Effect of Bovine Follicular Fluid and Follicle-Stimulating Hormone on Follicular Growth in Unilaterally Ovariectomized Prepuberal Heifers

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ABSTRACT

A study was conducted to determine if charcoal-extracted follicular fluid inhibits FSH-induced follicular development in prepuberal heifers. Thirty-six prepuberal heifers were allotted by breed and weight to a $2 \times 2$ factorial experiment involving charcoal-extracted follicular fluid and FSH treatments. Heifers were unilaterally ovariectomized and injected (intravenously; 10 ml) every 8 h for 88 h with either charcoal-extracted follicular fluid or saline. Follicle-stimulating hormone (2 mg) or saline was injected (intramuscularly) every 8-h starting 24 h after initiation of charcoal-extracted follicular fluid to 88 h following unilateral ovariectomy. Plasma samples were collected at 8-h intervals from 48 h prior to unilateral ovariectomy to 96 h following unilateral ovariectomy when the remaining ovary was removed. Follicular fluid and total ovarian weight increased following FSH treatment. The increases were not inhibited by charcoal-extracted follicular fluid or saline. Plasma concentration of FSH, but not LH, declined following charcoal-extracted follicular fluid administration. In summary, charcoal-extracted follicular fluid did not inhibit FSH-induced follicular development in prepuberal heifers when charcoal-extracted follicular fluid was administered at a dosage that reduced circulating concentration of FSH by approximately 40%.

INTRODUCTION

Folliculogenesis is regulated by extraovarian and intraovarian mechanisms. Recent attention has focused on follicular secretion of non-steroidal factors that may be involved in intraovarian, interfollicular, and intrafollicular communication (22, 26). One of these factors is inhibin, which selectively blocks the release of FSH from the anterior pituitary (7). Injection of charcoal-extracted follicular fluid (CFF) inhibits follicular maturation and delays estrus in cattle (15), sheep (20, 21), pigs (24), and horses (1). Administration of CFF selectively suppresses FSH in ovariectomized heifers (13) and blocks both compensatory ovarian hypertrophy (COH) and the transient rise in FSH associated with COH in unilaterally ovariectomized (ULO) prepuberal heifers (16) and gilts (24). Whether the suppression in follicular growth following CFF treatment of ULO animals is due to a selective inhibition of FSH (inhibin) or to a direct effect on the ovary is unclear since follicular fluid (FF) also contains a number of low molecular weight proteins (nonsteroidal factors) that may regulate a variety of follicular events (26). Several of these may affect both secretion of FSH and the responsiveness of the ovary to FSH (6, 18).

The objective of the study was to determine if bovine CFF inhibits follicular development in ULO prepuberal heifers. In addition, the direct action of CFF on the ovary was investi-
gated in ULO heifers treated with FSH. The prepuberal ULO model was selected since it had been successfully used in heifers (16) and gilts (24) to demonstrate CFF inhibition of COH and follicular growth.

MATERIALS AND METHODS

General

Thirty-six prepuberal beef heifers were allotted by breed (Angus, Hereford, Charolais, and Simmental) and weight (X ± SD, 207 ± 23 kg) to four treatment groups: saline-saline (saline; n = 9), CFF-saline (CFF; n = 9), saline-FSH (FSH; n = 9) and CFF-FSH (n = 8; one animal was removed due to ovulation as determined by presence of corpora lutea). All animals were ovarioctomized unilaterally at time 0. Heifers received injections (i.v.; 10 ml) of saline or CFF and injections (i.m.; 1 ml) of saline or FSH (2.0 mg; Armour Standard) at 8-h intervals to 88 h following ULO. Injections of CFF or saline were initiated immediately following ULO, and injections of FSH or saline began 24 h later. The injections of FSH were delayed 24 h relative to injections of CFF to assure exposure of the ovary to CFF prior to FSH stimulation. The remaining ovary was removed 96 h following ULO. Removal of the right or left ovary in each ULO treatment was determined randomly prior to ULO. FSH was of porcine origin (Burns-Biotech Lab, Inc., Omaha, NE, Lot #592J84) and had biological LH activity of 1.9%, as determined by a rat interstitial cell bioassay (8).

