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EFFECT OF CHARCOAL TREATED BOVINE FOLLICULAR
FLUID ON SERUM FOLLICLE-STIMULATING HORMONE
AND LUTEINIZING HORMONE IN CASTRATED RATS AND
HEIFERS: A DEMONSTRATION OF INHIBIN-ACTIVITY

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# EFFECT OF CHARCOAL-TREATED BOVINE FOLLICULAR FLUID ON SERUM FOLLICLE-STIMULATING HORMONE AND LUTEINIZING HORMONE IN CASTRATED RATS AND HEIFERS: A DEMONSTRATION OF INHIBIN-ACTIVITY

By

Andrea Day Curato

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# ABSTRACT

## EFFECT OF CHARCOAL-TREATED BOVINE FOLLICULAR FLUID ON SERUM FOLLICLE-STIMULATING HORMONE AND LUTEINIZING HORMONE IN CASTRATED RATS AND HEIFERS: A DEMONSTRATION OF INHIBIN-ACTIVITY

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The objective of these studies was to determine if charcoal-treated bovine follicular fluid selectively suppressed the secretion of follicle-stimulating hormone in castrate rats and heifers. To meet this objective follicular fluid was collected from ovarian follicles of cattle. Steroids were removed from follicular fluid with charcoal, and charcoal-treated follicular fluid was injected into heifers ovariectomized three days earlier and into castrated male and female rats. It was demonstrated (1) that bovine follicular fluid lowered concentrations of follicle-stimulating hormone, but not luteinizing hormone in heifers and female rats; and (2) that bovine follicular fluid blocked the castration rise in follicle-stimulating hormone but not luteinizing hormone in male rats. Since total amounts of estradiol and progesterone were low in bovine follicular fluid after charcoal extraction, factors other than these steroids, perhaps inhibin, altered secretion of follicle-stimulating hormone in heifers and rats.

.....In dedication to

hopes and dreams....

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# ABBREVIATIONS

| | |
|---|---|
| bFF | bovine follicular fluid |
| $^{o}$C | degrees centrigrade |
| cm | centimeter(s) |
| $E_2$ | estradiol |
| FF | follicular fluid |
| FSH | follicle-stimulating hormone |
| g | gram(s) |
| GnRH | gonadotropin-releasing hormone |
| h | hour(s) |
| i.v. | intravenous |
| kg | kilogram(s) |
| LH | luteinizing hormone |
| mg | milligram(s) |
| min | minutes |
| µl | microliter(s) |
| ml | milliliter(s) |
| mm | millimeter(s) |
| n | number of animals per treatment group |
| NIAMDD | National Institutes of Arthritis, Metabolic, and Digestive Diseases |
| ovx | ovariectomized |
| P | progesterone |
| pFF | porcine follicular fluid |
| PGF | prostaglandin F2α |
| ps | pig serum |
| PBS | phosphate buffered saline |
| RIA | radioimmunoassay |
| RP1 | reference preparation-1 |
| T | testosterone |
| TSH | thyroid-stimulating hormone |
| x g | times gravity |

# INTRODUCTION

The hypothalamus synthesizes and releases gonadotropin-releasing hormone (GnRH). GnRH acts on the pituitary to stimulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These gonadotropins are transported via the blood to the gonads where FSH and LH regulate spermatogenesis, folliculogenesis, steroidogenesis, and ovulation. Increasing output of estradiol ($E_2$) and progesterone (P) from the gonad feeds back on the hypothalamus to partially regulate pituitary secretion of gonadotropins. Although $E_2$ and P exert a negative feedback effect on LH, these steroids do not fully account for the negative feedback control of FSH. A gonadal factor called inhibin has been implicated in the control of secretion of FSH from the pituitary gland.

The objective of my research was two fold: first, to demonstrate the presence or absence of an inhibin-like substance in bovine follicular fluid (bFF) and second, to determine if bFF could selectively inhibit the secretion of FSH in ovariectomized (ovx) heifers. My first objective was studied by employing two documented *in vivo* assay systems for the detection of inhibin-activity (female rats Marder et al., 1977; male rats, Hermans et al., 1980). My second objective was measured by injecting bFF into ovx heifers and measuring changes in concentrations of LH and FSH in serum.

# REVIEW OF LITERATURE

## Definition of Inhibin

The concept of inhibin had its beginning in 1923 when Mottram and Cramer described the effects of X-irradiating testes. Seminiferous tubules were shrunken and disorganized while interstitial cells appeared normal, and the pituitary showed histological changes resembling those after castration. Based on these data, Mottram and Cramer postulated that a hormone from the seminiferous tubles was a major inhibitor of pituitary function and this hormone had a role in the regulation of sperm production. It was not until 1932 that the term inhibin was used by McCullagh to describe a water soluble testicular extract, which prevented hyperfunction of the pituitary gland in castrated rats. McCullagh also described a fat soluble entity, separate from inhibin, known today as androgen or testosterone, that was responsible for the maintenance of the secondary sex glands.

Although the structure of testosterone was reported in 1935 (David et al.), the structure of inhibin remains unknown. Recent interest in inhibin occurred with the demonstration that a non-steroidal inhibitor of FSH was present in bull semen (Franchimont, 1972) and in ovine rete testis fluid (Setchell and Sirinathsinghji, 1972).

Main et al. (1979) stated the inhibition hypothesis as two pituitary gonadotropins acting on the testes, FSH stimulating the seminiferous tubles, and LH stimulating the Leydig cells. The Leydig cells produce androgens (particularly testosterone) which control LH secretion by negative feedback, and the seminiferous tubles produce inhibin which controls FSH secretion by negative feedback. This hypothesis is often challenged by the "androgen only" hypothesis. Here, gonadotropin control is believed regulated only by androgen feedback and not inhibin (see Setchell et al., 1977, for review). However, in contrast to preparations of inhibin, neither androgens nor other steroids lower FSH in castrated animals to normal levels without either suppressing LH or causing hypertrophy of secondary sex organs.

There has been increasing evidence which supports the presence of a specific non-steroidal inhibitor of FSH in follicular fluid. This inhibitor of FSH secretion was first detected in bovine follicular fluid (FF; de Jong and Sharpe, 1976) and subsequently in porcine (Marder et al., 1977; Schwartz and Channing, 1977; Welchen et al., 1977), equine (Miller et al., 1979b), and human FF (Chari et al., 1979; Chappel et al., 1980b).

