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# Concentrations of Insulin-Like Growth Factor-I in Blood and Ovarian Follicular Fluid of Cattle Selected for Twins[1]

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## ABSTRACT

Recent studies have implicated insulin-like growth factor I (IGF-I) as an intraovarian regulator of follicular growth and differentiation. Therefore, we investigated the possibility that cattle selected for twin births may have increased concentrations of IGF-I within the ovarian follicle and(or) in peripheral blood. The estrous cycles of 14 cows with histories of producing twins and 12 control monotocous cows were synchronized with 35 mg of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Blood and follicular fluid were collected 48–50 h post-administration of $PGF_{2\alpha}$ (follicular phase of the estrous cycle). Concentrations of IGF-I were measured by RIA after acid-ethanol treatment of serum or follicular fluid. Twin-producing cows had a greater ($p < 0.05$) number of large (≥4 mm) follicles and 47% greater ($p < 0.05$) concentrations of IGF-I in peripheral blood than control cows. Cattle selected for high twinning frequency also had greater ($p < 0.05$) concentrations of IGF-I (±SE) in the two largest follicles than control (unselected) cows (327 ± 28 vs. 243 ± 29 ng/ml). IGF-I concentrations in pooled small (1–3.9 mm) follicles were less ($p < 0.05$) than in large follicles but did not differ between control and twin-producing cattle. In addition, the percentage of IGF-I concentrations measured in follicular fluid to that of serum was lower ($p < 0.05$) in small follicles than in large follicles, and was greater ($p < 0.05$) in large follicles of control (93.2 ± 5.3%) than twin-producing (76.2 ± 4.4%) cattle. Moreover, concentrations of IGF-I in serum and follicular fluid were correlated positively ($r = 0.69$, $p < 0.01$). Concentrations of estradiol and progesterone in follicular fluid of small or large follicles did not differ between control and twin-producing cattle. Collectively, the present study provides evidence suggesting that natural twinning in cattle is associated with increased concentrations of IGF-I in both blood and follicular fluid. These data are consistent with the hypothesis that IGF-I plays a role in the regulation of folliculogenesis and is a mediator of a genetic component of multiple ovulations in cattle.

## INTRODUCTION

Insulin-like growth factor-I (IGF-I) has been postulated to be one of several intraovarian regulators of ovarian follicular growth and differentiation (for reviews, see Adashi et al., 1985; Hammond et al., 1988b). Specifically, in vitro studies have established that ovarian granulosa cells can secrete IGF-I and that IGF-I is stimulatory to granulosa cell mitosis and steroidogenesis (Adashi et al., 1985; Hammond et al., 1988b; Schams et al., 1988; Spicer et al., 1988). Although IGF-I concentrations in follicular fluid increase with follicular size (Spicer et al., 1988; Hammond et al., 1988a) and follicular fluid concentrations of IGF-I and progesterone are positively correlated across individual follicles (Spicer et al., 1988), no evidence exists to support the idea that levels of IGF-I in blood may regulate follicular growth and differentiation in cattle.

Because concentrations of IGF-I in peripheral plasma may be determined genetically within several mammalian species (Buonomo et al., 1987; Blair et al., 1988), we investigated the possibility that cattle selected for natural twinning may have increased IGF-I concentrations in the ovarian follicle and(or) blood. To this end, we measured concentrations of IGF-I, progesterone, and estradiol ($E_2$) in follicular fluid, and concentrations of IGF-I and progesterone in serum collected simultaneously from cattle selected and unselected for the natural production of dizygotic twin births (Echternkamp et al., 1985).

## MATERIALS AND METHODS

### *Animals*

The purebred and crossbred Simmental and Charolais cows used in the present study were acquired from two cattle populations at the Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, Nebraska. The 14 twin-producing cows (average parity = 6.8) were obtained from a population of cattle selected for the natural occurrence of twin ovulations and births (Echternkamp et al., 1985; Echternkamp and Gregory, 1987) and had produced one or more sets of dizygotic twins with a combined twinning frequency of 29.8%. The 12 contemporary monotocous cows (control cows) were acquired from another population of cattle at the Research Center subjected to the same management protocols and environmental conditions; twinning

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frequency for the entire control population was 3.7%. Control cows (average parity = 6.2) were selected to match individual twin-producing cows for breed, age, parity, and body size and condition (6 or greater body condition score). All cows were cyclic and six or more months postpartum; calves were weaned at least three months before cows were slaughtered. Approximately 90 days before slaughter, cows were moved to small pastures and fed 8.9 kg dry matter (DM)/day of a diet composed of 45.3% corn silage and 53.6% alfalfa silage (DM basis), supplemented with calcium, phosphorous, vitamins, and trace minerals to provide 100% of NRC nutritional recommendations (NRC, 1984) for a 650-kg, nonlactating beef cow, resulting in a positive gain in body weight. Cows were fed daily, with the last feeding occurring 16–17 h before slaughter. The two populations of cows were housed together for 30 days before slaughter.