Preparation of Charcoal-Extracted Follicular Fluid

Follicular fluid was obtained by aspirating bovine follicles (<20 mm in diameter) collected at a local abattoir. The FF was kept on ice until centrifugation at 1700 × g for 1 h to remove remaining charcoal fragments. The CFF was frozen (−20°C) until used in the experiment. In addition, negative results were obtained when samples of CFF were cultured for the presence or absence of Serratia liquifaciens bacteria present in porcine FF that has been shown to secrete an FSH-B1 (28). Therefore, collection and processing of the FF was sufficient to prevent or remove the S. liquifaciens contamination. Concentrations of estradiol-17β [E2 (17)] and progesterone [P; (4)] in the pooled CFF preparation were 248 and 348 ng/ml prior to extraction and .08 and 47 ng/ml after extraction, respectively.

Blood Collection and Ovariectomy

Blood samples were collected every 8 h via jugular venipuncture into 20-ml heparinized tubes beginning 48 h prior to ULO and continuing until removal of the second ovary (96 h after ULO). Plasma samples were collected before injections and stored at −20°C until concentrations of FSH (11), LH (30), and E2 (17) were measured by radioimmunoassay. Intraassay coefficients of variation for FSH, LH, and E2 were 3.5, 5.0, and 7.1%, respectively. Interassay coefficients of variation were 13.0, 6.0, and 15.6% for FSH, LH, and E2, respectively.

Heifers were tranquilized with 10 mg of acepromazine maleate and locally anesthetized with 50 ml of 2% lidocaine hydrochloride. Ovaries were removed using an ecrasseur via a paralumbar incision using aseptic technique. After removal, ovaries were kept on ice and transported to the laboratory. Ovaries were weighed and diameter of surface follicles measured (to the nearest millimeter) using hand calipers. Follicular fluid from individual follicles measuring 5 mm or greater was aspirated. Follicular fluid from follicles <5 mm was aspirated and pooled by ovary. Estradiol-17β and P concentrations in FF were measured, as previously described, as well as the concentration of testosterone [T; (9)].

After aspiration of FF from individual follicles, ovaries were sectioned into .5-mm slices using a hand microtome, blotted dry, and reweighed to determine FF weight (total ovarian weight - blotted dry weight). Ovarian slices were lyophylized and reweighed.
Figure 1. Change in follicular fluid weights (mean ± SE) between the ovary removed at unilateral ovariectomy and the ovary removed 4 d after treatment from heifers administered saline, charcoal-extracted bovine follicular fluid (CFF), FSH, or CFF-FSH.

Statistical Analysis

The main linear statistical model was a completely randomized design in which treatments were arranged factorially (2 x 2) containing CFF and FSH as the main effects and their interaction. Using this model, two analyses were made depending upon whether data were single or repeated measurements. Single measurements included total ovarian weight, follicular fluid weight, lyophilized ovarian weight, and follicular fluid steroid concentrations. Because variances among treatments were not homogeneous, all ovarian measurements were analyzed after appropriate transformation of the original data (29). Repeated measurements of plasma concentrations of FSH, LH, and E2 were analyzed as a split-plot in time design (12). The main plot for plasma FSH, LH, and E2 was the same as the ANOVA for ovarian traits. The subplot contained the effect of time and the interaction of time with the main plot effect. The effect of heifer within CFF and FSH treatment was used as an error term to test the main plot effect. Mean separation was performed using Fisher’s least significant difference procedure (29). Concentrations of steroids in FF were analyzed as a 2 x 2 arrangement as previously described for ovarian traits, except that FF steroids in 10- to 13-mm follicles were analyzed as a one-way ANOVA comparing saline, FSH, and CFF-FSH treatments because numbers of 10- to 13-mm follicles in CFF-treated animals were not sufficient for comparison. Variances among concentrations of FF steroids in follicles were not homogeneous; therefore, log10 transformation of data was used (29). All analyses were performed using least squares procedures for unequal subclass numbers (25).

RESULTS

Ovarian Measurements

Compensatory Ovarian Hypertrophy. To examine effects of COH as well as treatment effects, comparison of ovarian measurements (total ovarian weight, follicular fluid weight, and lyophilized ovarian weight) was made between the first ovary removed prior to treatment and the second ovary removed at termination of the study. Follicular fluid weight of the second ovary increased 11% in saline-treated heifers and decreased 14% in CFF-treated heifers relative to the first ovary (nonsignificant; Figure 1). Total ovarian weight and follicular fluid weight were increased (P<.05) in FSH and CFF-FSH groups. The failure to observe a significant difference in COH may be expected due to the short interval (4 d) between the removal of the first and second ovary. It has been well-established that COH occurs in prepuberal (16) and puberal (19) heifers and prepuberal gilts (24) when the time interval from removal of the first ovary to removal of the second ovary is 7 to 13 d. Significant COH likely would have been observed in this study if a similar time period had been used. Trends observed in saline-treated controls (11% increase in follicular fluid weight) indicated the phenomenon was occurring. Since our objective was to examine size and number of follicles, we were unable to use a longer time period from beginning of FSH treatment to removal of the second ovary because estrus and ovulation would have occurred in FSH-treated animals.
Ovarian Measurements. In the remainder of the manuscript, comparison of treatment effects on ovarian measurements are based upon data from the second ovary. Follicular fluid and total ovarian weights were increased \( (P<.05) \) by FSH but not CFF treatment (Figure 2), and the CFF-FSH interaction was not significant. Neither FSH nor CFF affected lyophilized ovarian weight (Figure 2).