Although inhibin is defined as a water-soluble gonadal substance that exerts a specific inhibition of FSH release from the pituitary gland (deJong, 1979a), LH release from the pituitary gland can also be affected (Legace et al., 1978; Shander et al., 1980; Scott and Burger, 1981; Rush et al., 1980). This suggests that a non-steroidal inhibitor of LH secretion is present in the gonads.

## Sources and Origin of Inhibin

In males, inhibin-activity is found in different components of semen, spermatozoa (Fachini et al., 1963; Lugaro et al., 1969; 1973), seminal plasma (Franchimont et al., 1975a, 1977; Chari et al., 1978b), epididymal homogenates (LeLannou and Chambon, 1977), rete testes fluid (Setchell and Sarinathsinghji, 1972; Setchell and Jacks, 1974; Davies et al., 1976, 1978), and testicular lymph (Baker et al., 1976, 1978). Ovine (Lee et al., 1974; Nandinini et al., 1976), bovine (Keogh et al., 1976), and rat (Moodbidri et al., 1980) testicular tissue also contain inhibin. The Sertoli cell is believed directly involved in the snythesis and secretion of inhibin (Steinberger and Steinberger, 1976). However, Sertoli cells may require the presence of germinal cells for the induction of inhibin secretion (Rich and DeKretser, 1977).

In females, inhibin-activity is found in ovarian FF (de Jong and Sharpe, 1976; Hopkinson et al., 1977; Marder et al., 1977; Schwartz and Channing, 1977; Welchen et al., 1977; Channing et al., 1980), ovarian homogenates (Chappel, 1979), and ovarian venous plasma (DePaulo, et al., 1979; Shander et al., 1980; Channing et al., 1980). Granulosa cells in the ovary may be the source of inhibin in females (de Jong and Sharpe, 1976). For example, when media (charcoal extracted to remove steroids) from cultured granulosa cells is added to a pituitary cell culture system, concentration of FSH in media is reduced (Welchen et al., 1977; Erickson and Hsueh, 1978). Media from undifferentiated granulosa cells also reduces secretion of FSH from cultured pituitary cells (Erickson and Hsueh, 1978). Thus, granulosa cells acquire the ability to produce inhibin early in the development of a follicle.

## Measurement of Inhibin-Activity

### Detection of Inhibin-Activity Using Cultures of Pituitary Cells

Baker et al. (1976) developed the first monolayer pituitary cell culture to assay for inhibin-activity. Either basal concentrations of GnRH-stimulated release of gonadotropins are measured after addition of preparations containing suspected inhibin-activity. Culture systems differ in sex and age of rats used, concentration of pituitary cells in culture, composition of culture media, and amount of time of exposure to the test material (Erickson and Hsueh, 1978; Franchimont, 1979; de Jong et al., 1979b; Steinberger, 1979; DePaulo et al., 1979; Scott et al., 1980; Scott and Burger, 1981).

A pituitary cell culture is a very sensitive method of detecting inhibin-activity. As little as 0.5 μl of charcoal-treated bovine FF (Franchimont et al., 1979) or 1.0 μl of charcoal-treated porcine FF (Shander et al., 1980b) reduces the capacity of pituitary cells in culture to release FSH. Shander and coworkers (1980) have demonstrated in pituitary cell cultures that $E_2$, P, and testosterone (T), either alone or in combination have no selective negative effects on the release of FSH.

## In Vivo Detection of Inhibin

The suppression or blockage of a post-castration rise in concentration of FSH in castrated animals or suppression of FSH in intact animals is taken as direct evidence of inhibin-activity. The model system of Marder et al. (1977) is one of the most sensitive and repeatable *in vivo* methods for detection of inhibin. On the morning of metestrus, as determined by vaginal cytology, rats are bilaterally ovx and injected intravenously (tail vein) 3.5 h later with the test substance, such as FF. Rats are killed 5.5 h following injection to obtain trunk blood for quantification of LH and FSH. Concentrations of LH are unaffected by injection of follicular fluid whereas FSH is suppressed in a dose dependent fashion. For example, 23 μl of bovine FF has been shown to suppress concentrations of FSH in the serum 50 percent below control levels (Grady et al., 1982).

### Radioligand Assays for Detecting Inhibin

Antibodies to semipurified inhibin has been produced in rabbits (Franchimont et al., 1975c; Vaze et al., 1979; Shashidhara Murtly et al., 1979; Sairam et al., 1978). Adult intact rats exhibit a dose-dependent increase in serum FSH when injected with increasing amounts of inhibin antisera. This suggests that endogenous inhibin is neutralized by the antibody and therefore incapable of negative feedback control of FSH secretion (Franchimont et al., 1975c; Vaze et al., 1979). Franchimont et al. (1975c) developed a radioimmunoassay (RIA) for ovine rete testis fluid and Vaze et al. (1979) developed an RIA for human seminal plasma. Antibodies to the ovine fluid do not cross react with GnRH, somatostatin, or pituitary hormones, but do cross react with bovine testicular extract and bovine follicular fluid inhibin preparations. Antibodies to human seminal plasma inhibin do not cross react with prolactin, LH, or FSH from humans, sheep, and rats, thyrotropin stimulating hormone (TSH), GnRH, or steroids from humans, ram testicular extracts, or bull semen. However, the antibody of Vaze et al. does cross react with monkey semen, rat serum, and FF from cows, sheep, and pigs. The above assays were not developed to measure inhibin in serum, but the assay of Vaze et al. is sensitive enough to measure inhibin in the peripheral circulation of rats, providing evidence that inhibin does enter blood circulation. However, one universal inhibin molecule has not been found, therefore bioassays are necessary to monitor attempted purification regimens and refinement of RIAs for inhibin.

