DEVELOPMENT AND APPLICATION OF A BIOASSAY FOR FOLLICLE-STIMULATING HORMONE

A thesis presented in partial fulfilment of the
requirements for the degree of

Master of Science
in
Physiology

at Massey University, Palmerston North,
New Zealand

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2000

Abstract

Follicle-stimulating hormone (FSH) is involved in the regulation and maintenance of vital reproductive processes, such as gametogenesis, follicular development and ovulation. Produced in the anterior pituitary, FSH is a glycoprotein hormone that exists as a family of isohormones. Follicle-stimulating hormone concentrations have traditionally been measured by radioimmunoassay (RIA). However, results generated using RIA are a determination of the immunological activity of FSH. The potential of FSH to generate a biological response cannot be measured by RIA. Therefore, the identification of physiologically significant differences in the activity of these isoforms requires the use of assay systems that can differentiate between the biological activity of the FSH isoforms.

Commonly used assays for measuring the biological activity of FSH are based on the measurement of aromatase activity in cultured rat Sertoli cells following stimulation with FSH. However, these assays have an inherently high ethical cost involved due to the use of primary tissue culture. In addition, the variation in these assays associated with differences between animals is difficult to eliminate. Recently a bioassay for human FSH has been described based on FSH stimulation of cyclic AMP production by a Chinese hamster ovary (CHO) cell line stably expressing the human FSH receptor (FSH-R). The purpose of this study was to evaluate the potential usefulness of this CHO FSH-R cell line expressing the human receptor for FSH to be used as a bioassay to measure the biological activity of ovine FSH. The receptor cell line bioassay described in this study is based on the ability of FSH to stimulate cAMP production by cultured CHO FSH-R cells. Optimisation of the culture system to enable the bioactivity of ovine FSH to be measured by bioassay was undertaken. This involved optimising the density of cultured cells, the time in culture and time exposed to FSH and the most suitable dose range for FSH. The influence of matrix effects, such as those exerted by serum was also investigated. The specificity of the assay towards FSH was also determined as was the sensitivity, accuracy and precision of the assay. No stimulation of cAMP production was seen in CHO FSH-R cells following treatment with α-FSH, β–FSH, LH, TSH, GH, prolactin or vasopressin at concentrations up to 10 μg/ml.

Although the methodology used differed slightly depending on the presence or absence of serum, all assayed were performed using the following methods and materials. Freshly thawed FSH-R cells were bulked up in culture, and aliquots of 1×10^5 to 5×10^5 cells/well dispensed into 48 well

culture dishes and incubated overnight at 37°C. The assay culture media was then replaced with 0.25 ml fresh media (α-MEM + 0.1% BSA + 0.25 mM 3-isobutyl-1-methyl-xanthine) containing varying doses of NIH-FSH-RP2 (RP2) FSH preparations or FSH containing samples, and the cells incubated for 4 hours at 37°C. The assay culture media was then removed and stored frozen at -20°C until assayed for cyclic adenosine monophosphate (cAMP) by RIA.

Once optimal assay conditions were determined, the CHO FSH-R cell bioassay was used to measure FSH concentrations in ovine serum, pituitary extracts and medium from cultures of ovine pituitary cells. It was found that the concentrations of FSH in serum from intact sheep was close to the detection limit of the assay. Thus, while FSH concentrations could be measured in serum from some sheep, other animals had concentrations that were too low to be accurately measured by the bioassay in its present form. The assay was, however, well suited to measuring FSH concentrations in serum from sheep that had elevated concentrations of FSH. In one study, FSH concentrations measured by the bioassay were compared to those measured by RIA in sheep that had been ovariectomised and then hypophysectomised. It was found that the profile of FSH concentrations following hypophysectomy was similar whether measured by RIA or by bioassay (R^2=0.7513), though absolute concentrations sometimes differed. This suggested that the immunoassay and bioassay were not always measuring the same characteristics of FSH. The assay was also used to measure FSH concentrations in samples of ovine hypophyseal venous blood. However, the results obtained for these samples indicated a poor correlation between FSH concentrations obtained by bioassay and RIA. Levels of bioactive FSH in hypophyseal venous blood fluctuated markedly and were up to 10-fold higher than the associated RIA concentrations.

The CHO-cell bioassay was also found to be very suitable for measuring pituitary concentrations of FSH. In one study, pituitary extracts underwent chromatography and the separated isoforms of FSH were analysed by bioassay and RIA. Again, there was excellent correlation (R^2=0.9328) between the concentrations of FSH measured both assay types. However, some differences were apparent suggesting a discrepancy in the biological and immunological characteristics of different FSH isoforms. The bioassay was also used to measure FSH concentrations in media from pituitary cells in tissue culture where serially diluted samples displayed good parallelism with the RP2 FSH standard curve.

Results of this study demonstrate that the CHO FSH-R cell bioassay is suitable for measuring the biological activity of ovine FSH in a variety of biological fluids. The use of a permanent cell line

eliminates the high ethical cost associated with primary tissue culture that other bioassay systems have. The inherent variation associated with culture systems utilising tissue from different sources is also avoided. The sensitivity of the bioassay is suitable for measuring FSH in surgically altered sheep or hypophyseal blood concentrations where FSH levels are generally higher than those in the peripheral circulation. In addition to blood samples, the bioassay is also excellent for monitoring FSH activity in pituitary extracts and in media from tissue culture. However, the sensitivity of the bioassay currently does not always allow measurement of bioactive FSH concentrations in serum samples with low FSH levels.

In summary, the CHO FSH-R cell bioassay described in this study offers a useful alternative to RIA and other bioassays for monitoring the biological activity of ovine FSH and its isoforms in various biological fluids. It is concluded that this convenient and robust bioassay may have considerable application in future investigations of ovine FSH bioactivity.

Acknowledgements

Firstly, I would like express my deepest thanks to Associate Professor Keith Lapwood for his guidance, support, and most of all, for his patience. I am profoundly indebted to Dr. Keith Henderson for all the help he has given me. Suffice it to say, this project could not have begun, let alone be completed, without his expert advice, knowledge and support, both as a supervisor and as a friend. My heartfelt gratitude goes out to Dr. Ken McNatty for his inspirational enthusiasm, encouragement and for allowing me the opportunity and independence to undertake this project during my time at AgResearch. The Reproductive Physiology Group at AgResearch, Wallaceville was a wonderful place to work and is composed of a fantastic group of people, which is a reflection on the leadership you provide.

This work would not have been possible without the generous gift of the FSH receptor cell line from Dr. Christopher Albanese. I would like to thank Dr. Iain Clarke for the use of some extremely valuable serum samples. Similarly, I owe thanks to Dr. Lloyd Moore for the provision of pituitary extract samples and associated RIA data. I would also like to thank Norma Hudson for the provision of serum samples and RIA data. My thanks also goes out to Winny Ng Chie for the provision of RIA data (look, no hyphen!!!). I could not have hoped to work with anyone more pleasant and knowledgeable about radioimmunoassays than Stan Lun. Indeed, I would like to thank everyone in the Reproductive Physiology Group, AgResearch, Wallaceville, for their helpfulness and good will over the years. Special thanks goes to Jeff (Hellboy) Stewart for his friendship and for listening to me prattle on about nothing while at AgR.

How could I ever give enough thanks to my wife, Tina? I owe you everything. Without your love, dedication and support, I would have given up a very, very long time ago. To my mother and father – there are no parents in this world who have been more loving and self-sacrificing than you. It may have taken me a while to realise, but it's something that will remain with me always.

