Fullerton, CA) while cell viability was estimated using Trypan Blue dye exclusion dye (Invitrogen; (Jimenez-Krassel et al. 2002)). Cells (50,000 live cells per well) from each pool were plated in 96-well Falcon Primaria plates and cultured at 37°C in a humidified atmosphere (5% CO₂ and 95% air). During serum-free culture, 75% of media was removed and replaced with fresh media on days 2 and 4 of culture, and cultures were terminated after 6 days of culture. All hormone, growth factor and mRNA measurements were made on Day 6 of culture. Each experiment used a single pool of granulosa cells from 8 to 10, 3 to 5 mm follicles per pair of ovaries obtained from a total of 3 to 5 cows (or 3 to 5 pairs of ovaries) in the Low or High AFC Group. During each experiment, 6 culture wells were utilized for each treatment in the High and Low AFC
Group. Prior to hormone analyses, media samples from 2 culture wells were combined within each treatment and each combined sample measured in duplicate. Prior to mRNA analyses, granulosa cells from all 6 wells per treatment were combined and the combined sample measured in duplicate. Each experiment was repeated 3 times on 3 different days.

Estradiol, Progesterone and AMH Assays

Commercial RIA kits (Diagnostic Products Corp., Los Angeles, CA) previously validated by our laboratory (Jimenez-Krassel et al. 2003; Kobayashi et al. 2004; Jimenez-Krassel et al. 2009) were used to measure concentrations of estradiol and progesterone in each sample of spent media. Estradiol assay sensitivity was 0.5 pg/ml and progesterone assay sensitivity was 0.05 ng/ml (Sen et al. 2007; Jimenez-Krassel et al. 2009). Intra- and inter-assay coefficients of variation for both assays were < 10%.

A commercial human MIS/AMH ELISA kit (DSL-10-14400, Beckman Coulter, Inc., Brea, CA), which was validated for use in cattle (Ireland et al. 2008), was used to measure AMH concentrations in each sample of spent media per kit instructions. The two-site AMH assay does not cross-react with other members of the TGFβ superfamily including TGFβ, BMP4, or activin (Kevenaar et al. 2006). The inter-assay and intra-assay coefficients of variation were < 6%. AMH concentrations in different volumes of spent media collected from granulosa cells treated with 0 or 25 ng/ml ovine FSH (oFSH) were parallel with the AMH standard curve, and AMH concentrations were undetectable in media without cells (data not shown).
Messenger RNA Analyses

Total RNA was isolated from granulosa cells using the RNeasy mini kit (Qiagen, Valencia, CA) per kit instructions. RNA was treated with DNase to remove genomic DNA contamination and reverse transcribed (Bettegowda et al. 2006). Expression of all genes was analyzed by real-time quantitative PCR (Bettegowda et al. 2007). Primers were designed using either Primer Express (Applied Biosystems, Foster City, CA) or PerlPrimer (Marshall 2004) for bovine sequences in Genbank, and the amplicon sizes ranged from 73 to 269 bp (Table 1). Copies of CYP19A1, FSHR, AMH, and OXT mRNA were quantified using the standard curve method for absolute quantification (Li et al. 2000; Whelan et al. 2003). The mean mRNA abundance for each target gene was normalized against level of the constitutive housekeeping gene, beta-actin (β-actin), which is unchanged in the present study (data not shown) and during antral follicle development in the bovine (Ireland et al. 1994). Data were expressed as copies of target gene per 10,000 copies of ACTB.

Study 1: Effect of FSH on Estradiol and AMH Production and Abundance of mRNAs for CYP19A1, AMH, and FSHR in Granulosa Cells

To determine if FSH action on granulosa cells differed between cattle with low versus a high AFC, granulosa cells from both AFC groups were treated with 0, 0.01, 0.05, 0.1, 0.5, 1, 10, and 25 ng/ml oFSH (AFP7558C; National Hormone and Pituitary Program, Baltimore, MD) for 6 days. To terminate cultures, 75% of the media was removed from wells and stored at -20º C until measurement of estradiol and AMH. Wells were washed with 150 µl DPBS twice and incubated with trypsin-EDTA.
(0.125mg/well). Cells were then removed from each well by trituration, and numbers of cells determined using the Coulter counter. In an additional set of wells, granulosa cells were lysed after media removal and pooled, total RNA was isolated from pools of granulosa cells, and abundance of mRNAs for CYP19A1, AMH, and FSHR determined.