### *Sample Collection*

Two groups of control (n = 12) and twin-producing (n = 14) cattle, exhibiting regular estrous cycles, were synchronized with a single injection (i.m.) of 35 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse, Upjohn Co., Kalamazoo, MI) during the midluteal phase between 11 August 1987 and 19 April 1988. Ovaries were collected immediately at slaughter, 48–50 h after $PGF_{2\alpha}$ injection, via a midventral incision. The diameters of all follicles ≥4 mm on the ovarian surface were recorded and their follicular fluid was collected individually. Follicular fluid from all follicles 1–3.9 mm in diameter was collected and pooled within each cow. Blood samples were collected at time of $PGF_{2\alpha}$ injection and immediately before slaughter via jugular venipuncture to assess concentrations of progesterone (to verify luteal regression) and IGF-I in serum/plasma. Blood serum/plasma (hereafter referred to as serum) and follicular fluid were stored at −20°C until concentrations of $E_2$ and/or progesterone were quantified by RIA as described previously (Spicer et al., 1988).

### *IGF-I RIA*

Immunoreactive IGF-I in follicular fluid and serum was quantified by RIA after an acid-ethanol extraction procedure described previously (Spicer et al., 1988), except that a new antibody (UBK487; National Hormone and Pituitary Program, Baltimore, MD) was used. Intra- and interassay coefficients of variation were 4.6% and 16.3%, respectively. Briefly, aliquots of follicular fluid or serum were diluted 1:4 with 87.5% acidic ethanol (0.25 N HCl final concentration) and incubated for 16 h at 4°C. The samples were then centrifuged for 30 min at 1 200 × *g* at 4°C and neutralized with 0.855 M Tris. This extraction procedure resulted in parallelism between the IGF-I standard (Amgen, Thousand Oaks, CA) and bovine serum, plasma, and follicular fluid (Fig. 1). Recovery of IGF-I standard added to assay tubes containing extracted follicular fluid was excellent (Fig. 2).

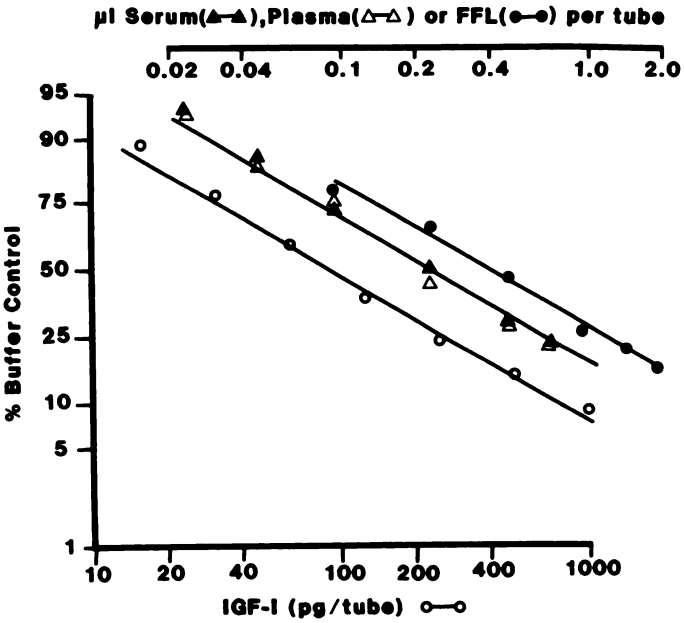


FIG. 1. Competition of acid-ethanol-treated bovine serum, plasma, and follicular fluid (*FFL*) in the IGF-I RIA. A pool of serum and plasma (collected simultaneously from 8 cyclic cattle) and follicular fluid (>10-mm follicles collected from cyclic cattle) were treated with acid-ethanol as described in *Materials and Methods*. Increasing volumes of these extracts were lyophylized and reconstituted in assay buffer and expressed in microliter follicular fluid equivalents added per tube as percentage of total binding (Buffer Control) of [$^{125}$I]iodo hIGF-I (Amgen Biologicals, Thousand Oaks, CA) on a log-logit plot. Displacement curve for authentic hIGF-I (Amgen Biologicals) is also shown.