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Chapter One

INTRODUCTION

1 Introduction

Follicle-stimulating hormone (FSH) is a key glycoprotein hormone involved in the regulation and maintenance of essential reproductive processes, including gametogenesis, follicular development and ovulation (Scaramuzzi *et al*, 1993; Linsay, 1991) and acts by binding to specific receptors localized in the gonads. In the male, FSH acts on the Sertoli cells in the seminiferous tubules of the testis, to stimulate spermatogenesis. However, the mechanisms of FSH stimulation of spermatogenesis remains unclear. The target for FSH action in the female are the granulosa cells of follicles, where it stimulates production of estrogens and progestins, as well as various nonsteroidal substances (Dahl and Hsueh, 1988).

1.1 Structure of FSH

The two pituitary gonadotrophins, FSH and luteinizing hormone (LH) are members of the glycoprotein protein hormone family, which also include pituitary thyroid stimulating hormone (TSH), and the placental gonadotrophin chorionic gonadotrophin (CG), found only in equids and primates. All glycoprotein hormones are composed of a common α-subunit that is noncovalently associated with a hormone-specific β-subunit. In addition, all the glycoprotein hormones consist of a mixture of molecular isoforms. The nature of the microheterogeneity of glycoprotein hormones arise from differences in glycosylation of the hormones, including the degree of terminal sialylation and/or sulfation (Ulloa-Aguirre *et al*, 1995). The resulting acidic-base charge provides the major basis for separation by chromatographic techniques, such as isoelectric focusing or chromatofocusing (Simoni *et al*, 1994). Therefore, oligosaccharide chains of varying stages of completion, and different oligosaccharide structures are found on a single gonadotrophin. Although the heterogeneity of FSH is well established, regulation of FSH heterogeneity in the circulation is poorly understood. In addition, the functional significance of such a variety of isoforms for FSH remains unclear. Structural differences in these isoforms alter a number of physical and physiological properties of the hormone, including changes in receptor-binding activity, plasma half-life, and *in vivo* and *in vitro* bioactivity. Therefore a mixture of circulating FSH isoforms reaches target tissues within the gonads to influence a variety of physiological endpoints.

1.1.1 Glycosylation

Unlike other hormones now known to be glycoproteins, in which a single protein chain is glycosylated, both the α– and β-subunits of the glycoprotein hormones, including FSH, are glycosylated. For the α subunit of FSH (92-96 amino acids in length), the pattern of glycosylation is very consistent, with two N-linked glycosylation sites at asparagine (Asn) 52 or 56 and Asn78 or 82. Patterns of glycosylation on the β-subunit, however, are more variable than those found on the α-subunit. The β-subunit, comprised of 111-112 amino acids, exhibits two glycosylation sites at Asn6, 7, or 13 and Asn23, 24, or 30 (Ulloa-Aguire *et al*, 1995). Glycosylation and the processing of incorporated oligosaccharides involves a complex biosynthetic pathway that begins in the rough endoplasmic reticulum, and continues through the Golgi apparatus until the mature hormone is transported to secretory granules. The numerous carbohydrate intermediates resulting from processing in the Golgi apparatus are responsible for many of the FSH glycoforms secreted by the pituitary.

The oligosaccharide structures on FSH are highly variable and play a key role in determining the biological properties of the hormone. Most oligosaccharide chains in FSH are double branching structures with one end terminating in a negatively charged group (GalNAc-SO_4 or Gal-sialic acid) and the other branch terminating in either a negatively charged group or in non-charged mannose (Ulloa-Aguire *et al*, 1995). Tribranched oligosaccharides of different types containing sialic acid residues are also found. The main chemical basis for the isoform differences and extensive charge heterogeneity of FSH is attributed to variations in the structure and distribution of sialylated oligosaccharides. Follicle-stimulating hormone, in general, is rather acidic (Simoni *et al*, 1994), with a median isoelectric point (pI) approximately 4-5. Enzymatic desialylation of ovine pituitary FSH results in a redistribution of the isohormones towards less acidic forms (Keel and Schanbacher, 1988). Isoforms of FSH identified by charge-based fractionation differ from each other not only with respect to the degree of terminal sialylation and sulphation, but also in the inner carbohydrate moieties of the oligosaccharide side chains. Therefore, many FSH isoforms may have the same charge yet differ in oligosaccharide composition.

1.1.2 FSH Isoform Distribution

Although the factors affecting final gonadotrophin isoform distribution are complex and multifactorial, endocrine changes clearly regulate the proportions of FSH isoforms both within the pituitary and in the peripheral circulation. Differences in circulating FSH iosforms may be mediated by a number of different factors such as: 1) regulated preferential secretion of one or more FSH isoforms; 2) differences in the removal of carbohydrate moieties from the FSH molecule by circulating enzymes; and 3) changes in metabolic clearance (Ulloa-Aguire *et al*, 1995).

1.2 Synthesis and Release of FSH

Follicle stimulating hormone is the primary hormone involved in the emergence of waves of follicles, and its decline is associated with selection of a dominant follicle. The dominant follicle then becomes dependent on LH for further development when concentrations of FSH are minimal. The patterns of secretion of the two gonadotrophins are highly divergent, yet they can be produced in the same cell under the control of a single releasing hormone. The synthesized gonadotrophins are stored in secretory granules within the cytoplasm, and are secreted by exocytosis (Roche, 1996).

In the pituitary, the major hormone responsible for stimulating gonadotrophin release is gonadotrophin-releasing hormone (GnRH). Specific receptors on gonadotrophs respond to GnRH, triggering the release of intracellular Ca^{2+}, causing a transient release of both FSH and LH. The gonadotrophs become insensitive to GnRH, therefore the pulsatile nature of its release is necessary to prevent down regulation of its receptors. Without continual pulsatile stimulation by GnRH, secretion of LH and FSH declines rapidly, as do concentrations of messenger RNAs for the subunits of gonadotrophins in the anterior pituitary gland.

Other hormones that influence the secretion of FSH include estradiol, progesterone, inhibin; and activin. Inhibin and estradiol act directly at the anterior pituitary to decrease expression of the gene encoding the FSH subunit by reducing both the transcription and stability of mRNA, thereby negating the GnRH effects on FSH release. Estradiol causes a major decrease in FSH, while LH is initially decreased and then increased. Inhibin also suppresses FSH release, but without affecting LH. A summary of factors that influence FSH secretion is shown in Figure 1.1