## Chemical Characteristics of Inhibin

### The Nature of Inhibin

Testosterone (T), $E_2$, dihydrotestosterone, and P in semipurified inhibin preparations are not present in sufficient amounts to explain the inhibitory effect on FSH *in vivo* or *in vitro* (Franchimont et al., 1975a, 1979; Hopkinson et al., 1977). Low doses of androgen preferentially suppress serum LH over FSH (Franchimont et al., 1975b). However, androgens are capable of suppressing the GnRH-stimulated release of both FSH and LH (Eddie et al., 1978, 1979). The dose response lines differ from responses obtained with inhibin. Small asmounts of $E_2$ and androgens stimulate the secretion of FSH from rat pituitary cells in culture (Labrie et al., 1978), while inhibin suppresses the secretion of FSH. Follicular fluid preparations (steroid free) retain their ability to selectively suppress FSH both *in vivo* and *in vitro* (Steinberger and Steinberger, 1976; Erickson and Hsueh, 1978; Welchen et al., 1977; de Jong et al., 1978; Setchell et al., 1977), suggesting a nonsteroidal factor is responsible for the suppression of FSH. However, steroids do have roles in regulation of LH and FSH secretion (see review, Barraclough et al., 1979).

## Purification and Characterization of Inhibin

Many attempts have been made to purify the inhibin molecule (see de Jong et al., 1981). Various methods consist of precipitation, ultrafiltration and dialysis, gel filtration and electrophoresis, ion exchange chromaatography, isotachophoresis, precipitation with antibodies, and affinity chromatography. The biochemical data obtained so far, do not agree on a single molecular weight for inhibin. Estimates range from 1,000 to 90,000 daltons. The large number of inhibin sources and the variety of methods used for purification have resulted in confusion with respect to the characterization of inhibin and its biological similarities across species. de Jong et al. (1981) reported that most results suggest a molecular weight between 15,000 and 25,000 daltons and that inhibin may be a hydrophobic glycoprotein with an isoelectric point between pH 5.0 and 6.0.

## Sites of Inhibin Action

### The Pituitary as a Site of Inhibin Action

It is difficult to make conclusive arguments for or against any one inhibin target site considering that the source of origin and chemical nature are questionable. Nevertheless, there is increasing evidence that the pituitary is the site of action.

Release and synthesis of FSH, but not LH, is reduced when charcoal-treated media from granulosa (Erickson and Hsueh, 1978) or Sertoli cell cultures (Chowdhury et al., 1978), is added to pituitary cells in culture. This provides evidence for direct action of inhibin on the pituitary. A GnRH-induced release of FSH, but not LH, in rats is reduced with injections of charcoal-treated porcine FF (Rush and Lipner, 1979; De Paulo et al., 1979). Similar results are demonstrable *in vitro* (Shander et al., 1980b). However, inhibin is unable to reduce concentrations of FSH in the face of high concentrations of GnRH suggesting that inhibin activity can be overcome with GnRH at the pituitary (Rush and Lipner, 1979).

## The Hypothalamus as a Site of Inhibin Action

Since the release of LH and FSH is mediated by GnRH, the possibility exists for a hypothalamic site of inhibin action. Media from rat testes organ culture reduces the amount of GnRH released from rat hypothalami in culture (Demoulin et al., 1980). Six hours after an injection of semi-purified inhibin into the third ventricle, release of FSH is maximally suppressed in castrated rats (Lumpkin et al., 1981). However, the pituitary responds to GnRH stimulation at this time, suggesting that an inhibin-hypothalamic interaction was responsible for reduced FSH secretion from the pituitary. Reduced secretion of FSH may be mediated by lowered secretion of GnRH and differences in threshold responsiveness to GnRH for the secretion of FSH and LH. This threshold hypothesis is supported with the finding that a 10-fold greater concentration of synthetic GnRH is required to stimulate a half maximal response in the release of FSH over that required for half maximal response in release of LH (Leung and Padmadabhan, personal communication). Thus it is possible that lowered GnRH reduces the release of FSH, but not LH, because the pituitary is more sensitive to GnRH for the release of LH.

## The Gonad as a Site of Inhibin Action

Spermatogenesis is negatively effected by inhibin *in vitro* and *in vivo* (Demoulin et al., 1980). In immature rats, inhibin injections resulted in atresia of follicles (Chari et al., 1981). Atresia may be a result of binding inhibition of FSH to its receptors on granulosa cells (Sato et al., 1980). Oocyte maturation inhibitor (OMI; Jagiello et al., 1974; Tsafirri and Channing, 1975) and an inhibitor of luteinization (Ledwitz-Rigby et al., 1977) are found in FF. These non-steroidal substances could be responsible in part for gonadal effects following treatment of animals with FF. Follicular fluid also contains a component capable of inhibiting the adenylyl cyclase system, inhibiting progesterone secretion, inhibiting luteinization without affecting the steroidogenic enzyme systems (Rigby et al., 1980; Ledwitz and Rigby, 1980) and reducing prostaglandin $F2_{\alpha}$ (PGF) from theca and granulosa cells (Kraiem et al., 1978). Preovulatory follicles lack this PGF inhibitor, supporting the idea that a decline in inhibitory action may be necessary for follicular maturation and, if not, atresia may result.

In summary, follicular fluid contains components that affect the hypothalamus, pituitary, and gonad. The components may be separate entities or a complex family of regulators required to maintain the cyclic waves of folliculogenesis. Inhibin is a possible member of this family.

## The Endocrine Role of Inhibin in Domestic Animals

Most of the evidence in support of inhibin has come from laboratory animal studies; however, inhibin-activity has been demonstrated in domestic animal species.

Bovine testicular extracts, when infused over a 24 h period, reduce concentrations of FSH within the next 24 h period in castrate rams (Keogh et al., 1976; Baker et al., 1976). Concentrations of LH increased slightly. Concentrations of FSH, but not LH in blood are reduced following the infusion of human follicular fluid from the follicular but not luteal phase of the menstrual cycle into the anterior pituitary gland of ovariectomized monkeys (Chappel et al., 1980b). Reductions in the concentration of serum FSH ranged from 30 to 70 percent. This variation may be due to differences in individual follicular fluids tested or differences in responses between monkeys. Charcoal-treated porcine FF injected early or midway in the menstrual cycle of monkeys reduces circulating serum concentrations of FSH, but not LH (Channing et al., 1981). Steroids may interact with inhibin. Removal of steroids with ethanol extraction or destruction of proteins by heating (100°C, 2 min) diminished the ability of equine follicular fluid to lower concentrations of FSH in ovariectomized mares as compared to the response to whole FF (Miller et al., 1979b). In fact, when charcoal-treated equine FF is injected in combination with $E_2$ (1 mg) the decrease in FSH is greater than that induced by either treatment alone (Miller et al., 1981).