In addition, recovery of the IGF-I standard added to follicular fluid before extraction was examined relative to duration of acid-ethanol exposure (Fig. 3). Incubation of follicular fluid with acid-ethanol for >16 h at 4°C or >4 h at

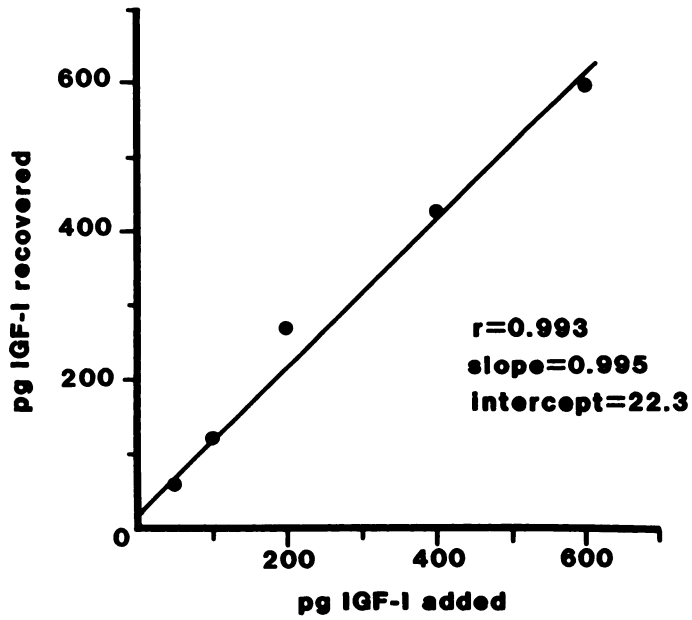


FIG. 2. Recovery of varying amounts of IGF-I (Amgen Biologicals) added to a constant volume of follicular fluid using the RIA for IGF-I.

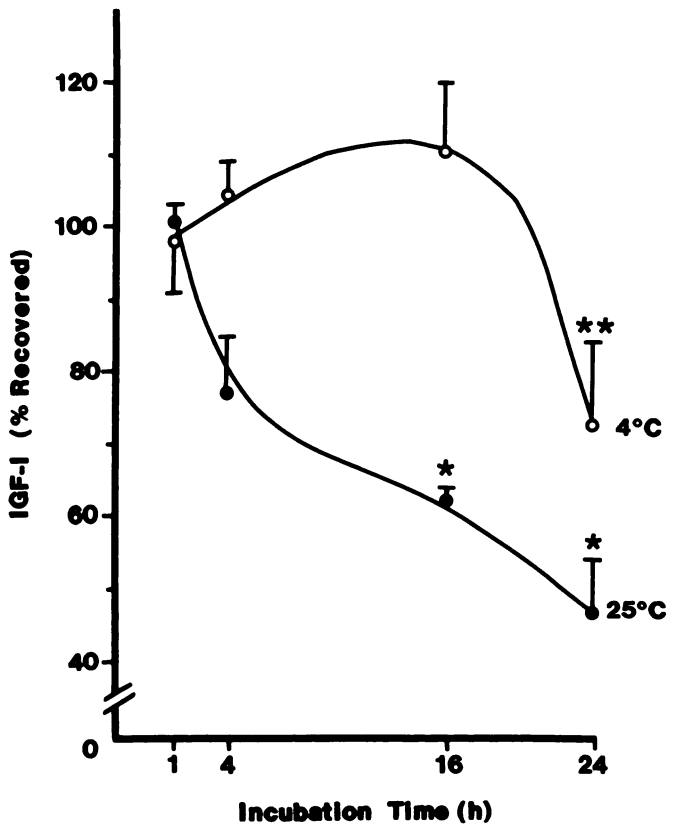


FIG. 3. Effect of incubation time and temperature during acid-ethanol extraction on recovery of IGF-I (Amgen Biologicals) added to a constant volume of follicular fluid. Two hundred or 400 ng/ml equivalent of IGF-I (Amgen Biologicals) was added in duplicate to 100 μl of bovine follicular fluid, incubated for 30 min at 25°C to equilibrate, and extracted with acid-ethanol at the indicated times and temperatures (4°C, o—o; 25°C, •—•). Each point is the average of the 200 and 400 ng/ml value expressed as percentages recovered. $*p < 0.05$ vs. 1 h at 25°C. $**p < 0.05$ vs. 16 h at 4°C.