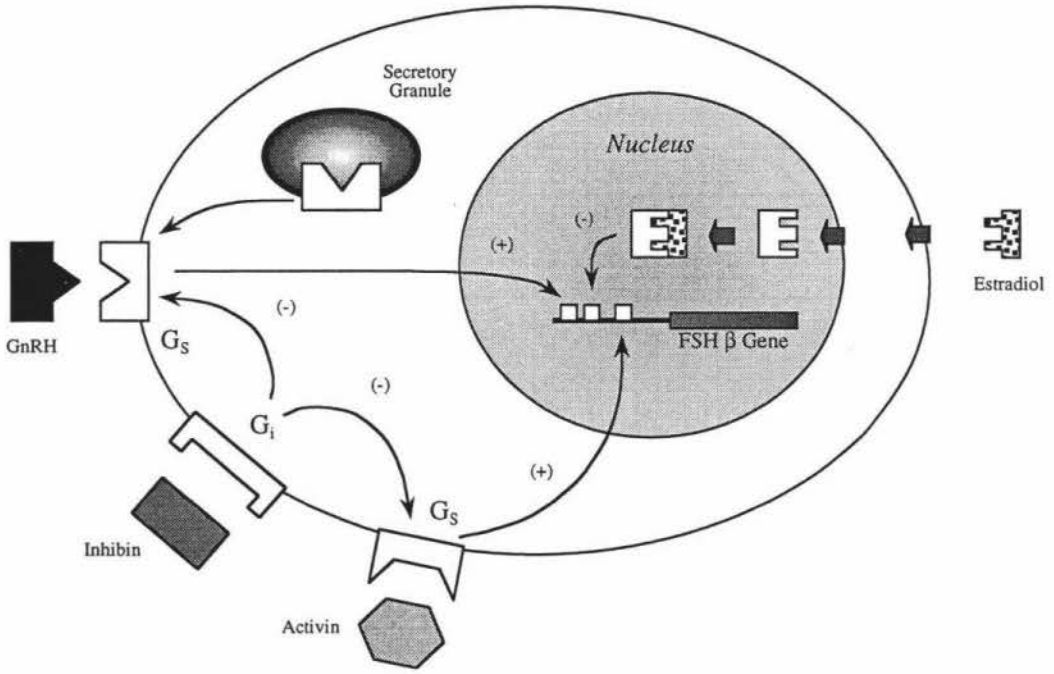


Fig 1.1 Schematic representation of factors known to regulate synthesis and secretion of FSH. Binding of GnRH to its receptors stimulates both protein kinase C (PK_C) and protein kinase A (PK_A) second messenger systems, thereby activating the stimulatory G protein (G_s). This, in turn, leads to the activation of the genes encoding α- (not pictured) and β- FSH subunits. Inhibin likely binds with an inhibitory G protein (G_i) coupled receptor. This may lead to inhibition of the stimulatory activity of GnRH and activin on FSH synthesis and secretion. Interaction of estradiol with its receptor inhibits transcription of the gene for β-FSH subunit. However, activation of the estradiol receptor leads to an increase in transcription for the gene encoding the receptor for GnRH (not shown) (Nett, 1990).

1.3 Physiology of FSH

In the female, the primary role of gonadotrophins is to initiate and sustain follicular functions. The initial stages of folliculogenesis occur independently of gonadotrophic hormones. In the adult ovary, folliculogenesis starts when follicles leave the pool of resting follicles to enter the growth phase. From there, the development of the follicle is characterized by a dramatic course of cellular proliferation and differentiation.

1.3.1 Follicular Development

Follicles in the ovary begin to form during fetal development and most remain in a resting stage until they either degenerate or are activated by various signals to enter the growth phase. These follicles constitute the stock of the resting follicles. Three types of resting follicles (primordial, intermediary, small primary) are evident in most mammals and can be distinguished by the size of the follicle and the structure and number of the granulosa cells surrounding the oocyte. With increasing age, the population of resting follicles progressively decreases. The age-related depletion of the resting follicle stock occurs as a result of two processes: atresia and entry into the growth phase. In sheep, the first follicles begin development at day 70 of gestation and by birth, at day 148, antral follicles are well developed. In adult ewes the estimated growth time for follicles is six months. Formation of the antrum occurs in ovine follicles when they reach 0.2mm in diameter and further increase in follicle size is attributed to an increase in granulosa cell numbers and a build up of follicular fluid. The granulosa cells reach a maximum growth rate when the follicle is approximately 1.0mm in diameter, after which the number of cell divisions declines until there is almost no mitotic activity in the mature preovulatory follicle. The follicular fluid is a derived from serum and is enriched with secretory products from follicle cells such as glycosaminoglycans, insulin-like growth factor I (IGF-1), inhibin, transforming growth factor β (TGF_{β}), prostaglandins and steroids. These substances may amplify or inhibit the actions of gonadotrophins such as LH and FSH.

Models of follicular growth and development based primarily on follicle size, number and morphological characteristics have been superseded by those based more on the functional capacities of the functional cells of the follicle. In one such model (Scaramuzzi *et al*, 1993),

follicles can be quiescent (i.e. primordial), committed to growth, ovulatory or atretic. Committed follicles can be non-responsive to gonadotrophins, and those that are responsive can be independent of or dependent on FSH and LH.

The role of gonadotrophins in initiating follicular growth is unclear. However, at advanced stages of follicular growth, antral follicles become dependent on FSH support for terminal follicular growth, leading to the emergence of waves of follicles. At this stage, an inappropriate stimulation, particularly by LH, can cause atresia. (Findlay, 1994). The decline of FSH is associated with selection of a dominant follicle that becomes dependent on LH for ovulation when concentrations of FSH are minimal. In addition, the reinitiation of growth in primordial follicles and acquisition of responsiveness of granulosa cells to FSH can occur independently of gonadotrophin support, indicating a role of autocrine and paracrine factors in folliculogenesis

In this model, the follicles committed to growth can be differentiated along a hierarchy according to their developmental status. Granulosa cells clearly undergo differentiation as the committed follicle move through the functional stages towards ovulation or atresia (Findlay, 1994). The stages of differentiation can be determined using parameters such as expression of gonadotrophin receptors, production of cyclic adenosine monophosphate (cAMP) in response to secretagogues, and production of steroids (estradiol, progesterone and 20α-hydroxyprogesterone) and peptide hormones (inhibin, oxytocin). Relatively undifferentiated granulosa cells, found in early committed follicles, lack any significant differentiation in terms of ability to respond to FSH and LH and to produce steroids and peptides (Scaramuzzi *et al*, 1993). Granulosa cells of small-medium bovine follicles demonstrate responsiveness to FSH and LH, but will not spontaneously luteinize *in vitro* unless treated with gonadotrophin. However, fully differentiated granulosa cells found in ovulatory follicles are generally capable of spontaneous luteinization *in vitro* (Findlay, 1994).

1.3.2 Control and Regulation of Folliculogenesis

The initiation of follicular growth is still not fully understood and there are two schools of thought on whether the initial stimulus for growth comes from the oocyte or the granulosa cells. While FSH is thought to influence the number of follicles that commence growth, gonadotrophins may not be essential in initiating follicle growth as follicles with four or five layers of granulosa cells have been observed in sheep following hypophysectomy. Although it is uncertain whether preantral follicles require gonadotrophins for follicular growth, it is evident that growth related processes are slowed down in their absence (Peters and McNatty, 1980).

1.3.2.1 Role of Estrogen in Follicles

Follicle-stimulating hormone is thought to stimulate the differentiation of the granulosa cells and promote antrum formation. To prevent the follicle from undergoing atresia, continuous presence of FSH is required. In human and sheep, FSH is present in some, but not all antral follicles. In follicles which have a higher concentration of androgen than estrogen (androgenic follicles), FSH is not present in detectable amounts. These follicles always undergo atresia.

Estrogen, primarily in the form of 17β-estradiol, is found in antral follicles arising mainly from the theca and granulosa cells. In the sheep ovary, over 95% of estradiol produced originates from granulosa cells, while the output of estradiol from theca interna cells is negligibl (McNatty and Henderson, 1987). Estradiol in conjunction with FSH enhances mitotic activity and influences the number of LH and prolactin receptors on granulosa cells. The concentration of estradiol in some follicles can be as high as 40,000 times that of estradiol in plasma in sheep. The estradiol/androgen balance in the follicle is kept in favour of estradiol by synthesis of estradiol from androgen. This ability to metabolize androgen to estrogen is maintained by FSH, thus follicles deficient in FSH lose this ability and eventually become androgenic and degenerate. Follicles which have been FSH-estradiol primed can then respond to a preovulatory rise in LH which subsequently leads to ovulation and corpus luteum formation.

As a result of the elevated levels of circulating estrogen, FSH production from the pituitary falls resulting in lowered peripheral concentrations of FSH. Without exposure to sufficient levels of FSH, the smaller antral follicles are no longer able to maintain their levels of estrogens and consequently undergo atresia. Therefore the dominant follicle stimulates its own growth and simultaneously deprives the others of FSH, accelerating their demise. The remaining healthy follicles already have high level of estrogens stored and continue to accumulate FSH.