**Study 2: Effect of FSH on Progesterone Production and Abundance of OXT mRNA**

Previous studies (Wrathall et al. 1993; Berndtson et al. 1995) report that relatively high doses of FSH increase progesterone production and abundance of OXT mRNA, which are well established biomarkers of luteinization (Murphy 2000) during culture of bovine granulosa cells. Therefore, to determine if the doses of FSH used in the present study potentially induced luteinization of granulosa cells, which could confound interpretation of results, progesterone concentrations and abundance of OXT mRNA were measured in media and RNA obtained from Study 1.

**Statistics**

All statistical analyses were performed using Statistical Analysis System (SAS 9.1 Institute, Cary, NC). Responsiveness of pools of granulosa cells from cows with Low or a High AFC to different doses of FSH were analyzed statistically using multivariate ANOVA. Main effects included follicle class (Low or High AFC) and FSH dose (0, 0.01, 0.05, 0.1, 0.5, 1, 10, 25 ng/ml). However, the effect of different doses of FSH was usually biphasic with relatively low FSH doses either increasing or not altering while higher doses increased or decreased amounts of many of the biomarkers of FSH action (estradiol, progesterone, AMH and mRNAs for CYP19A1, AMH, FSHR and OXT).
Thus, if the overall ANOVA was significant ($P < 0.05$), linear regression analysis (SAS 2004) was used to determine if a significant ($P < 0.05$) linear increase or decrease in the FSH biomarker occurred before or after the FSH dose resulting in the peak response to FSH (usually 0.1 or 0.5 ng/ml). Tukey-Kramer test was used to determine if a significant ($P < 0.05$) difference existed between means (SAS 2004). Data were log transformed when necessary to meet the assumptions of normality, but non-transformed means ($\pm$SEM) are reported for all studies.

Results

**Study 1: Effect of FSH on Estradiol and AMH Production and Abundance of mRNAs for CYP19A1, FSHR and AMH in Granulosa Cells in Individuals with Low Versus a High AFC**

After 6 days of culture, the basal capacity of untreated granulosa cells (0 FSH dose) to produce estradiol was 53% greater and the average capacity of granulosa cells for all FSH doses combined (0 to 25 ng/ml) to produce estradiol and express CYP19A1 mRNA was 4.3- and 7.8-fold greater, respectively, in the High versus the Low AFC Group (Fig. 1). In the High AFC Group, peak estradiol production and expression of CYP19A1 mRNA in granulosa cells occurred in response to the 0.5 ng/ml dose of FSH, and doses of FSH from 0 to 0.5 ng/ml increased whereas FSH doses > 0.5 ng/ml decreased estradiol production and expression of CYP19A1 mRNA in a linear dose response fashion (Fig. 1). In contrast, in the Low AFC Group, peak estradiol production and expression of CYP19A1 mRNA occurred at the 0.1 ng/ml FSH dose, and doses of
FSH from 0 to 0.1 ng/ml increased whereas FSH doses > 0.1 ng/ml decreased estradiol production and expression of CYP19A1 mRNA in a linear fashion (Fig. 1).

The basal expression of FSHR mRNA in untreated granulosa cells was ~3-fold greater and the average expression of FSHR mRNA for all FSH doses combined was 3.6-fold greater in the High versus the Low AFC Group (Fig. 2). The peak expression of FSHR mRNA in granulosa cells occurred in response to the 0.1 ng/ml dose of FSH for both AFC Groups (Fig. 2). Although doses of FSH from 0 to 0.1 ng/ml did not alter (P > 0.16) expression of FSHR mRNA in either AFC Group, FSH doses > 0.1 ng/ml decreased (P ≤ 0.05) expression of FSHR mRNAs in a linear fashion in both AFC Groups (Fig. 2).