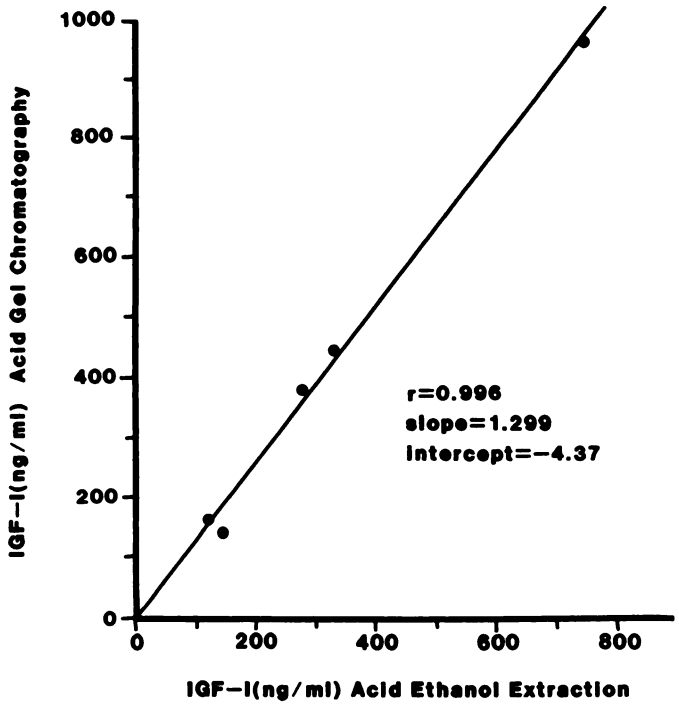


FIG. 4. Comparison of results obtained by acid-ethanol extraction and chromatography in 1 M acetic acid. Bovine follicular fluid samples were incubated for 16 h at 4°C in acid-ethanol or 1 M acetic acid. After incubation, samples in acid-ethanol were then run in the IGF-I RIA as described in *Materials and Methods*. Samples were separated on a Sephadex G-75 (46 cm × 1.6 cm) column, fractions were neutralized, and IGF-I peak was quantified in the IGF-I RIA.

25°C caused significant losses in recoverable IGF-I (Fig. 3). Also, results obtained by acid-ethanol extraction (16 h at 4°C) were highly correlated with those derived from samples chromatographed in 1 M acetic acid on a 46-cm gel filtration column (Sephadex G-75, Sigma Chemical Co., St. Louis, MO) but were 22% lower than gel filtration values (Fig. 4). A typical elution profile of immunoreactive IGF-I in bovine follicular fluid using 1 M acetic acid is presented in Figure 5. The acid-ethanol treatment (16 h at 4°C) also removes over 95% of the binding protein activity present in bovine follicular fluid and serum (Fig. 6).

### *Statistical Analysis*

Follicular fluid hormone data were grouped into two sizes (1–3.9 mm or ≥4 mm) for control and twin-producing cattle and subjected to Least-Squares analysis of variance (SAS, 1988) with "animal group" and "size of follicle" as main effects. Concentrations of IGF-I in blood and number and diameter of follicles were subjected to Least-Squares analysis of variance with "animal group" as main effect. Rela-

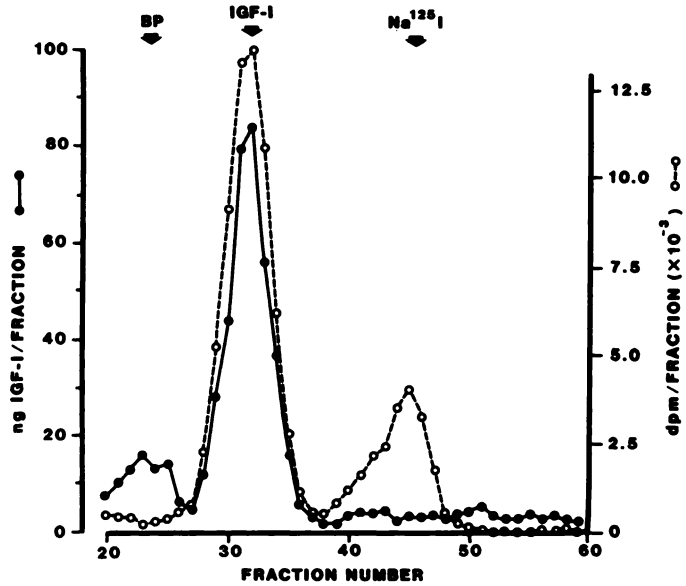


FIG. 5. Elution profile of IGF-I from follicular fluid as determined by RIA after gel filtration chromatography as described in *Figure 4*. Elution profile of an aliquot of [$^{125}$I]iodo-IGF-I (Amgen Biologicals) added to the follicular fluid sample is also given (o—o). *BP* = binding protein peak. The relative elution volume for BP and IGF-I was 1.32 ± 0.03 and 1.86 ± 0.03, respectively (n = 8).