Eventually the heightened level of circulating estrogen, secreted by the developing follicle, stimulates an increase in LH production, which accumulates in the follicle. Exposure of the follicle to high levels of LH initiates the final stages of oocyte maturation, inhibition of estrogen secretion and further granulosa cell divisions. Increased prostaglandin synthesis and initiation of the corpus luteum formation from the granulosa cells also occur due to the presence of LH.

1.3.2.2 Activin and Inhibin in Follicles

Inhibin, activin, and their binding protein, follistatin, have direct and indirect effects on granulosa and theca cells that can influence follicular development and steroidogenesis (Roche, 1996). Inhibin is a glycoprotein that has been isolated from follicular fluid as a heterodimer consisting of a common α-subunit combined with one of two β-subunits, βA (inhibin-A) or βB (inhibin-B) (Gougeon, 1996). The βA- and βB-subunits combine to give three forms of activins: activin (βAβB), activin A (βAβA), and activin B (βBβB). Follistatin is a monomeric glycosylated protein that is the major high-affinity binding protein for both inhibin and activin in follicular fluid.

A characteristic feature of activin and inhibin are the opposite effects that these peptides exert on certain follicular functions. On one hand, inhibin stimulates androgen production in theca interna cells, while activin exerts a negative influence on androgen production in the same cells. On the other hand, inhibin appears to be a potent inhibitor of ovarian cell proliferation in mice, whereas activin has been shown to stimulate *in vitro* proliferation of immature rat granulosa cells (Li *et al*, 1995). The effects of follistatin on steroid synthesis by preovulatory granulosa cells are indirect. Follistatin inhibits the suppressive effects of activin, as it binds with high affinity to activin and with lower affinity to inhibin (Gougeon, 1996).

Activin has been shown to play an important role in regulating granulosa cell development and functional integrity in ovarian follicles. Receptors and mRNA for activin have been found in oocytes, granulosa cells and theca interna cells of the rat (Cameron *et al*, 1994). Activin increases the number of FSH receptors in rat granulosa cells and stimulates the reorganization of follicular structures from monolayer cultures of immature granulosa cells in both rats and humans (Li *et al*, 1995). Inhibin has both autocrine and paracrine effects; it increases LH-induced androgen synthesis in thecal cells, and production of inhibin is stimulated by steroids and FSH (Roche, 1996). Thus, inhibin and activin provide a mechanism by which follicles can communicate directly with the pituitary in a feedback relationship with FSH. Therefore, within individual follicles a sequential change of inhibin, activin and their binding proteins may ultimately determine the different fates of the follicles committed to growth.

1.4 Biological Features of FSH Isoforms

Due to structural differences, FSH isoforms differ in their ability to bind to target cell receptors, survive in the circulation, and stimulate a biological response *in vitro* and *in vivo*.

Removal of the carbohydrate moieties in FSH has been found to increase the receptor binding ability and immunological activities of the hormone in radioreceptor and radioimmunoassays (Manjunath *et al*, 1982). In contrast, the deglycosylated FSH molecules have a greatly diminished capacity for stimulation of cAMP by gonadal cells (Manjunath *et al*, 1982). In addition, when combined with intact FSH, the deglycosylated FSH has been shown to interfere with the ability of the intact hormone to elicit steroidogenesis and these hormones are potent and specific antagonists in *in-vitro* (Manjunath *et al*, 1982).

The protein domain of the FSH molecule binds to specific plasma membrane FSH receptors, while the carbohydrate moieties of FSH are believed to interact with membrane components either within or adjacent to the receptor molecule. In general, certain peptide regions are thought to be critical for receptor binding, whereas the oligosaccharides are thought to be important for signal transduction. Indeed, receptor binding and signal transduction has been shown to be dissociable functions involving different sites on the FSH glycoprotein (Valove *et al*, 1994).

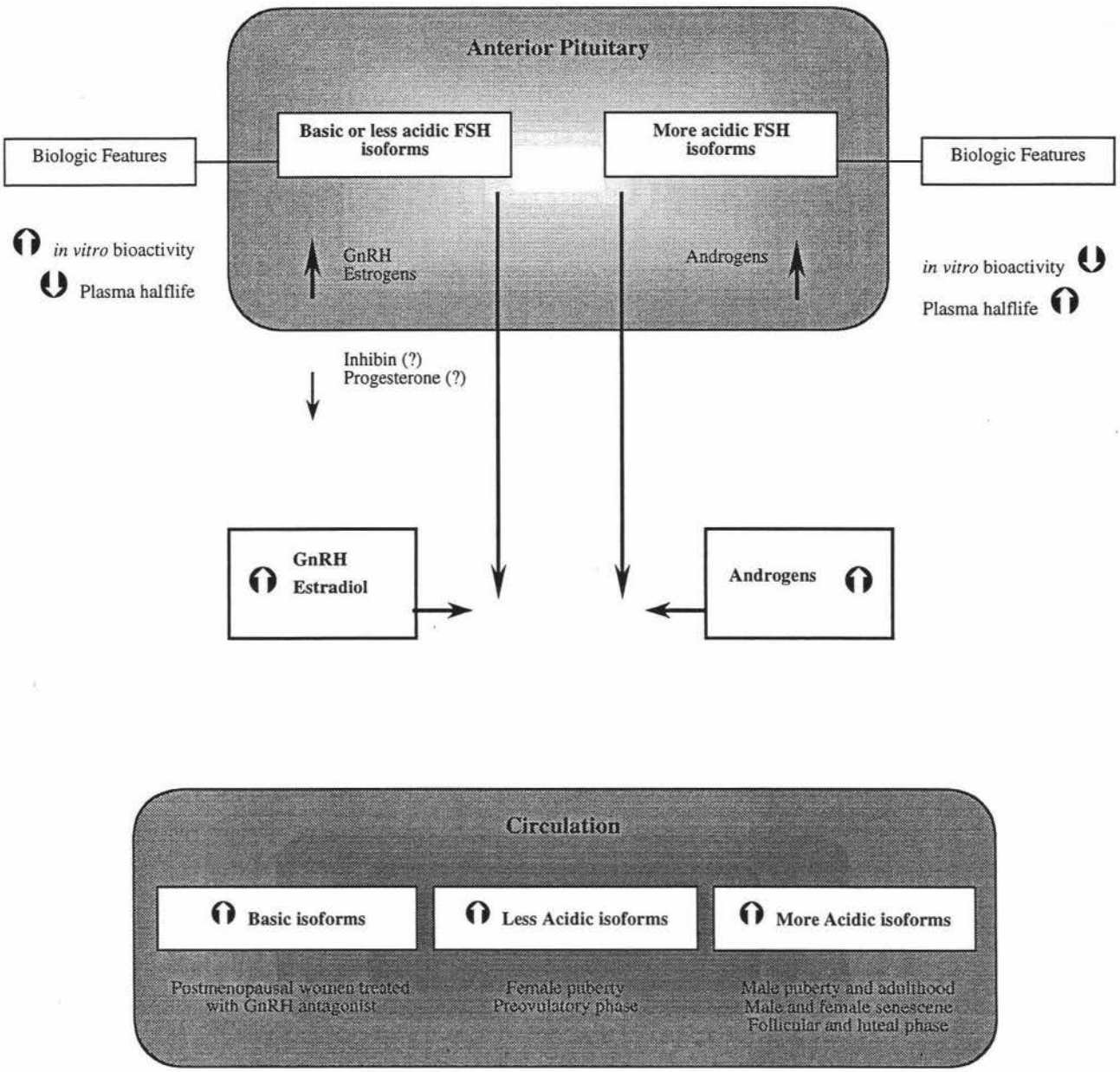


Fig 1.2 Hormonal factors influencing the synthesis and release of different FSH isoforms from the pituitary gland and the distribution of these isoforms associated with various physiological conditions (Roche, 1996).