The basal capacity of untreated granulosa cells to produce AMH was ~2-fold greater and the average concentration of AMH and expression of AMH mRNA for all FSH doses combined were 3- and ~4-fold greater, respectively, in the High versus the Low AFC Group (Fig. 3). In both AFC Groups, peak AMH production by granulosa cells occurred in response to the 0.1 ng/ml dose of FSH, and FSH doses from 0 to 0.1 ng/ml increased whereas FSH doses > 0.1 ng/ml decreased AMH production in a linear fashion (Fig. 3). For the High AFC Group, the peak expression of AMH mRNA in granulosa cells occurred in response to the 0.5 ng/ml dose of FSH, and FSH doses from 0 to 0.5 ng/ml increased whereas FSH doses > 0.5 ng/ml decreased expression of AMH mRNA in a linear fashion (Fig 3, lower panel). In contrast, for the Low AFC Group, the peak expression of AMH mRNA was at the 0.05 ng/ml dose of FSH; and, although FSH doses from 0 to 0.05 ng/ml did not alter AMH mRNA expression, FSH doses > 0.05 ng/ml decreased expression of AMH mRNA in a linear fashion (Fig. 3, lower panel).
Study 2: Effect of FSH on Progesterone Production and Abundance of OXT mRNA in Individuals with Low Versus a High AFC

In contrast to estradiol production, which was much greater for granulosa cells in the High versus the Low AFC Group, basal capacity of untreated granulosa cells to produce progesterone was ~2-fold greater and the average progesterone production by granulosa cells for all FSH doses combined was 45% greater in the Low versus the High AFC Group (Fig. 4). Although FSH doses from 0 to 0.1 ng/ml increased estradiol and AMH production (Figs. 1, 2), they had no effect on progesterone production (Fig. 4). However, FSH doses from 0.5 to 25 ng/ml increased progesterone production in a linear fashion in both AFC Groups (Fig. 4) while simultaneously decreasing estradiol and AMH production and expression of CYP19A1, FSHR and AMH mRNAs (Figs. 1-3).

The average expression of OXT mRNA for all FSH doses combined was 24% greater in Low versus the High AFC Group (Fig. 4, lower panel). Similar to progesterone production, expression of OXT mRNA was unaltered in response to FSH doses from 0 to 0.1 ng/ml in both AFC Groups. Although alterations in OXT mRNA expression were highly variable in response to FSH doses from 0.5 to 25 ng/ml, the average for OXT mRNA expression in granulosa cells treated with doses of FSH from 0.5 to 25 ng/ml combined was greater than the average for OXT mRNA expression for FSH doses from 0 to 0.1 ng/ml combined (Fig. 4, lower panel).

Discussion
The most significant finding of the present study indicates that granulosa cells are refractory to FSH action in cattle with low versus a high AFC. This observation provides new insight into the potential mechanisms involved in ovarian dysfunction in individuals with a low AFC because it implies that the diminished ovarian function (Ireland et al. 2011), poor responsiveness to gonadotropin stimulation (Aflatoonian et al. 2009; Hsu et al. 2011), and reduced fertility (Pöhl et al. 2000; Nahum et al. 2001; Kupesic et al. 2002; Durmusoglu et al. 2004; Frattarelli et al. 2004; Muttukrishna et al. 2005; Cushman et al. 2009; Mossa et al. 2010a) in cattle and women with a low AFC may be linked to an inherently diminished capacity of granulosa cells to respond to FSH stimulation.

Determination of the precise mechanisms that cause granulosa cells in cattle with a low AFC to become refractory to FSH stimulation was beyond the scope of this study. Nevertheless, results of the present in vitro study, coupled with our previous in vivo results using the bovine model and results of others, imply that chronically enhanced FSH secretion negatively impacts gonadotropin responsiveness resulting in ovarian dysfunction in cattle with a relatively low AFC (Ireland et al. 2011). It is well established that FSH receptors are exclusive to granulosa cells in females (Hunzicker-Dunn et al. 2006), and FSH is required for development of antral follicles (Richards et al. 1976; Kumar et al. 1997) including the occurrence of follicular waves during estrous cycles in cattle (Turzillo et al. 1993). However, FSH secretion is inversely associated with the high variation in AFC and ovarian function in young adult cattle (Ireland et al. 2011) and chronically heightened in older cattle (Malhi et al. 2005) and women (Reame et al. 1998; Welt et al. 1999) with a reduced ovarian reserve and suboptimal fertility compared with their younger counterparts. Moreover, relatively high FSH concentrations
in the presence of insulin in rodents (Eppig et al. 1998), heightened secretion of FSH in transgenic rodents (McTavish et al. 2007), and superovulation of cattle (Lonergan et al. 1994; Blondin et al. 1996) diminish developmental competence of oocytes and fertility. Taken together, these findings imply that chronically heightened FSH secretion may have a long-term negative impact on ovarian function and fertility in individuals with a relatively low AFC.