Gonadotropin secretion is regulated by feedback of ovarian products. Basal concentrations of LH are regulated by $E_2$ and P in rats (Grady et al., 1981), sheep (Goodman et al., 1981), and cattle (Kesner et al., 1981). Although $E_2$ and P are involved in the regulation of basal FSH secretion, some other ovarian factor, possibly inhibin, is required for complete control (Chappel, 1980a, b; Goodman et al., 1981; Grady et al., 1981). The preovulatory surge of gonadotropins in cattle is a result of increasing $E_2$, decreasing P, and a possible increase of GnRH (Kesner et al., 1981). Concentrations of LH remain at baseline throughout the bovine estrous cycle, with the exception of the periovulatory period. However, concentrations of FSH increase and then return to baseline following ovulation (Dobson et al., 1978; Roche and Ireland, 1981). Inhibin may be present during both releases of FSH. The GnRH surge may overcome inhibin's ability to suppress FSH during the periovulatory period, while the gonadotropin surge may shut down the ovary so that its products are not secreted or are secreted at very low levels. Therefore, FSH could be released in the absence of negative feedback from a combination of steroids and inhibin. Inhibin's presence throughout the remainder of the cycle may act in concert with $E_2$ and P to keep concentrations of FSH at baseline.

Gonadotropin stimulation of the ovary is necessary for the initiation and maintenance of folliculogenesis (Richards, 1978). Systemic administration of bovine follicular fluid to sheep and cattle following prostaglandin synchronization reduces the total number of surface follicles and reduces the size of the largest follicle (Miller et al., li79a). Concentrations of circulating gonadotropins were not measured, therefore, events cannot be attributed to reduced concentrations of FSH. However, in monkeys, porcine FF reduces concentrations of FSH during the early follicular phase and reduces recruitment and selection of the ovulatory follicle. Reduced concentrations of FSH during the late follicular phase results in atresia of the pre-existing dominant follicle (Dizerega et al., 1981). These results suggest that growing follicles require continued FSH support throughout the cycle. Lowering FSH by injecting FF, therefore, results in interrupted folliculogenesis. Preantral follicles may enter the growing pool of follicles following the preovulatory surge of FSH (Matton et al., 1981) or following the cyclic four-day increases in FSH reported by Schams and Schallenberger (1976).

Since FSH controls growth of ovarian follicles, a better understanding of the role of inhibin on negative feedback control of FSH secretion will improve our ability to regulate estrous cycles in cattle. However, no studies have examined the effects of inhibin on secretion of FSH in cattle. Thus, we proposed to determine whether FF from bovine follicles contains inhibin-activity using two *in vivo* bioassays and to examine whether FF from bovine follicles suppressed FSH secretion in heifers.

# MATERIALS AND METHODS

Bovine ovaries were collected in pairs from non-pregnant cows at a local slaughterhouse and stage of the estrous cycle was estimated based on gross appearance of the corpus luteum (Ireland et al., 1980). Follicular fluid was aspirated from follicles greater than 10 mm in diameter and assigned to one of the following pools: preovulatory (day 18-1), postovulatory (day 2-6), or mid-cycle (day 7-17). Follicular fluid was centrifuged (5°C, 10 min, 248 x g) to remove follicular cells and frozen (-20°C). After thawing, FF was stirred with 25 mg of washed Norit charcoal per ml at 37°C for 20 min and centrifuged at 105,700 x g for 30 min. This supernatant was used for all subsequent experiments. Charcoal treatment of bovine follicular fluid (bFF) reduced steroid content 99 percent (Table 1). Steroids were quantified by single antibody radioimmunoassay (RIA) as previously described for estradiol (Wetteman et al., 1972), progesterone (Convey et al., 1976), and testosterone (Haynes et al., 1977). Protein in follicular fluid was quantified using the method of Lowry et al. (1951) before and after charcoal extraction of steroids (Table 2). Porcine FF contained 75.40 mg protein per ml of fluid.

TABLE 1. Concentrations of Steroids (ng/ml) before and after Charcoal Extraction of Steroids from Bovine Follicular Fluid

| | $E_2$ | | P | | T | |
|---|---|---|---|---|---|---|
| | Before | After | Before | After | Before | After |
| Preovulatory | 108.09 | 0.45 | 312.00 | 1.33 | 17.64 | * |
| Postovulatory | 127.14 | 0.26 | 171.00 | 1.47 | 18.62 | * |
| Midcycle | 98.51 | 0.50 | 226.00 | 1.82 | 14.32 | * |

*Concentrations of testosterone were not detectable (lower limit of assay, 0.5 ng/ml).

TABLE 2. Concentrations of Protein (mg/ml) before and after Charcoal Extraction of Steroids from Bovine Follicular Fluid

| | Before | After |
|---|---|---|
| Preovulatory | 97.81 | 96.04 |
| Postovulatory | 94.71 | 96.48 |
| Midcycle | 96.04 | 95.59 |

## Experiment 1: Effect of Charcoal-Treated Bovine Follicular Fluid on Serum FSH in Rats Ovariectomized at Metestrus

This study was done in collaboration with Dr. Rosemary Grady and Dr. Neena Schwartz, Northwestern University, Evanston, Illinois. Portions of the Grady et al. (1982) results have previously been reported.

### Experimental Design

This experiment was designed to determine if the pools of charcoal-treated bFF were capable of exhibiting inhibin-activity. A colony of female Sprague-Dawley rats were housed under 14 h light, 10 h dark with feed and water *ad libitum*. Rat vaginal smears were examined daily to determine the length and stage of cycle. Rats having regular 4 - 5 day cycles were bilaterally ovariectomized under ether anesthesia at metestrus. Three-and-one-half hours following ovx, rats received bFF, porcine FF (pFF) or porcine serum (PS) via a tail vein at one of the following levels of protein, 2.25, 4.50, or 9.00 mg. Volume equivalents of these fluids were 23, 47, or 94 ul of bFF, 30, 60, or 119 ul of pFF, and 90 ul of PS (9.00 mg protein). Porcine FF and PS have been previously characterized and shown to selectively suppress concentrations of FSH in ovx rats (Marden et al., 1977; Lorenzen et al., 1978) and therefore served as reference standards (n = 3 or 4 rats). Preovulatory, postovulatory and midcycle bFF were the unknown test fluids (n = 3 or 4 rats). The doses chosen were previously determined in a pFF dose-response trial using PS as a control fluid. Level of protein as no effect on concentrations of gonadotropins when PS is injected so the highest level served as a control in this study. At 5.5 h after treatment the rats were decapitated and trunk blood collected. Plasma was obtained by centrifugation (5°C, 20 min, 1548 x g) and frozen (-20°C) until assayed. RIA was used to detrmine concentration of FSH in plasma. NIAMDD kit instructions were followed and NIAMDD-FSH-RP1 was used as the reference standard.