Although total surface follicular numbers were similar among groups, FSH caused a shift from small to large follicles (Figure 3). Heifers treated with FSH (FSH and CFF-FSH) had more \( (P<.05) \) follicles 7 to 9 mm and 10 to 13 mm in diameter and fewer \( (P<.05) \) follicles \( \leq 3 \) mm in diameter than heifers not receiving FSH (saline and CFF). The CFF treatment did not inhibit FSH-induced follicular growth. Exogenous FSH treatment stimulated follicular growth in approximately 50% of the prepuberal heifers treated \( (n=17) \). Ovarian response to FSH was similar between groups receiving FSH \( (5 \text{ to } 9) \) alone or in combination with CFF \( (5 \text{ of } 8) \).

Endocrine Measurements

Plasma Hormones. Mean plasma concentrations of FSH, LH, and \( E_2 \) were similar among groups prior to ULO. Plasma concentration of FSH decreased \( (P<.05) \) more than 40% and was lower \( (P<.05) \) the first 24 h following CFF treatment than that following saline treatment (Figure 4). Circulating FSH remained low throughout the experimental period for heifers receiving only CFF. Plasma concentration of FSH increased \( (P<.05) \) following initiation of FSH treatment 24 h after ULO but did not change in saline-treated heifers. The increase in plasma FSH in heifers receiving exogenous FSH may have in part been due to the cross-reactivity \( (3\%) \) of the bovine FSH antisera with porcine FSH.

Plasma \( E_2 \) concentrations were low among treatment groups and there was no interaction between main effects. However, plasma \( E_2 \) tended to increase \( (P<.10) \) in heifers that received FSH \( (2.4 \pm 0.35 \text{ pg/ml}) \) compared with those not receiving FSH \( (1.4 \pm 0.25 \text{ pg/ml}) \).

Follicular Fluid Steroids. Treatment with FSH \( (P<.05) \), but not CFF, affected \( E_2 \) concentration but not P and T in FF of small \((<5 \text{ mm})\) follicles. Intrafollicular concentration of \( E_2 \) was greater \( (P<.05) \) in small follicles of animals that received FSH (FSH and CFF-FSH groups) than those that did not (saline and CFF groups; Table 1). Concentrations of \( E_2 \) and P but not T in medium follicles \((5 \text{ to } 6 \text{ and } 7 \text{ to } 9 \text{ mm}) \) were affected \( (P<.05) \) by both FSH and CFF and the FSH-CFF interaction was significant \( (P<.05) \). Intrafollicular concentration of \( E_2 \) was greater \( (P<.05) \) and P lower \( (P<.05) \) in medium follicles \((5 \text{ to } 6 \text{ and } 7 \text{ to } 9 \text{ mm}) \) of heifers given FSH (FSH and CFF-FSH treatment; Table 1). In addition, mean concentration of \( E_2 \) in medium follicles \((5 \text{ to } 6 \text{ and } 7 \text{ to } 9 \text{ mm}) \) from the CFF only group was considerably \( (P<.05) \) lower and progesterone was higher \( (P<.05) \) than that of the saline group (however, there was only an \( n=2 \) for the CFF group). Because large follicles were of insufficient quantity in CFF-treated animals, main effects of FSH and CFF could not be examined. Comparison of saline, FSH, and CFF-FSH-treated animals revealed no differences in intrafollicular \( E_2, P, \)
and T in large follicles (10 to 13 mm; data not shown).

**DISCUSSION**

Charcoal-extracted follicular fluid treatment during the preovulatory period delays the onset of estrus in ewes (20, 21) and heifers (15). Because CFF specifically inhibits FSH secretion, the delayed interval to estrus was presumably due to reduced concentration of FSH in plasma or an inability of FSH to stimulate folliculogenesis.