Studies comparing in vitro bioactivity with immunoreactivity appear to indicate that less acidic, less glycosylated FSH isoforms have a higher biopotency *in vitro*, but have a shorter half-life (Stanton *et al*, 1992). Although heterogeneity has been studied extensively in the pituitary, regulation of FSH heterogeneity in the circulation is poorly understood due in part to the relatively low concentrations of circulating FSH. Despite this limitation, studies examining the correlation between qualitative differences in pituitary FSH content with age, sex and stage of the estrous cycle indicate that endocrine changes clearly regulate the proportions of FSH isoforms both within the pituitary and in the peripheral circulation (Roche, 1996). A majority of studies show that estradiol is associated with increased release of less acidic isoforms of FSH, and androgens are associated with increased release of more acidic forms of FSH. The onset of GnRH induced puberty in sheep and the preovulatory phase in normal cycling women is accompanied by major shifts in the distribution pattern of circulating FSH isoforms, and an associated increase in FSH bioactivity (Padmanabhan *et al*, 1992). A summary of factors thought to influence the synthesis and distribution of different FSH isoforms is shown in Figure 1.2.

1.5 FSH Mode of Action

Follicle-stimulating hormone, like all protein hormones, mediates its effects on target cells via specific protein receptors located on the cell surface. Receptors for FSH have been studied in both males and females. The primary location for FSH receptors in males is the Sertoli cells within the seminiferous tubule. In females, the major site of FSH receptor expression is the granulosa cells.

In both sexes, the secondary messenger cAMP mediates the action of FSH through its receptors. Treatment with membrane soluble cAMP analogs or cAMP-inducing agents stimulates multiple physiological responses in granulosa cells. Furthermore, the action of FSH in granulosa cells is enhanced with the addition of phosphodiesterase inhibitors that reduce cAMP breakdown (Welsh *et al,* 1985; Simoni *et al*, 1997). Follicle-stimulating hormone induced increase of intracellular cAMP in granulosa or Sertoli cells activate PKA, which results in subsequent phosphorylation of structural proteins, enzymes, and transcriptional activators.

Binding of FSH to its receptors *in vitro* is a process highly dependent on temperature and salt concentration and slowly becomes non-reversible at 37°C (van Loenen *et al*, 1994a). However, only a few minutes are required to attain steady state receptor activation, as measured by cAMP production in Sertoli cells, whereas the development of high-affinity receptor binding requires several hours (van Loenen *et al*, 1994b). The binding of FSH to granulosa cell membrane preparations is fully reversible only when hormone-receptor association occurs at 4°C and requires strong acidic treatment. The molecular basis for the slow formation of an irreversible interaction between FSH and its receptors is unknown. Whether the stabilization of FSH receptors occurs *in vivo* is also not known, although, in such a case, it could be an important step for hormone-receptor internalization.

1.5.1 FSH Receptor Structure

FSH receptors belong to the family of G protein-coupled receptors, characterized by complex transmembrane proteins consisting of seven hydrophobic helices inserted into the plasmalemma and by intracellular and extracellular domains of variable dimensions depending on the ligand (Simoni *et al*, 1997). Early experiments indicate that FSH receptors are oligomeric glycoproteins, consisting of four disulfide-linked monomers (Reichert and Dattatreyamurty, 1989). It is thought that FSH receptor binding is dependent on the integrity of the disulfide bonds, which stabilize the receptor conformation. The intracellular portion of FSH receptors is coupled to a G Protein and, upon receptor activation of the extracellular domain, initiates a cascade of events that ultimately leads to the specific biological effects of FSH in target tissues.

Depending on the species, the calculated molecular mass of the mature receptor protein ranges between 75 and 76.5 kDa, based on the cDNA sequence (Simoni *et al*, 1997). Further characterization of the amino acid sequence and hydropathy plot analysis suggests that FSH receptors consist of a large hydrophilic domain followed by hydrophobic regions 21-24 amino acids long, which span the plasma membrane seven times. At the C terminus, the sequence predicts a highly basic cytosolic segment.

Beside the transmembrane domain with the typical seven membrane-spanning helices, the glycoprotein hormone receptors are characterized by a very large extracellular domain in the N

terminus region, required for interaction with complex ligands. The extracellular domain displays several significant primary and secondary structural features. It is composed of several imperfectly replicated leucine-rich units of approximately 24 residues each, which, because of there amphiphatic character, are thought to mediate interaction between the hydrophilic hormone and the hydrophobic membrane (Gromoll et al, 1996). The secondary structure is determined by eight highly conserved cystein residues, a structural feature that can also be found in LH receptors and TSH receptors. This suggests a crucial role for the cystein residues in maintaining conformational (Gromoll et al, 1996). The extracellular domain of FSH receptors also has three potential sites for N-linked glycosylation conserved in all species at amino acid positions 191, 199, and 293. Additional species specific glycosylation sites may also be present. Although it was suggested that the glycosylation pattern of FSH receptors may affect hormone binding, recent studies indicate that glycosylation is rather required for correct folding of the receptor protein and transportation to the membrane (Davis *et al*, 1995).

1.5.2 Signal Transduction

FSH stimulation of cAMP production involves three distinct plasma membrane components: the hormone receptor, the nucleotide regulatory or G protein (guanine nucleotide binding protein), and the catalytic moiety of adenylyl cyclase. The receptor and cyclase are responsible for hormone recognition and enzymatic activity, respectively, and the G protein, in the presence of GTP, modulates their activity. After interaction with the hormone, the receptor becomes coupled to the G protein. The FSH activated G protein, termed G_s, stimulates adenylyl cyclase activity and, in turn, production of cAMP (Zhang *et al*, 1991). Association of the receptor to G_s can only occur when the receptor is in an active state. This implies that the receptor exists in an equilibrium between two forms, inactive and active, the second resulting in a conformational change of the inactive form. A summary of events that take place following receptor binding and activation is detailed in Figure 1.3. The interaction between FSH and its receptor determines receptor isomerisation to the active form and initiates a cascade of events collectively indicated as signal transduction.