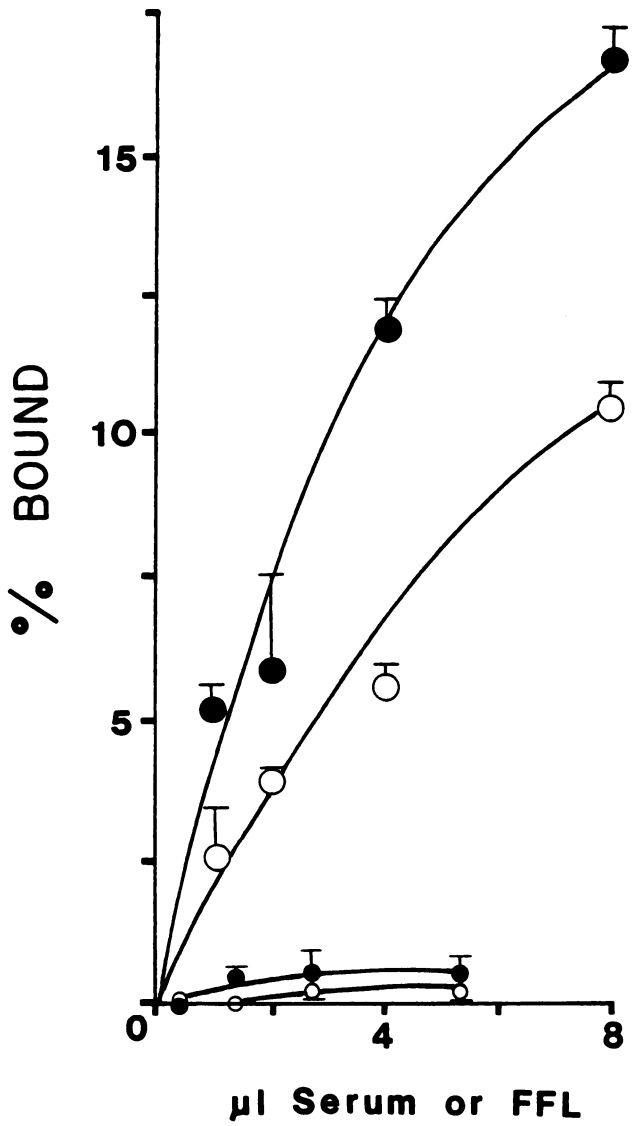


FIG. 6. Binding protein activity in bovine serum (o—o) and follicular fluid (•—•, *FFL*) before and after acid-ethanol treatment. Binding protein activity was determined by incubation with $^{125}$I-labeled IGF-I by the method of Moses et al. (1979). *Large circles* represent unextracted serum or FFL, and small circles represent extracted serum or FFL. Each point is the mean ±SE of duplicate determinations. The serum and FFL samples were a pool from six cows.

tionships among the variables measured were evaluated by regression and simple correlation (Pearson correlation coefficients) analysis (SAS, 1988).

## RESULTS

*Numbers and size of follicles.* The number of large follicles (≥4 mm in diameter) per pair of ovaries was greater ($p < 0.05$) in twin-producing than in control cows (2.3 ± 0.3 vs. 1.6 ± 0.2, respectively). Average diameter of large (≥4 mm) follicles did not differ ($p > 0.10$) between control and twin-producing cows (12.9 ± 1.0 vs. 11.2 ± 1.0 mm, respectively). In addition, diameter of the largest (16.1 ± 0.9 vs. 16.6 ± 1.3 mm, respectively) and second largest (6.4 ± 0.9 vs. 8.3 ± 1.0 mm, respectively) follicle did not differ ($p > 0.10$) between control (n = 12) and twin-producing (n = 14) cows.

*Concentrations of IGF-I in serum and follicular fluid.* Twin-producing cows (n = 14) had 47% greater ($p < 0.05$) concentrations of IGF-I in serum (collected 48–50 h post-PGF$_{2\alpha}$ injection) than control cows (n = 12) (436 vs. 297 ng/ml) (Fig. 7). Results for serum and plasma IGF-I were pooled in Figure 7, since we found that IGF-I concentrations did not differ in serum and plasma samples collected simultaneously from the same animal (see Fig. 1).