Granulosa cells from the high and low AFC cattle responded in a dose response fashion to FSH stimulation, but granulosa cells from the Low AFC Group responded minimally to FSH and produced much lower amounts of estradiol and AMH, and CYP19A1 and AMH mRNA compared with cells in the High AFC Group in the present study. Although number of FSH receptors was not determined in the present study, the overall abundance of FSH receptor mRNA was also much lower in granulosa cells of cattle with low versus a high AFC. Thus, the refractoriness of granulosa cells in the Low AFC Group to FSH action could be explained by a reduced number of FSH receptors. In addition, high gonadotropin concentrations desensitize or uncouple gonadotropin receptors from their respective signaling systems not only in granulosa cells, but also in thecal and luteal cells (Conti et al. 1976; Amsterdam et al. 2002), which in turn may hinder ovarian function. Thus, the chronically heightened FSH and LH secretion, such as that observed for cattle (Ireland et al. 2011) and women (Haadsma et al. 2007; Jayaprakasan et al.) with a low AFC, may potentially desensitize and therefore disrupt the gonadotropin signaling cascade in granulosa, thecal, and luteal cells. This possibility could explain why expression of the FSHR and FSH-induced expression of CYP19A1 mRNA, which are well established FSH target genes (Hunzicker-Dunn et al. 2006), and
estradiol production, the hallmark of fully functional FSH signaling cascade (Hunzicker-Dunn et al. 2006), were markedly diminished in granulosa cells of cattle with low versus a high AFC in the present study. Moreover, an inherently dysfunctional gonadotropin signaling cascade caused by chronically heightened gonadotropin secretion could also explain why responsiveness of luteinized granulosa (Jimenez-Krassel et al. 2009), thecal (Mossa et al. 2010b) and luteal cells (Jimenez-Krassel et al. 2009) to LH, circulating progesterone (Jimenez-Krassel et al. 2009), testosterone (Mossa et al. 2010b), and AMH (Ireland et al. 2008) concentrations during estrous cycles, responsiveness to superovulation (Ireland et al. 2007), and intrafollicular concentrations of androstenedione and estradiol in ovulatory follicles (Mossa et al. 2010b) are greatly diminished in cattle with low versus a high AFC. Although it is unknown if granulosa, thecal or luteal cells are refractory to gonadotropin stimulation in women with a low AFC, granulosa cells obtained from the largest follicles of women that respond poorly to ovarian stimulation protocols also have a reduced expression of FSHR mRNA compared with higher responders (Cai et al. 2007).

The present in vitro studies also showed that responsiveness of granulosa cells to FSH was biphasic, and that relatively high doses of FSH trigger alterations in differentiation of granulosa cells resembling luteinization. In support of these results, previous studies using bovine granulosa cells also show that high doses of FSH increase progesterone and oxytocin production (Wrathall et al. 1993; Berndtson et al. 1995) and high doses of FSH induce ovulation in rodents (Hsueh et al. 1988). The suppression in estradiol production and expression of CYP19A1 and FSHR mRNAs, coupled with the simultaneous increase in progesterone production and abundance of OXT mRNA,
observed in the present study are well established hallmarks of luteinization in bovine
granulosa cells (Murphy 2000) and for other species including humans (Khan-Dawood et
al. 1998). Moreover, AMH expression is limited or undetectable in corpora lutea
compared with non-luteinized follicles in rats, horses, and women (Baarends et al. 1995;
Meduri et al. 2007; Ball et al. 2008). This finding implies that the decrease in AMH
production following treatment with relatively high doses of FSH observed in the present
study may have been caused by FSH-induced luteinization of granulosa cells. Taken
together, in addition to a low AFC and diminished ovarian reserve, another potential
explanation for the refractoriness of individuals with a low AFC to gonadotropin
stimulation is the possibility that the chronically high gonadotropin secretion, such as that
observed in cattle with a low AFC (Burns et al. 2005; Jimenez-Krassel et al. 2009),
coupled with the relatively high doses of FSH used during superovulation protocols
(Lonergan et al. 1994; Blondin et al. 1996), may initiate significant alterations in
differentiation of granulosa cells similar to the early stages of luteinization, as observed
in granulosa cells treated with relatively high FSH doses in the present study.