In this study, administration of CFF in combination with FSH did not inhibit the ability of exogenous FSH to stimulate follicular growth, since FF weight, surface diameter, numbers of follicles, circulating concentrations of E₂ and intrafollicular steroid profiles were not different between animals that received FSH alone or in combination with CFF. In addition, McNeilly (21) demonstrated that estrus was not delayed when bovine CFF was given in combination with FSH (50 μ g/h for 48 h) during the preovulatory period in ewes.

**Figure 3.** Numbers of follicles per ovary (least squares means) 96 h following unilateral ovariectomy of prepuberal heifers administered saline (control; n = 9), charcoal-extracted bovine follicular fluid (CFF; n = 9), FSH (n = 9), or CFF-FSH (n = 8).
Follicular fluid contains substances that might act in the ovaries to inhibit FSH stimulation of follicular cells. Previous studies (6, 27) have demonstrated that bovine FF contains a nonsteroidal substance(s) (FSH-BI) that inhibits FSH binding to granulosal cells in culture. There are both high and low molecular weight FSH-BI. The concentration of low molecular weight FSH-BI has been correlated positively with the relative degree of follicular atresia (27). This suggests that low molecular weight FSH-BI may be related to the physiological state of the follicle and thus modulate follicular responsiveness under physiological conditions. Other nonsteroidal factors that may have a direct effect on the ovary have been identified in FF. These factors include mitotic inhibitor [MI; (5)] and follicle regulatory protein [FRP; (18)].
TABLE 1. Intrafollicular concentrations (ng/ml) of estradiol-17β (E2), progesterone (P), and testosterone (T) in surface follicles less than 5, 6 to 6 and 7 to 9 mm in diameter from unilaterally ovariectomized prepuberal heifers treated with charcoal-extracted, bovine follicular fluid (CFF), FSH, or CFF-FSH.

<table>
<thead>
<tr>
<th>Follicle size</th>
<th>Treatment</th>
<th>E2</th>
<th>SE</th>
<th>P</th>
<th>SE</th>
<th>T</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>X</td>
<td>SE</td>
<td>X</td>
<td>SE</td>
<td>X</td>
<td>SE</td>
</tr>
<tr>
<td>&lt;5 mm (pooled/animal)³</td>
<td>Saline (n = 5²)</td>
<td>1b</td>
<td>.4</td>
<td>108</td>
<td>54</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>CFF (n = 8)</td>
<td>1b</td>
<td>.3</td>
<td>81</td>
<td>31</td>
<td>31</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>FSH (n = 4)</td>
<td>24a</td>
<td>9</td>
<td>77</td>
<td>56</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>CFF-FSH (n = 4)</td>
<td>28a</td>
<td>9</td>
<td>45</td>
<td>33</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>5 to 6 mm³,4,5</td>
<td>Saline (n = 5)</td>
<td>2b</td>
<td>1</td>
<td>52b</td>
<td>29</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>CFF (n = 2)</td>
<td>1c</td>
<td>0</td>
<td>232a</td>
<td>138</td>
<td>1</td>
<td>.4</td>
</tr>
<tr>
<td></td>
<td>FSH (n = 4)</td>
<td>66a</td>
<td>15</td>
<td>54b</td>
<td>24</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>CFF-FSH (n = 4)</td>
<td>28a</td>
<td>4</td>
<td>13b</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>7 to 9 mm³,4,5</td>
<td>Saline (n = 11)</td>
<td>19</td>
<td>4b</td>
<td>78b</td>
<td>53</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CFF (n = 2)</td>
<td>.2c</td>
<td>2</td>
<td>723a</td>
<td>92</td>
<td>.3</td>
<td>.2</td>
</tr>
<tr>
<td></td>
<td>FSH (n = 25)</td>
<td>69a</td>
<td>15</td>
<td>67b</td>
<td>16</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CFF-FSH (n = 24)</td>
<td>41a</td>
<td>5</td>
<td>34b</td>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

a,b,c Means within a follicle size in a column with different superscripts differ (P<.05; analysis performed on log₁₀ transformed data).

1 Values reported as means ± SE of original data.
2 n = Minimum number of follicles used in determination of intrafollicular steroid concentration.
3 Main treatment effect of FSH (P<.05).
4 Main treatment effect of CFF (P<.05).
5 Interaction of CFF and FSH (P<.05).

Although CFF is a crude preparation, purified preparations were and are unavailable for large scale in vivo studies using livestock. Although bovine CFF in the present study with cattle and others with sheep (21) did not block the FSH-induced follicular growth, ovine CFF has been shown to inhibit pregnant mare serum gonadotropin (PMSG)-induced ovarian growth in ewes (2, 3). Conversely, results of McNeilly (21) and the present study suggest that bovine CFF does not inhibit ovarian development in FSH-stimulated ewes and heifers.