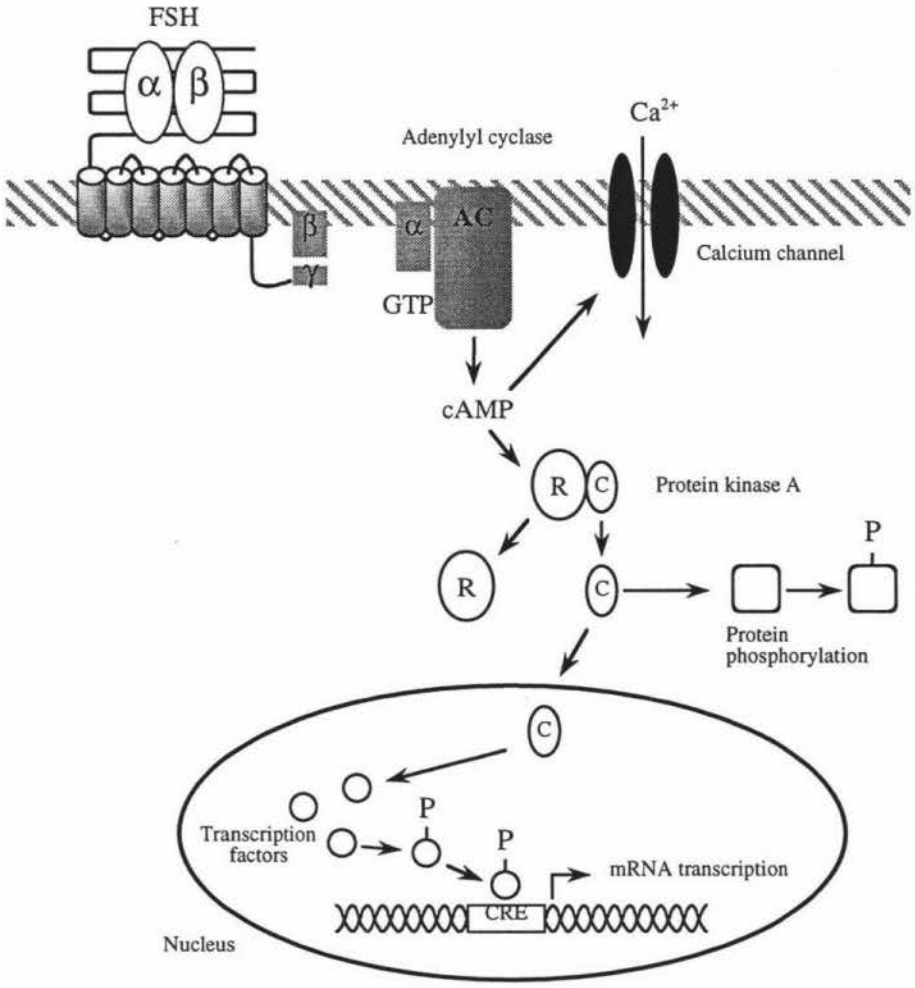


Figure 1.3 Model of signal transduction pathways of the FSH receptor. Upon binding of FSH to the FSH receptor, the G_{sa} subunit dissociates. In conjunction with GTP, this complex directly activates adenylyl cyclase, which produces cAMP from ATP. PKA is activated by cAMP, which leads to the dissociation of the catalytic subunit (C) from the regulatory subunit (R). The active catalytic site can activate proteins and transcription factors by phosphorylation. In addition, the production of cAMP leads to a rise in intracellular Ca^{2+} via the gating of calcium channels

On stimulation by the occupied and activated receptor molecule, the G protein releases GDP and binds GTP. In its GTP-bound conformation, the activated G protein is capable of stimulating adenylyl cyclase. This active complex exhibits GTPase activity, and hydrolysis of the bound GTP to GDP terminates the action of the G protein.

The activation of adenylyl cyclase by FSH and the subsequent synthesis of cAMP therefore triggers a cascade of events that ultimately lead to numerous physiological events in the target tissues including stimulation of granulosa cell proliferation, antrum formation, steroidogenesis, inhibin production, and accumulation of plasminogen activator (Dahl and Hsueh, 1988).

1.5.3 Receptor Desensitization

In the absence of FSH, cultured granulosa cells rapidly lose FSH binding sites, but incubation in the presence of FSH also results in a dose- and time-dependent loss of receptors in a process referred to as down-regulation. Sertoli cells incubated in the presence of FSH exhibit a decrease in adenylyl cyclase sensitivity with a decrease of cAMP production preceding the FSH receptor loss. By modulating the number and functionality of FSH receptors, depending on the presence or absence of FSH, target cells are able to protect themselves from overstimulation.

In addition to an increase in phosphodiesterase activity and consequent cAMP degradation, an early event after receptor stimulation is agonist-induced receptor desensitization, due to uncoupling of the FSH receptor from G_s (Ford and LaBarbera, 1988). Uncoupling occurs through enzymatic phosphorylation of the C-terminal, intracellular domain of G protein-coupled receptors.

As part of the desensitization process, FSH receptor numbers decrease through internalization of hormone-receptor complexes or reduced receptor protein synthesis. Receptor internalization is evident after 1-4 hours and precedes the decrease of receptor protein synthesis. Over longer periods of time, the presence of FSH results in a dose- and time-dependent decrease of functional FSH binding sites in porcine granulosa cells maintained in culture for up to 6 days (Simoni *et al,* 1997). However, at the end of this period, levels of FSH receptor mRNA increase.

1.6 Expression of FSH Receptor in Cell Lines

The use of recombinant DNA technology led to major advances in the understanding of the structure of FSH receptors. Due to the similarity of cloned LH receptors and TSH receptors and the observation that all three glycoproteins act on their respective receptors via the cAMP pathway, it was assumed that the structural design of FSH receptors should also be similar to that of the other glycoprotein hormone receptors. Since Sertoli cells are a specific target of FSH action and do not bind LH, cDNA probes corresponding to selected regions of the LH receptor were used to screen a rat Sertoli cell cDNA library. The isolated cDNA were then transfected into human embryonic kidney cells, which then displayed an FSH-dependent and saturable increase in intracellular cAMP. In contrast, no cAMP stimulation was observed when using human (h) CG or hTSH, indicating the successful cloning of rat FSH receptor (Sprengel *et al,* 1990).

After cloning of the cDNA, the FSH receptors were expressed in a number of cell lines. Both rat and human receptor cDNA were successfully introduced into eukaryotic cell lines and shown to be functional, thus producing a number of recombinant lines useful for studying FSH receptor properties such as hormone binding, signal transduction, and desensitization. Thus, with the advent of FSH receptor cell lines the establishment of novel bioassays for FSH has been made possible.

1.6.1 Assays for FSH

The development of a detection and measurement system is a critical step in identifying hormones and understanding their functions. Generally, the first assays developed were performed *in vivo*, as functions that were possibly regulated by the hormone could be used as endpoints in assays to monitor the purification of that hormone. The major disadvantage of *in vivo* assays the lack of precision, as animal to animal variance is usually high. However, testing hormone preparations in live animals is extremely important in that their functions may be modified by unknown factors *in vivo*, which cannot be predicted by other methods. The standard *in vivo* assay for FSH is the ovarian augmentation test whereby rat ovaries are collected after FSH

treatment and their weights measured. Any increase in ovarian weight is presumed to be due to follicular development induced by FSH (Mannaerts *et al*, 1991).

1.6.1.1 Radioimmunoassay vs. Bioassay

The development of radioimmunoassays (RIA) for gonadotrophins has proved to be a valuable tool for understanding the role of these hormones in reproduction. The major components of an RIA are (1) an antibody that is specific for the hormone to be examined, (2) a radioactively labeled hormone, and (3) the unknown sample or standard. Through competitive binding of the labelled hormone and the hormone in the sample to the specific antibody, the amount of hormone in the unknown sample can be quantified. However, immunological activity may not always correlate with biological activity as antibodies generated against FSH may not recognize all isoforms equally and different antibodies may also recognize different isoforms preferentially. Despite these disadvantages, RIA's are usually far more sensitive than bioassays and do not require the use of animals for primary tissue culture. Therefore, both RIA and specific *in vitro* bioassays are required to further understand gonadotrophin potency and physiology (Ulloa-Aguirre *et al*, 1995).

Considering that neither receptor binding assays (RBA) nor RIAs measure postreceptor events, *in vitro* bioassays have been used to study the biopotency of FSH isoforms at the target cell level. These assays make use of culture systems involving either primary cell cultures or immortalized cell lines. One advantage of using *in vitro* bioassays compared to *in vivo* assays is that many more experimental tests can be performed per experimental animal, as only a small amount of tissue is normally required for each test. In addition, the precision of *in vitro* assays is usually much higher than in *vivo* assays (Mannaerts *et al*, 1991; Braileanu *et al*, 1998).