Twin-producing cows also had 43% greater ($p < 0.05$) concentrations of IGF-I in the two largest follicles than control cows (327 vs. 243 ng/ml) (Fig. 7). Concentrations of IGF-I in pooled samples of small (<4 mm) follicles were similar in twin-producing and control cows (238 ± 38 vs. 177 ± 43 ng/ml, respectively). The percentage of concentration of follicular fluid IGF-I:serum IGF-I averaged 74.4 ± 3.1% for all follicles sampled, and was lower ($p < 0.01$) in small follicles than in large follicles (Fig. 8). A significantly lower percentage of follicular fluid:serum IGF-I was found in large follicles (≥4 mm) but not small follicles of twin-producing (76.2%) than in control (93.2%) cows (Fig. 8). Also, concentrations of IGF-I in serum versus follicular fluid of all follicles ≥4 mm were correlated positively ($r$ = 0.691, $p < 0.001$).

*Follicular fluid steroids.* Concentrations of $E_2$ or progesterone in small and large follicles or the two largest follicles did not differ between control and twin-producing cows (Table 1). Pooled across populations and follicle-size groups, the percentage of follicular fluid:serum IGF-I was correlated with follicular fluid $E_2$ concentrations ($r$ = 0.280),

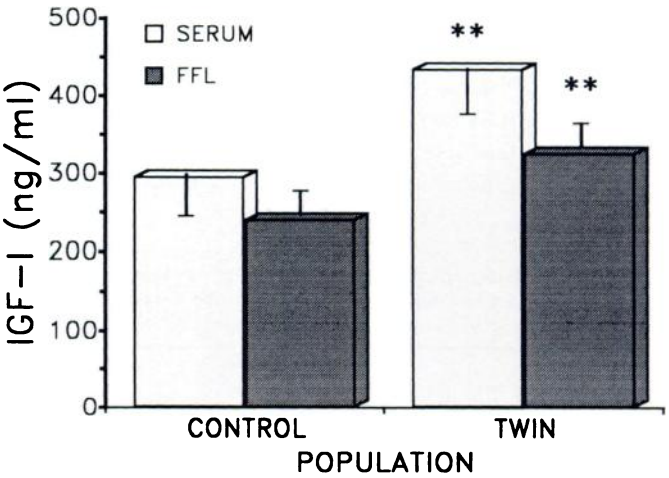


FIG. 7. Concentrations of IGF-I in serum and follicular fluid (*FFL*) of control and twin-producing (TWIN) cattle. Six plasma and 8 serum samples from 14 twin-producing cows, and 6 plasma and 6 serum samples from 12 control cows were collected 48–50 h post-PGF$_{2\alpha}$ administration and IGF-I concentrations were determined as described in *Materials and Methods*. Concentrations of IGF-I in follicular fluid of the two largest follicles were determined. ** Means differ from their respective control means ($p < 0.05$).

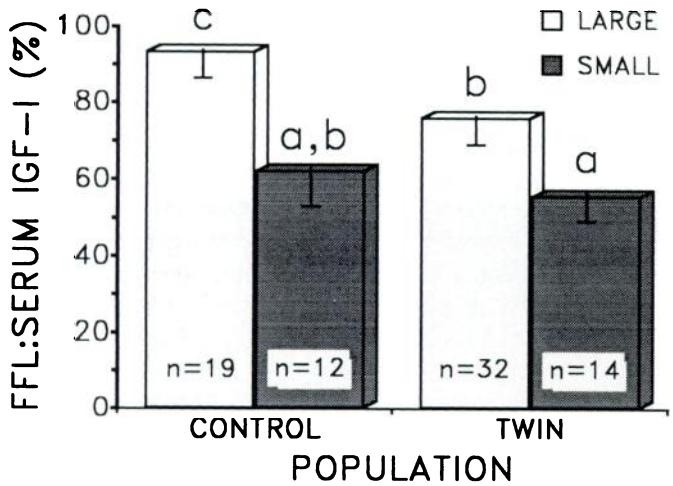


FIG. 8. Average percentage of the concentration of IGF-I in follicular fluid (*FFL*): serum for small (<4 mm) and large (≥4 mm) follicles of control and twin-producing (TWIN) cows. The number of samples incorporated into each mean is inside the *bar*. [abc] Means with different superscripts differ ($p < 0.05$).