Concentrations of LH in serum of rats at metestrus do not rise withn 9 h post ovariectomy (Marder et al., 1977; Campbell et al., 1977) and Lorenzen et al. (1978) found that procine FF has no effect on LH. Therefore, concentrations of LH were not assayed for in the metestrous rat model system.

## Statistical Analysis

The hormone data were found heterogeneous in variance and was not correctable by transformation. Therefore, specific comparisons were made using Behren's test statistic (Gill, 1978). Level of significant difference was tested against Bonferroni-t values since all comparisons were non-orthogonal (Gill, 1978).

## Results

Injections of PS control do not effect castration concentrations of FSH in plasma (Marder et al., 1977; Lorenzen et al., 1978). Injections of PS result in concentrations of FSH similar to those obtained following injection of phosphate buffered saline (PBS) injections containing the same concentration of protein (unpublished data). Responses to the lowest does of pFF and bFF, were not significantly different from the PS control with the exception of postovulatory bFF which reduced concentrations of FSH ($P < 0.05$; Table 3). As level of protein increased in injections of pFF and bFF, concentrations of FSH in plasma decreased. Postovulatory bFF had the greatest negative effect on concentrations of FSH and bFF was more potent than pFF at the two lower doses.

## Experiment II: Effect of Charcoal-Treated Bovine Follicular Fluid on the Castration Rise in Serum FSH and LH in Male Rats

## Experimental Design

The assay of Hermans et al. (1980) was employed to examine effects of bFF on the postcastration increase in FSH in 50-day-old male Sprague-Dawley rats (200-250 gm) housed under 12 h light, 12 h dark. Rats were bilaterally castrated or sham operated under ether anesthesia. Immediately following surgery each rat received a subcutaneous injection of 500 μl per 100 g

TABLE 3. Effects of Bovine and Porcine Follicular Fluid on Castration Levels of FSH (ng/ml) in Female Rats Ovariectomized at Metestrus

| Treatment | Amount of charcoal-treated follicular fluid or serum | | |
|---|---|---|---|
| | 2.25[1] | 4.50 | 9.00 |
| PS[2] (control | ND | ND | 500.9±62.2 |
| pFF | 349.7±23.6(30)[3] | 265.8± 7.3(47)[a] | 140.5±13.3(72)[c] |
| bFF | | | |
| preovulatory | 293.3±21.0(42)[4] | 215.1± 6.9(57)[b] | 156.6±14.1(69)[b] |
| postovulatory | 251.4± 8.4(50)[b] | 170.6±14.4(66)[b] | 138.4± 7.5(72)[c] |
| midcycle | 304.3±42.4(39) | 212.7±16.7(58)[b] | 137.5± 8.2(73)[c] |

[a] Milligrams of protein.

[b] PS porcine serum, pFF = porcine follicular fluid, bFF = bovine follicular fluid, ND = not determined.

[c] Mean (± standard error) ng of FSH in serum.

[d] Percent suppression as compared to PS control in parentheses. Injections of PS at lower levels results in concentrations of FSH similar to those when 9mg protein given. a = $P < 0.10$, b = $P < 0.05$, c = $P < 0.01$ as compared to PS controls.

body weight of one of the following: preovulatory bFF (n = 10), postovulatory (n = 10), midcycle bFF (n = 9), or serum from an ovx cow (n = 13) as a control fluid. Follicular fluid was collected and prepared as described earlier. Ovx serum was charcoal-treated as described for bFF. Rats were killed and trunk blood collected 8 h following treatment. Plasma was collected after centrifugation (5°C, 20 min, 1548 x g) and frozen (-20°C) until assayed for gonadotropin content. Concentration of FSH and LH were determined by RIA (NIAMDD kit directions; Niswender et al., 1968) with the exception that the second antibody was replaced by 2% Protein A (IgSL10, The Enzyme Center, Boston, MA.) in 1% bovine serum albumin in PBS to separate free from bound hormone. On the third day of incubation, 150 μl of 2% Protein A was added to each assay tube and incubated at room temperature for 30 min. Sodium chloride solution (2 ml, 0.9%) was added to terminate the separation reaction.

## Statistical Analysis

Hormone data were heterogeneous in variance and was not correctable by transformation. Therefore, the same procedure as discussed for Experiment 1 was used.

## Results

Serum LH increased ($P < 0.01$) from 32 ng/ml in the sham operated group (N = 10) to 103 ng/ml in the castrated control group (n = 10) (Table 4). Concentrations of LH in the group receiving control serum were not different from the castrate group. The concentration of FSH increased ($P < 0.01$) from 424 ng/ml of serum in the sham operated group to 555 ng/ml of serum in the castrate control group. The concentration of FSH in the group receiving control serum were not different from the castrate group. All bFF pools blocked ($P < 0.01$) the castration rise in FSH, but were not different in their capacity to suppress FSH. Concentrations of LH were unaffected by bFF as compared with castrate controls.