To evaluate the potency of individual FSH isoforms, bioactivity and activities in either RIAs or receptor binding activity have been compared as the receptor-binding activity versus immunoactivity (RBA/RIA) or the bioactivity versus immunoactivity (B/I) ratio. In early studies, incubation of rat granulosa cells with equivalent concentrations of six different hamster FSH isoforms showed that the less acidic isoforms stimulated plasminogen activator release more than the highly acidic isoforms (Ulloa-Aguirre *et al*, 1988).

1.6.1.2 FSH *in vitro* Bioassays

Numerous *in vitro* assays for FSH have been developed utilizing testicular tissue or granulosa cell preparations for binding assays. These include the stimulation of plasminogen activator in granulosa *cells* (Beers and Strickland, 1978) and estrogen production by Sertoli cells controlled by FSH sensitive aromatase (Padmanabhan *et al*, 1987). However, these assays are ethically costly, as large numbers of animals are required to provide tissue for primary cell culture. In addition, due to the requirements of primary cell cultures, disparities between cultures are difficult to avoid because of variation between individual animals. As a result, an acceptable level of sensitivity may be difficult to obtain in such bioassays.

Recently, the development of a Chinese hamster ovary cell line that stably expresses the human FSH receptor has enabled the development of an FSH bioassay for human FSH (Albanese *et al*, 1994). This bioassay effectively overcomes the main problems associated with more traditional *in vitro* bioassays, namely the ethical cost involved and sensitivity. Therefore, the feasibility of using this cell line for the development of an *in vitro* bioassay for ovine FSH will be examined in this study.

Given the disadvantages of FSH bioassay systems requiring primary tissue culture, this study examines the use of an alternative bioassay system that does not suffer from the same drawbacks. Therefore, described in this study are preliminary experiments used to investigate the FSH stimulated cAMP response of a receptor cell line described by Albanese *et al* (Albanese *et al*, 1994) followed by the validation of an *in vitro* FSH bioassay using ovine sera, purified ovine pituitary extracts, and pituitary cell culture media. The potential use of the bioassay as a tool for investigating heterogeneity in FSH is examined.

Chapter Two

MATERIALS AND METHODS

2 Methods and Materials

2.1 Cell Culture

2.1.1 Biological Materials

Chinese hamster ovary cells that express the human FSH receptor (CHO FSH-R) were a gift from Dr. C. Albanese at Massachusetts General Hospital, Boston, USA and the characteristics of these cells have been described in detail previously (Albanese *et al*, 1994).

2.1.2 Culture Conditions

Cell cultures were performed using standard aseptic techniques with sterile glass and plasticware used throughout, except where noted. All cultures were incubated at standard culture conditions (37°C in a 5% CO_2 atmosphere and with 100% air humidity) in a Cóntherm model 190 CO_2 incubater.

2.1.3 Initiation of Culture

The CHO FSH-R cells were taken from liquid nitrogen, thawed and the culture initiated. Cell numbers were gradually expanded using the following protocol.

A vial of frozen CHO-FSH/R cells was thawed at 37°C for 5 min. Thawed cells were diluted with 12 ml α-Minimum Essential Medium (α-MEM) (Gibco/BRL, Grand Island, USA) culture medium supplemented with 10% fetal calf serum v/v (FCS) (Gibco/BRL), 100 units/ml penicillin (Gibco/BRL), 100 µg/ml streptomycin (Gibco/BRL); and 100 µg/ml geneticin (Gibco/BRL), subsequently referred to as culture medium, in a 15 ml tube and centrifuged at 100 G for 5

minutes. The supernatant was discarded and the cells resuspended in 24 ml culture media. Cells were then distributed equally into each well of a Nunc (Rochester, NY, USA) 6 well culture dish. All culture dishes were then incubated in standard culture conditions until cell growth had reached a stage where they formed a confluent monolayer.

2.1.4 Trypsinisation of Cells

Once the CHO FSH-R cells formed a confluent monolayer the cultures were passaged by removing the cells from the culture flask surface and reseeding new tissue culture flasks at a cell density of 50,000 – 200,000 cells/ml.

Removal of the cells was begun by washing the cells twice in ice-cold 50 mM phosphate buffered saline (PBS) and then incubating them in ice cold PBS + 0.25% trypsin w/v (Gibco/BRL) at a depth of 2 mm for 2 minutes at room temp. This solution was then poured off after which the cells were incubated for a further 5 minutes at 37°C.

Any remaining trypsin was neutralized by the addition of 50 ml culture medium. Cells were then dislodged from the surface of the flask by gentle agitation in culture medium. Detached cells were transferred to a 50 ml Falcon tube and centrifuged at 100 G. for 10 min. The supernatant was then discarded and the cells re-suspended in 10 ml culture medium. New Nunc 800 ml culture flasks were then re-seeded at a cell density of 50,000 – 200,000 cells/ml.

Cultures were allowed to grow until they formed a confluent monolayer, after which they were dislodged from the culture flask using trypsinisation and re-seeded into new tissue culture flasks, frozen down for storage, or dispensed into multiwell culture dishes for use in the FSH bioassay.

All stock solutions of antibiotics were suspended in MilliQ water and sterilised through a 0.2 μm filter.

2.1.5 Cryostorage of Cells

For long-term storage, CHO-FSH/R cells were kept frozen in liquid nitrogen.

CHO-FSH/R cells were bulked up in 800 ml Culture flasks, trypsinized and harvested into α-MEM + 50% FCS v/v. The volume of media used was adjusted to give approximately 2.0 x 10^7 cells/ml.

Cells were then diluted by an equal volume of ice cold α-MEM + 30% dimethyl sulphoxide (DMSO) (Sigma, St. Louis, MO, USA) + 10% FCS v/v and dispensed into 1.5 ml Nunc cryostat vials in 1 ml aliquots containing approximately 1.0 x 10^7 cells. Cells were gradually cooled to –70°C over 24 hours in a polystyrene container placed in a –70°C freezer. The cells were then submerged in liquid nitrogen for storage.

2.2 FSH Bioassay Methodology

The FSH bioassay was based on the ability of preparations of FSH to stimulate a dose dependent accumulation of cAMP by cultured CHO FSH-R cells.

Cultured CHO FSH-R cells were trypsinized and distributed into Costar (Corning, NY, USA) 48 well culture dishes in 1ml of culture medium and incubated for 16 hr in standard culture conditions.

After incubation, the CHO FSH-R cells were prepared by washing twice with 250 ml 50 mM PBS + 100 units/ml penicillin + 100 µg/ml streptomycin, followed by the addition to all wells of α-MEM + 100 units/ml penicillin + 100µg/ml streptomycin + 0.25 mM 3-isobutyl-1-methyl-xanthine (IBMX) (Sigma) + 0.1% bovine serum albumin w/v (BSA) (ICP, Auckland, NZ), hence referred to as assay medium. The volume of assay medium used varied depending on the type of sample to be assayed and in some cases the expected range of FSH concentrations in the samples.

2.2.1 Assay of Samples

After the cells had been plated, washed, and immersed in assay medium, the bioassay was begun by adding the standards, controls, and samples to wells in triplicate and incubating for 4 hours in standard culture conditions. The volume of the additions to the wells varied depending on the type of sample to be assayed and the expected range of FSH concentrations in the samples. All additions were made to wells in a random position in the culture dish to minimise the effects of variation in culture conditions that may occur in different parts of the culture dish.

After incubation, the assay medium in each well was transferred from the plates to microtitre tubes and stored at –20°C for later assessment of extra-cellular cAMP content by RIA.

A standard curve was generated by plotting the concentration of FSH standard against cAMP production using a four parameter logistic curve fitting algorithm. FSH concentrations of the samples were then determined by interpolation from the standard curve.