Although an FSH-BI has been demonstrated in vitro in bovine FF, several factors may have prevented exogenously administered FF from having a direct effect on the ovary. Intrafollicular concentrations of FSH-BI or any other nonsteroidal factor may not have been increased sufficiently to exert ovarian action by peripheral injections of FF. However, similar results were obtained in a subsequent study (23) in which a greater quantity of CFF (20 ml) was administered.

The biological activity of the CFF is not in question since inhibin-like activity was present as evidenced by the selective depression of plasma FSH but not LH. Contamination of the FSH preparation with LH may mask effects of CFF on FSH-induced follicular growth. However, results were similar in a subsequent study in which a purified FSH preparation was used (<1% LH; (23)). In addition, species differences may exist between sheep and cattle, as direct ovarian action was demonstrated in studies using ovine CFF (2, 3) but not bovine CFF (21, 23) (current study).

In the present study, CFF in the absence of exogenous FSH depressed the circulating concentration of FSH without altering that of LH. This selective effect by CFF on FSH agrees with other studies in heifers (16) and gilts (24). The increase in concentration of LH following FSH treatment in the current study (Figure 3) was likely due to LH contamination (1.9%) of exogenous FSH or indirect effects of FSH and not a direct stimulation of LH by FSH, since no difference in LH (B. Flowers, personal communication) or FSH (10) concentrations were observed within 48 h following administration of PMSG to gilts. The increase in plasma FSH following exogenous porcine FSH treatment is
thought to have resulted from the 3% cross-reactivity of porcine FSH in the bovine FSH radioimmunoassay used. There is the possibility of an endogenous release of FSH; however, this cannot be determined from this study.

Heifers treated with FSH had increased total ovarian and follicular fluid weights but no change in total numbers of follicles. Treatment with FSH did not affect lyophylized ovarian weight, suggesting that the increase in ovarian weight was due to an increase in follicular fluid and not stromal tissue. Thus, FSH shifted the diameter of antral surface follicles from small (≤3 mm) to larger (7 to 9 mm and 10 to 13 mm) follicles. The slight elevation in plasma concentration of E₂ following FSH was probably due to the FSH-induced increase in follicular growth resulting from the increased production of E₂ by granulosal cells of larger follicles. Decreases in follicular fluid concentration of E₂ in medium follicles (5 to 6 mm, 7 to 9 mm) in heifers that received only CFF but not FSH injections may be a result of low number of follicles in the CFF group (n = 2). However, if this observation is true, it may be explained by increased atresia, possibly due to a decrease in circulating FSH concentration or a direct ovarian action by a substance similar to FRP. Follicle regulatory protein is a factor that has been isolated from human and porcine FF, which inhibits aromatase activity of rat and human granulosal cells (18). Further support for increased atresia following CFF treatment is the elevation in FF concentrations of P in medium (5 to 6 mm and 7 to 9 mm) follicles in the CFF group; a characteristic of atretic bovine follicles (14). The similarity in E₂ concentration observed in both medium (5 to 6, 7 to 9 mm) and large follicles (10 to 13 mm) as well as follicular populations between heifers that received CFF and FSH when compared with those that received only FSH would substantiate the conclusion that CFF-induced atresia is caused by suppression of FSH release from the pituitary. Further support for this conclusion is the finding that concentration of P in medium follicles from ovaries of heifers given CFF in combination with FSH were similar to those receiving FSH alone. Thus, treatment with FSH in an exogenous source resulted in stimulated follicular growth even when given in combination with CFF.

Treatment with FSH singly or in combination with CFF elevated intrafollicular E₂ in both small (<5 mm) and medium (5 to 6, 7 to 9 mm) follicles. Thus, FSH apparently increased follicular growth by stimulating small follicles to grow or by rescuing large follicles from atresia. Because CFF was given 24 h prior to FSH, previous exposure of follicles to CFF before FSH may have initiated atresia. Our study suggests an increase in follicular growth induced by FSH, and possibly a rescue from atresia in small and medium follicles following exogenous FSH treatment.

In summary, FSH-induced enhancement of antral follicular development is characterized by a shift in follicular size but not number. The FSH-induced follicular growth was not inhibited by a dosage of bovine CFF that reduced circulating concentrations of FSH by 40%. This study suggests that the action(s) of CFF on follicular growth (21, 27) are mediated by decreased circulating FSH and not due to a localized action in the ovaries.

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