$p < 0.05$) but not progesterone concentrations ($r = 0.185$, $p > 0.10$). Follicular fluid concentrations of IGF-I and $E_2$ were correlated ($r = 0.269, p < 0.05$), but IGF-I and progesterone were not correlated ($r = 0.103, p > .20$). For large follicles (≥4 mm) pooled across populations, diameter of follicles was significantly correlated to concentrations of IGF-I ($r = 0.448, p < 0.001$) and $E_2$ ($r = 0.471, p < 0.001$) in follicular fluid but not to progesterone ($r = -0.174, p > 0.20$).

## DISCUSSION

The rapid accumulation of information on the insulin-like growth factors (e.g., IGF-I) has revealed a diversity of biological roles and sources for these tissue regulators (Daughaday, 1982; Underwood et al., 1986; Zapf and Froesch, 1986). Identification of the biological sources and mechanisms of the IGFs has been complicated or misinterpreted by discrepancies in quantitative assay procedures among laboratories. In the present study, IGF-I measurements were affected quantitatively by both extraction temperature and time. Based on our findings, the acid-ethanol extraction of IGF-I from bovine serum at 25°C for 24 h as reported previously (Brier et al., 1986; Elasser et al., 1989) would have reduced IGF-I concentrations approximately 54%, resulting in an underestimation of actual IGF-I concentrations.

Twin-producing cows used in the present experiment had a history of producing dizygotic twin calves at a frequency more than eight times greater than control cows of the same age and breeds (Echternkamp et al., 1985; Echternkamp and Gregory, 1987). Although breed differences in twinning frequency in cattle have been documented (Rutledge, 1975), physiological mechanisms (endocrine or otherwise) for genetic regulation of ovulation rate, and subsequently of twinning rate, are unknown. The significantly greater concentrations of IGF-I in serum and follicular fluid of cattle with a twinning record in the present study provide the first evidence for a link between IGF-I production and fecundity. Indirect support for such a relationship is the historical occurrence of the highest frequency of twin births in cattle breeds of larger body size and increased growth rate. Similarly, we have found a positive genetic correlative ($r = 0.78; p < 0.01$) between frequency of twin ovulations at 12–18 mo of age and body weight at 368 days of age in heifers from dams and sires with a high estimated breeding value for twinning (unpublished data). Previous studies have demonstrated possible genetic determinants of plasma concentrations of IGF-I in mice (Blair et al., 1988) and pigs (Buonomo et al., 1987), and our studies also provide evidence for a genetic determinant of systemic and follicular IGF-I concentrations in cattle.

Although serum concentrations of FSH do not differ between twin-producing and control cows (Echternkamp and Gregory, 1989), the specific mechanism whereby increased IGF-I may alter folliculogenesis is unknown. In view of the dramatic stimulatory effects of IGF-I on steroidogenesis and mitosis in cultured granulosa and thecal cells reported previously (Adashi et al., 1985; Cara and Rosenfield, 1988; Hammond et al., 1988b; Hernandez et al., 1988; Schams et al., 1988), we propose that an increase in levels of IGF-I in follicular fluid of twin-producing cows may enhance FSH stimulation of follicles within the ovaries and contribute to selection of two versus one ovulatory follicle in cattle. It appears that numbers of large follicles and growth of the second largest follicle are increased in twin-producing cattle. However, steroidogenesis within various sized follicles appears unchanged in twin-producing cows. Likewise, FSH stimulation in cattle significantly increases the number of follicles maturing but does not affect steroid concentrations among individual antral (>3 mm) follicles of the same size (Wise et al., 1986). The increased follicular growth observed in twin-producing cows may then allow for the appearance of two "co-dominant" ovulatory follicles rather than one.

TABLE 1. Concentrations (mean ±SE) of estradiol and progesterone in fluid of small (<4 mm), large (≥4 mm), and the two largest follicles from either unselected cows (CONTROL) or cows selected for twinning (TWIN).

| Follicle type[a] | | n[b] | Estradiol (ng/dl) | Progesterone (ng/ml) |
|---|---|---|---|---|
| Control | —small | 12 | 14.9 ± 10.3[c] | 42.7 ± 8.7 |
| | —large | 19 | 589.7 ± 117.3[d] | 116.1 ± 29.0 |
| | —two largest | 24 | 459.8 ± 105.6[d] | 99.9 ± 23.9 |
| Twin | —small | 14 | 6.8 ± 3.9[c] | 65.2 ± 15.2 |
| | —large | 32 | 391.4 ± 84.8[d] | 88.9 ± 19.6 |
| | —two largest | 27 | 441.6 ± 97.4[d] | 101.4 ± 22.1 |

[a]Follicular fluid from follicles 1–3.9 mm in diameter was pooled within each cow; fluid from follicles ≥4 mm in diameter was collected individually.
[b]n = number of follicular fluid samples.
[cd]Means within a column with different superscripts differ ($p < 0.05$).