The effects of FSH on AMH production are controversial. For example, FSH
treatment decreases circulating AMH concentrations in women (Eldar-Geva et al. 2005;
Dumesic et al. 2009) and inhibits AMH mRNA expression in rats (Baarends et al. 1995).
In contrast, results of studies using luteinized granulosa cells from humans show that
FSH does not alter expression of AMH mRNA (Voutilainen et al. 1987) or AMH
production (Pellatt et al. 2007). While the precise reason for the differences in FSH
action on AMH production between studies is unknown, several explanations are
plausible. For example, the decrease in circulating AMH concentrations following FSH
treatments in vivo (Eldar-Geva et al. 2005; Dumesic et al. 2009) could have been caused by the negative impact of potentially high physiological or pharmacological doses of FSH during ovarian stimulation on granulosa cell production of AMH as observed in the present study. In addition, capacity of granulosa cells to produce AMH in response to FSH may depend not only on follicle type (e.g. small antral or dominant, (Baarends et al. 1995; Weenen et al. 2004) used as a source of granulosa cells for culture studies, but also whether the FSH dose initiates granulosa cell luteinization as observed in the present study.

In conclusion, granulosa cells in cattle with a low AFC have an inherently diminished capacity to respond to FSH, which could contribute to or cause the decrease in ovarian function, responsiveness to gonadotropin stimulation and fertility in individuals with a low AFC. These observations potentially explain why cattle with a low AFC respond poorly to gonadotropin stimulation (Ireland et al. 2007), despite significant numbers of follicles remaining in a relatively depleted ovarian reserve and growing during follicular waves (Ireland et al. 2008), and also why relatively high FSH doses for example during IVF cycles in women (Kailasam et al. 2004; Klinkert et al. 2005; Verberg et al. 2009) or superovulation in cattle (Blondin et al. 1996; Ireland et al. 2007), could initiate luteinization of granulosa cells and potentially be detrimental to ovarian function.
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**FIGURE LEGENDS**

*Fig. 1. Effect of FSH on estradiol production and abundance of CYP19A1 mRNA in granulosa cells from cattle with low versus a high AFC*

Granulosa cells were treated with various doses of FSH for 6 days. Estradiol production (top panel) and abundance of CYP19A1 mRNA (bottom panel) were determined on Day 6 of culture in the High and Low AFC Group. For the top panel, bars represent the overall mean ± SEM for the mean estradiol concentrations in media for 3 pools of granulosa cells from 3 to 5 cows per pool. The mean estradiol value for each pool was generated after measurement of 3 replicate media samples per pool. Each replicate sample contained media from 2 culture wells combined. For the bottom panel, bars represent the mean ± SEM for abundance of CYP19A1 mRNA in granulosa cells in 3 pools of granulosa cells from 3 to 5 cows per pool. Each pool contained mRNA isolated from granulosa cells from 6 culture wells combined. Results of ANOVA indicated that overall concentration of estradiol (P < 0.001) and abundance of CYP19A1 (P < 0.001) mRNA for all doses of FSH (0 to 25 ng/ml) combined were higher for granulosa cells from the High versus the Low AFC Group. The A above bars at the 0 FSH dose indicates a significant (P<0.01) difference between means. The asterisks above bars indicate that estradiol production or abundance of CYP19A1 mRNA increased (P < 0.001) linearly in response to FSH doses from 0 to 0.1 ng/ml or 0 to 0.5 ng/ml in the Low or High AFC Group, respectively. The plus symbol above bars indicates that estradiol production or abundance of CYP19A1 mRNA decreased (P < 0.05) linearly in response to FSH doses > 0.1 ng/ml or > 0.5 ng/ml for the Low or High AFC Group, respectively.
Fig. 2. Effect of FSH on abundance of FSHR mRNA in granulosa cells from cattle with high versus a low AFC