## Experiment III: Effect of Charcoal-Treated Bovine Follicular Fluid on Serum FSH and LH in Ovariectomized Heifers

### Experimental Design

The objective of this experiment was to determine if charcoal-treated bFF would suppress FSH, but not LH, in ovx heifers. Eleven crossbred beef heifers (300-395 kg) were confirmed cycling by rectal palpation. Heifers were offered corn silage and water *ad libitum* in loose housing. Heifers were bled via indwelling teflon cannulas (STX053, Becton-Dickinson, Rutherford, N.J.). Cannulas were held in place with a neckwrap of 7.62 cm wide Elastoplast elastic adhesive tape (Beiersdorf, Inc., South Norwalk, CT). Cannulas were filled with a 3.5% sodium citrate solution between sampling to prevent

TABLE 4.--Effect of Charcoal-Treated Bovine Follicular Fluid or Serum from an Ovariectomized Cow on Concentration of Follicle-Stimulating Hormone and Luteinizing Hormone in Male Rats after Castration

| Treatment | LH(ng/ml) mean ± sem | FSH(ng/ml) mean ± sem |
|---|---|---|
| Sham | 32.0± 3.2 | 424.4±12.2 |
| Castrate | 103.2±10.0[b] | 555.7±19.1[b] |
| Ovx serum | 120.4±15.0 | 604.1±34.9 |
| Preovulatory bFF | 101.3±16.3 | 422.4±27.7[d,e] |
| Postovulatory bFF | 86.6±14.6 | 350.8±18.9[c,e] |
| Midcycle bFF | 116.1±13.7 | 401.2±17.7[c,e] |

[a] Rats received 0.5 ml per 100 g body weight of treatment fluids directly after surgery. Trunk blood was collected 8 h following treatment.

[b] $P < 0.01$ as compared to sham control group.

[c] $P < 0.01$ as compared to castrate control group.

[d] $P < 0.05$ as compared to castrate control group.

[e] $P < 0.01$ as compared to ovx serum control group.

coagulation. Blood was drawn every 15 min for 2 h preceeding ovx to determine precastration baseline concentrations of LH and FSH. Heifers were ovx by inserting an ecraseur through an incision in the dorsal wall of the vagina to remove the ovaries. Blood samples were then taken at 15 min intervals for 4 h beginning 66 h after ovx (or 4 h before injections of bFF or serum). This series of samples established post castration baseline concentrations of LH and FSH. Heifers received three 20 ml injections of pools of bFF removed from follicles at different stages of the estrous cycle as described earlier (preovulatory, n = 3 heifers; postovulatory, n = 3 heifers, or midcycle, n = 3 heifers) or ovx serum (n = 2 heifers) via jugular cannula at 6 h intervals. The first injection of bFF or control serum was given at 0 h which was 70 h after ovx. Hourly blood samples were drawn from 0 through 48 h. Blood was refrigerated (4°C) for 6 to 12 h, incubated at 37°C for 2 h, and returned to 4°C before centrifugation (5°C, 30 min, 1548 x g). Serum was decanted and assayed for LH and FSH as previously described by Convey et al. (1976), and Carruthers et al. (1980), respectively.

### Statistical Analysis

Serum hormone values from hourly blood samples for treatment and control groups were regressed over time.

Hormone data were found heterogeneous in variance and were not correctable by transformation. Therefore, specific comparisons were made using Behren's t (Gill, 1978). Level of significant difference was tested against Bonferroni-t values since all comparisons were non-orthogonal (Gill, 1978).

The FSH response curves for each treatment were divided into three parts: the decending portion (0 to 12 h after bFF), the flat portion (12 to 26 h after bFF), and the ascending portion (26 to 48 h after bFF) and analyzed by linear regression. Specific slope comparisons were made using Sheffe's interval (Gill, 1978).

## Results

Mean concentration of FSH in serum increased from a precastration baseline of 53 ng/ml to 116 ng/ml following castration. Castration increased mean LH in the serum from approximately 1 ng/ml to 3 ng/ml. Mean concentration of gonadotropins in serum were not affected by injections of control serum (Figures 1 and 2).

All groups of heifers responded similarly to the three pools of bFF through 26 h. Concentrations of FSH dropped as early as four hours following the first injection of bFF. Although the group receiving bFF from preovulatory follicles had higher postcastration concentrations of FSH, the descending slopes were not significantly

FIGURE 1. Serum follicle-stimulating hormone (ng/ml) in ovariectomized heifers given three intravenous injections (20 ml) of serum from an ovariectomized cow (control:-⊕-), or charcoal-treated follicular fluid from cattle. Follicular fluid was obtained from follicles ≥ 10 mm in diameter and pooled based on stages of estrous cycle: postovulatory follicular fluid = days 2 to 6 (-⊞-), midcycle follicular fluid = days 7-17(-▲-), preovulatory follicular fluid = days 18-1 (-✱-). Arrows indicate time of injections. Blood samples were taken at hourly intervals.

Figure 1.