Whether the increased concentrations of IGF-I in follicular fluid of twin-producing cattle (present study) were of systemic or follicular origin is uncertain. Recent studies in swine and rats have implicated IGFs as intraovarian or paracrine regulators of follicular growth and differentiation (for reviews, see Adashi et al., 1985; Hammond et al., 1988b). A paracrine role for IGFs in ovarian function has been further supported by the observations that granulosa cells can secrete immunoreactive IGF-I and/or IGF-II in vitro and that ovarian tissues contain mRNA for IGFs (Murphy et al., 1987; Ramasharma and Li, 1987; Voutilainen and Miller, 1987; Hammond et al., 1988b). However, concentrations of IGF-I are significantly lower in ovarian follicular fluid than in serum of pigs (Hammond et al., 1988a) and cattle (present study). Thus, IGF-I derived from serum must be considered a possible source of intraovarian IGFs. In support of this suggestion, concentrations of IGF-I in serum and follicular fluid were correlated positively ($r$ = 0.69). On the other hand, we found that small follicles contained significantly lower IGF-I concentrations than large follicles (present studies), indicating local control of IGF-I levels. These results could reflect local biosynthesis, alterations in diffusion barries between blood and follicular fluid (e.g., basement membrane), or changes in IGF-I binding proteins. The average percentage of concentration of follicular fluid IGF-I:serum IGF-I of 74% (present study) is similar to the percentage of follicular fluid:serum concentrations of $\beta_2$-glycoprotein (145,000 Daltons) measured in individual bovine follicles (Andersen et al., 1976), suggesting that a slight diffusion barrier to IGF-I and its binding protein complex ($\simeq$150,000 Daltons) may exist within ovarian follicles. A diffusion barrier to IGF binding proteins has been demonstrated in limb lymph in humans (Binoux and Hossenlopp, 1988). Certainly, it seems necessary to postulate amplified IGF-I production in extraovarian tissues to account for the higher levels of IGF-I in serum of twin-producing cows. Additional studies will be required to understand the relative importance of systemic and locally produced IGF-I.

Genetic regulation of ovulation rate in sheep is well documented (Cummins et al., 1983; Robertson et al., 1984; Bradford et al., 1986; McNatty et al., 1987) and includes evidence that the genetic determinant for ovulation rate differs among breeds and/or strains. For Booroola ewes, it has been hypothesized that reduced inhibin production, genetically regulated, causes increased FSH secretion and thus higher ovulation rates (Cahill et al., 1981; Cummins et al., 1983; Robertson et al., 1984; Webb and England, 1984). However, differential FSH secretion does not appear to account for increased ovulation rate in the high fecundity Finnsheep breed (Webb and England, 1984; Wheaton et al., 1988) or twin-producing cattle (Echternkamp and Gregory, 1989). Existence of similarities between twin-producing cows and Finnsheep ewes, but not Booroola ewes, in genetic regulation of ovulation rate is suggested by preliminary findings showing that increased frequency of multiple ($\geq$2) ovulations in prolific Finnsheep ewes, but not Booroola Merino ewes, is associated with increased concentrations of serum IGF-I (Zavy et al., 1989).

Concentrations of IGF-I in peripheral blood of cattle, as reported previously, are either similar to (Honegger and Humbel, 1986; Zangger et al., 1987; Ellenberger et al., 1989) or lower than (Brier et al., 1986; Housenecht et al., 1988; Elsasser et al., 1989) values reported in the present studies. Several environmental factors such as nutritional status, known to affect circulating concentrations of IGF-I in cattle (Brier et al., 1986; Housenecht et al., 1988; Elsasser et al., 1989), may be responsible for the lower concentrations reported previously. Alternatively, the 24-h 25°C extraction incubation used previously (Brier et al., 1986; Elsasser et al., 1989) would have dramatically underestimated IGF-I concentrations due to reduced recovery.

In summary, the present study provides evidence to suggest that twinning in cattle is associated with increased concentrations of IGF-I in both serum and follicular fluid. Thus, these data support the notion that IGF-I, of ovarian and(or) systemic origin, plays a role in the regulation of folliculogenesis and is a mediator of a genetic component of multiple ovulations in cattle.

## ACKNOWLEDGMENTS

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