Granulosa cells were treated with various doses of FSH for 6 days. Abundance of FSHR mRNA was measured on Day 6 of culture in the High and Low AFC Groups. Each bar represents the mean ± SEM for abundance of FSH mRNA in 3 pools of granulosa cells from 3 to 5 cows per pool. Each pool contained mRNA isolated from granulosa cells from 6 culture wells combined. Results of ANOVA indicated that overall abundance of FSHR mRNA for all FSH doses (0 to 25 ng/ml) combined was higher (P < 0.001) in the High versus the Low AFC Group. The above bars at the 0 FSH dose indicates a significant (P<0.05) difference between means. The plus symbol above bar indicates that FSHR mRNA abundance decreased (P ≤ 0.05) linearly in response to doses of FSH > 0.1 ng/ml.

Fig. 3. Effect of FSH on AMH production and abundance of AMH mRNA by granulosa cells from cattle with high versus a low AFC

Granulosa cells were treated with various doses of FSH for 6 days. AMH production (top panel) and abundance of AMH mRNA (bottom panel) were measured on Day 6 of culture in the High and Low AFC Groups. Bars represent the mean ± SEM for 3 pools of granulosa cells from 3 to 5 cows per pool. Each pool contained media or cells from 6 culture wells combined. Results of ANOVA indicated that overall AMH concentrations and abundance of AMH mRNA for all FSH doses (0 to 25 ng/ml) combined were higher (P < 0.001) for the High versus the Low AFC Group. In the top panel, the above bars at the 0 FSH dose indicates a significant (P<0.01) difference between means. In addition, the asterisk above bars indicates that AMH production increased (P < 0.001) linearly in response to FSH doses from 0 to 0.1 ng/ml. The plus symbol
indicates that AMH production decreased (P<0.05) linearly in response to doses of FSH > 0.1 ng/ml. In the bottom panel, the asterisk above bars in the High AFC Group indicates that abundance of AMH mRNA increased (P < 0.05) linearly in response to FSH doses from 0 to 0.5 ng/ml whereas the plus symbol above bar indicates that AMH mRNA abundance decreased (P < 0.05) linearly in response to FSH doses > 0.5 ng/ml. Also, in the Low AFC Group, the plus symbol indicated that AMH mRNA decreased (P < 0.05) linearly in response to FSH doses > 0.05 ng/ml.

Fig. 4. Effect of FSH on progesterone production and abundance of OXT mRNA in granulosa cells from cattle with high versus a low AFC

Granulosa cells were treated with various doses of FSH for 6 days. Progesterone production (top panel) and abundance of OXT mRNA (bottom panel) were measured on Day 6 of culture in the High and Low AFC Groups. For the top panel, bars represent the overall mean ± SEM for the mean progesterone concentrations in media for 3 pools of granulosa cells from 3 to 5 cows per pool. The mean progesterone value for each pool was generated after measurement of 3 replicate media samples per pool. Each replicate sample contained media from 2 culture wells combined. For the bottom panel, bars represent the mean ± SEM for abundance of OXT mRNA in granulosa cells in 3 pools of granulosa cells from 3 to 5 cows per pool. Each pool contained mRNA isolated from granulosa cells from 6 culture wells combined. Results of ANOVA indicated that overall concentration of progesterone and abundance of OXT mRNA for all FSH doses (0 to 25 ng/ml) combined were higher (P < 0.001) for granulosa cells from the Low versus the High AFC Group. In the top panel, the A above bars for the 0 FSH dose indicates a significant (P<0.01) difference between means. In addition, the asterisk above
bar indicates that progesterone production increased (P<0.001) linearly in response to FSH doses from 0.5 to 25 ng/ml in the High and Low AFC Groups. In the bottom panel, the average OXT mRNA abundance at FSH doses from 0.5 to 25 ng/ml combined was greater (P<0.05) than the average for OXT mRNA abundance at FSH doses 0 to 0.1 ng/ml combined.
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