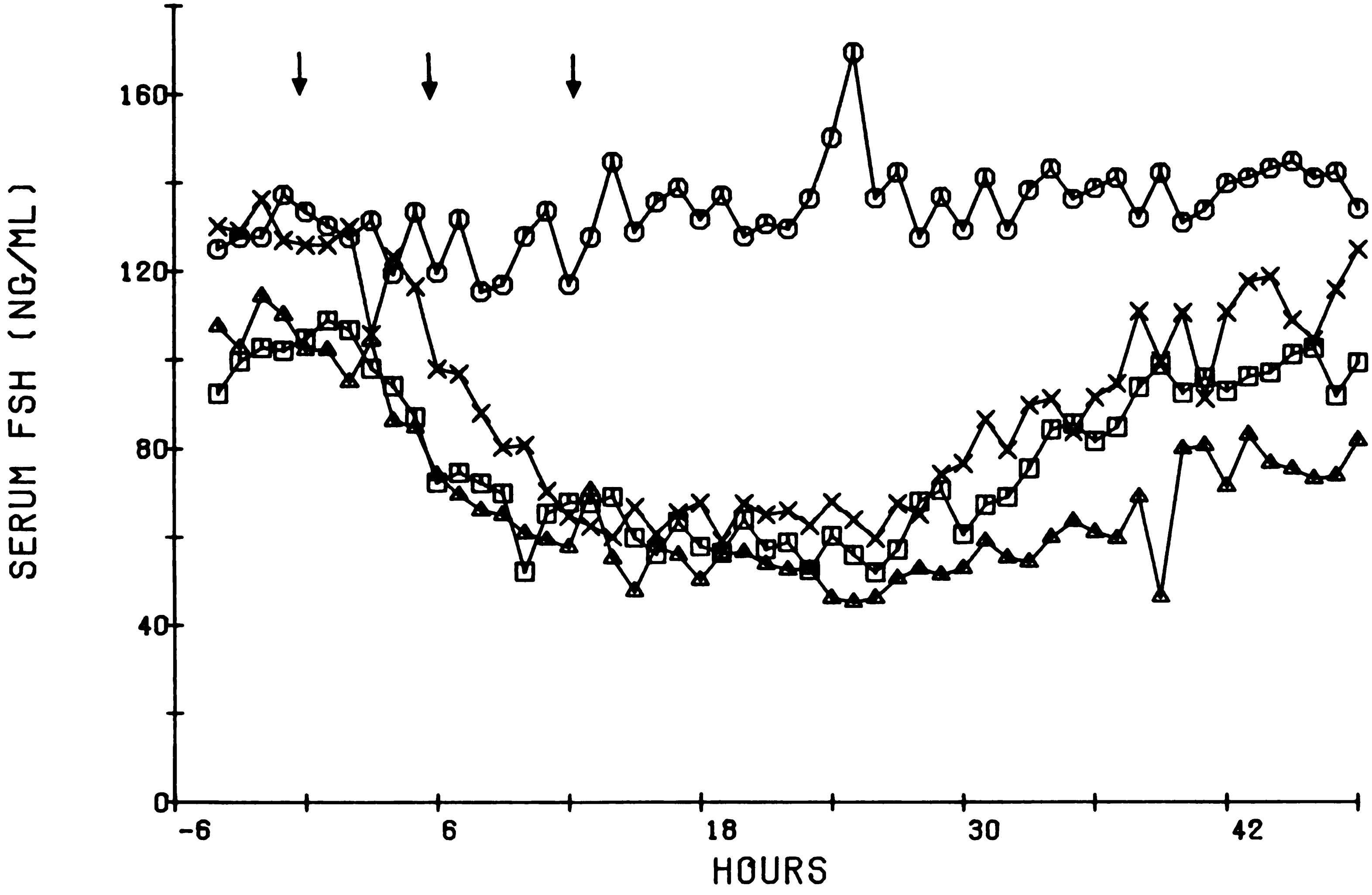
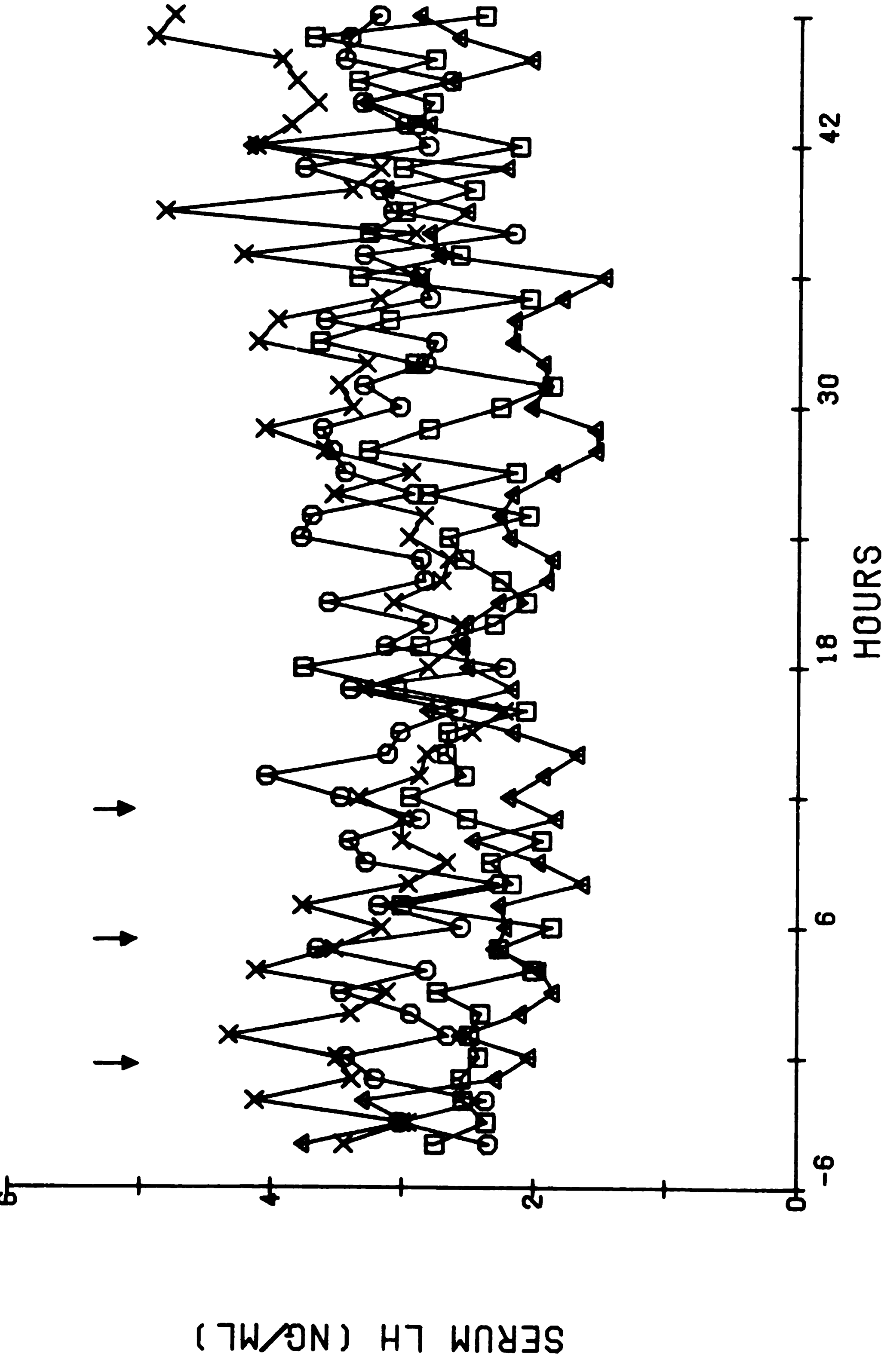


FIGURE 2. Serum luteinizing hormone (ng/ml) in ovariectomized heifers given three intravenous injections (20 ml) of serum from an ovariectomized cow (control: -⊕-), or charcoal-treated follicular fluid from cattle. Follicular fluid was obtained from follicles ≥ 10 mm in diameter and pooled based on stages of the estrous cycle: postovulatory follicular fluid = days 2-6 (-⊞-), midcycle follicular fluid = days 7-17 (-▲-), preovulatory follicular fluid = days 18-1 (-✕-). Arrows indicate time of injections. Blood samples were taken at hourly intervals.

Figure 2.



different. Concentrations of FSH continued falling after the second and third injections of bFF, reaching maximal suppression ($P < 0.005$) 6 to 12 hours following the third injection of bFF (Figure 1). Concentrations on FSH were suppressed to precastration baseline concentrations (53 ng/ml). Concentrations of FSH remained at precastration levels from 12 through 26 h. At 26 h the responses to bFF started to diverge. The ascending portions of the response curves were different ($P < 0.01$) between the midcycle bFF and the other two pools (pre- and postovulatory). By 36 h following the last injection of bFF, concentration of FSH in serum returned to castration levels for the groups of heifers treated with pre- and postovulatory bFF. The inhibitory effect of midcycle bFF on concentrations of FSH were more prolonged and concentrations had not reached postcastration levels by the end of blood sampling. Mean serum concentration of LH were unaffected ($P > 0.05$) by bFF (Figure 2).

# DISCUSSION

Selective suppression of FSH in the rat assay systems confirmed the results of Marder et al. (1977) in female rats and Hermans et al. (1980) in male rats, suggesting the presence of inhibin-activity in pools of bFF injected into heifers. DePaulo et al. (1979) found increased inhibin-activity in the ovarian venous plasma (OVP) of rats during diestrus, but the highest activity was found during early proestrus. In humans, FF from the follicular phase of the menstrual cycle contains increased inhibin-activity as compared to FF from the luteal stage (Chappel et al., 1980b; Channing et al., 1981). Inaccurate staging of ovaries, or overlapping of stage of the estrous cycle within pools of bFF could be responsible for the lack of differences in capacity of inhibin to suppress FSH in either of the rat bioassay systems. However, the possibility of no variation in inhibin-activity throughout the bovine estrous cycle cannot be ruled out. Inhibin-activity may always be present in bFF but selectively released into circulation at certain stages of the cycle in response to increased concentrations of FSH.

The female rat model was more sensitive than the male rat model, but this may have been a result of route of injection since female rats received 23-93 μl of bFF intravenously while male rats received volumes approaching 1,000 μl subcutaneously. In female rats, concentrations of FSH were more negatively effected than in male rats. Protein content of pools of bFF were similar (Table 2), however, pFF contained less total protein than bFF and was less potent suggesting that total amount of protein may be an index of inhibin-activity in follicular fluid.

Miller et al. (1979b) found the greatest suppression of FSH in mares 12 h following one injection of equine FF. In the present study, three injections of bFF may not have been necessary to reduce concentrations of FSH to precastration concentrations. It appears that the three injections of bFF prolonged the inhibition of FSH. Since Miller et al. (1979b) observed a rebound of FSH to castration levels within 12 h of maximal suppression. Midcycle bFF may have increased inhibin-activity in the cow, since concentrations of FSH were slower to recover when bFF injections were stopped (Figure 1). These results differ from results in Experiment I and II using rats where preovulatory bFF appeared to have more inhibin-activity. Differences may be attributed to the method of detection. First, bFF may have different effects in rats than in cows. Secondly the rat assays were endpoint determinations of bFF effects while the heifer experiment allowed mapping of effects over time.

Steroid concentrations were determined for each pool of bFF (Table 1). Charcoal extraction removed 99% of the total steroids in bFF. Total $E_2$ and P were 8 and 30 ng per 20 ml dose of FF. On a total blood volume basis (60 ml blood per kg of body weight, Altman, 1961), average circulating concentrations of $E_2$ and P would have been 0.4 pg/ml and 1.4 pg/ml, respectively, following each injection of 20 ml of bFF. Actual concentrations of steroids would have been lower if tissue uptake and metabolic clearance rates were taken into account. Thus, concentrations of $E_2$ and P were well below average circulating levels. Concentrations of progesterone range from 0.5 to 10 ng/ml serum (Glencross, 1973; Herriman et al., 1978) and estradiol ranges from 2 to 10 pg/ml serum (Glencross, 1972; Dobson and Dean, 1974) throughout the bovine estrous cycle. Roche and Ireland (1981) reported that exogenous administration of progesterone via vaginal coils in the artificial regulation of bovine estrous cycles had no effect on concentrations of FSH, but did affect LH. Kesner and Convey (1982) injected a large dose of $E_2$ (1 mg) that was effective in reducing concentrations of FSH in ovx cows to precastration concentrations, but lower levels were not tested. Moreover,

$E_2$ and P replacement in ovx ewes to normal levels would not reduce concentrations of FSH to precastration baseline levels (Goodman et al., 1981). Therefore, considering normal levels of $E_2$ and P are not capable of controlling basal secretion of FSH in sheep, it is doubtful that the low levels of $E_2$ and P injected could be responsible for the reduced concentrations of FSH. The ovarian transplant studies of Uilenbroek et al. (1978) have ruled out steroidal involvement in the selective suppression of FSH in the rat. Steroids were inactivated by hepatic metabolism, and replacement of $E_2$ and P to normal levels did not result in decreased FSH. Inhibin-activity of bFF has been destroyed with pronase (Hermans et al., 1980) and heat treatment (de Jong et al., 1981), suggesting a proteinaceous component.

In conclusion, bFF obtained from prevoulatory (day 18-1), postovulatory (day 2-6), and midcycle (days 7-17) stages of the estrous cycle contained inhibin-activity. However, the possibility of other steroids not accounted for could be responsible for the selective suppression of FSH. Purification and development of an RIA for inhibin are necessary to answer the question of what causes the selective suppression of FSH following the injection of bFF. Follicular fluid was capable of suppressing concentrations of FSH in ovx heifers to precastration baseline concentrations without altering mean serum concentrations of LH. Inhibin-activity was transient, and concentrations of FSH returned to castration levels in the absence of bFF injections. Injections of charcoal-treated bFF could potentially be a method of determining the role of FSH in folliculogenesis. Further investigations of the effects of bFF on gonadotropins in the intact heifer are necessary prerequisites in determining the potential of bFF in such studies.

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