2.2.2 FSH Standards

NIH-FSH-RP2 (RP2) standards from the National Hormone and Pituitary Program of the NIH (Bethesda, MA, USA) were prepared in 50mM PBS + 1% BSA w/v, 20 mM Tris-HCl, pH 8.6, serum from hypophysectomised sheep (hypox), or Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/BRL) + 10% hypox v/v depending on the subtrate of the sample to be measured. A variety of solutions were used to prepare the FSH standard to ensure all cells throughout an assay were exposed to similar conditions. Standards were prepared on the day of each assay with a volume of 10, 50 or 100 µl, depending on the volume of sample to be assayed

2.2.3 Internal Controls

Internal control samples were prepared by the addition of known quantities of FSH to a blank solution appropriate to the type of sample being assayed. All controls were prepared to the following specifications and stored at –70°C in 0.5 ml aliqouts.

Controls for assays of serum samples consisted of 0.5, 1.2, 3.0 and 8.0 ng/ml of RP2 in hypox. Controls for assays of purified pituitary extracts consisted of 2.5, 6.0, 16.0 and 40.0 ng/ml of RP2 in 20 mM tris-HCl, pH 8.6. Controls for assays of pituitary culture media consisted of 2.5, 6.0 and 16.0 ng/ml of RP2 in DMEM + 10% hypox v/v.

2.3 cAMP Radioimmunoassay

Frozen media from the FSH bioassay was thawed and assayed for cAMP by RIA. The cAMP RIA was developed by the Reproductive Physiology Group, Wallaceville Animal Research Centre, AgResearch (Henderson *et al*, 1987).

2.3.1 cAMP RIA Materials

Primary and secondary antibodies used in the cAMP RIA were developed in-house by the Reproductive Physiology Group. The primary antisera used is a rabbit anti-cAMP, while the secondary antisera is a sheep anti-rabbit IgG.

The radio-labeled tracer used in the cAMP RIA was produced as described in section 2.3.

2.3.2 cAMP Iodination Procedure

10 ml of Sephadex G10 (Pharmacia Fine Chemicals, AB, Uppsala, Sweden), which had previously been swelled overnight in 20 mM sodium acetate (NaAc) buffer, was dispensed on top of a 3-4 mm diameter glass ball in a 20 ml column. The column was then washed once with 1 ml 3% BSA w/v in 20 mM NaAc buffer followed by a wash with 50 ml of 0.1% BSA w/v in NaAc buffer (column buffer).

1 mCi of ^{125}I (New England Nuclear, Life Sciences Produces Inc, Boston, Ma, USA) in 10 µl was added to a 5ml polystyrene test tube containing 5µg cAMP (Sigma) in 50 µl of PBS. The

iodination reaction was then begun with the addition of 50 µl of 12.5 mM Cloramine T ($C_7H_7SO_2NNaCl.3H_20$) in 50 mM PBS. The tube was mixed and after 60 seconds the reaction was stopped by the addition of 100 µl of 12.5 mM sodium metabisulphite ($Na_2S_2O_5$) in 50 mM PBS. The content of the tube was then transferred to the column.

The column was then eluted with column buffer and fractions consisting of 26 drops (2 ml) were collected. Fractions 30-35 were pooled and kept for use in the cAMP RIA. All other fractions were discarded.

2.3.3 cAMP RIA Procedure

The cAMP RIA used is a competitive binding assay where a known quantity of ^{125}I labeled cAMP and an unknown quantity of unlabeled cAMP compete for binding to a primary antibody. cAMP bound to the primary antibody is then separated from the unbound fraction by precipitation using a secondary antibody, PEG 8000 (Union carbide Corp., Danbury, CT, USA) and centrifugation. The amount of radioactive cAMP bound to the primary antibody can then be quantified using a gamma radiation counter.

cAMP standards were added to in triplicates to 5ml polystyrene tubes in a volume of 100 µl at 0, 50, 100, 200, 500, 1000, 2000, 5000, 10000 and 20000 fMol/tube in fresh assay. 100, 1000, and 2000 fMol/tube cAMP internal controls in 100 µl of fresh assay medium was added into duplicate tubes and distributed randomly throughout the assay. Duplicate samples of 100 µl assay medium from the FSH bioassay were added to sample tubes.

To all standard, control and sample tubes was added 100 µl ^{125}I cAMP label in 50 mM NaAc + 3% w/v BSA with an activity of approximately 12,000 counts per minute (CPM). This was followed by the addition of 100 µl 1° antisera at a dilution of 1/20,000 in 50 mM NaAc + 0.1% BSA + 0.1% normal rabbit serum (NRS) (Gibco/BRL).

As a reference for the total CPM (Total), 100 µl of the same ^{125}I cAMP solution added to the previous tubes was also added in triplicate to 5 ml tubes. Non-specific binding (NSB) was measured using triplicate tubes with 100 µl of the same of ^{125}I cAMP solution and 100 µl of 50

mM NaAc + 0.1% BSA + 0.1% NRS without 1° antiserum. All tubes were shaken and then placed in a large air-tight container and incubated for 16 hours at 4°C.

After incubation, to all tubes except Totals was added 100 µl of 2° antisera at a dilution of 1/50 in 50 mM NaAc + 30% normal sheep serum (NSS) (Gibco/BRL). Tubes were then shaken and incubated at room temperature for 60 minutes.

After incubation, 1 ml of 10% w/v PEG 8000 in 50 mM PBS was added to all tubes except Totals. The tubes were then thoroughly mixed. All tubes except Totals were then centifuged in a refrigerated centrifuge at 8°C for 30 minutes at 4000 RPM (3992 G). The supernatant was then discarded and the CPM of all the tubes was measured on a Wallac Oy model 1261 Multigamma (Turku, Finland) gamma radiation counter.

A standard curve was generated by plotting cAMP concentration against bound CPM by using a four parameter logistical curve fitting algorithm in the program Multicalc (Wallac Oy, Turku, Finland). The concentrations of cAMP in the samples was then interpolated from the standard curve in Multicalc. Using the cAMP concentrations, the levels of FSH in the samples can be calculated.

2.4 Calculation of FSH Concentrations

FSH concentrations of the samples assayed in the bioassay were calculated using cAMP values of the assay medium determined by RIA. The cAMP concentrations of the FSH standard curve were analysed in the program SigmaPlot 2.0 (Jandel GmbH, San Raphael, CA, USA) using a four parameter logistic curve-fitting algorithm. From this analyses four parameters of the curve are obtained: the asymptotic maximum (a); the slope (b); the value at the inflexion point (c); and asymptotic minimum (d). Using these parameters, FSH concentrations of the unknowns can then be calculated in Excel (Microsoft Corporation, Redmond, WA, USA) using the formula:

$$y = \left(\left(\frac{a - d}{x - d} \right) - 1 \right)^{\frac{1}{b}} \times c$$

Chapter Three

PRELIMINARY EXPERIMENTS

3 Preliminary Experiments

A number of preliminary studies were conducted to evaluate the potential of the CHO FSH-R cell line for use in a viable bioassay for ovine FSH.

3.1 Time Course Evaluation

A pilot study was conducted to determine the basal and FSH stimulated accumulation of cAMP in cultured CHO FSH-R cells. The effects of different incubation times were also examined to ascertain the incubation time that provided an acceptable level of cAMP accumulation for assay purposes and that was also practical to use from a logistical point of view.

3.1.1 Experimental Method

Except where noted, the methods and materials used were as described in section 2.2.

Dispersed CHO FSH-R cells were plated into Nunc 24 well culture dishes at cell densities of either $1x10^5$ per well or 5×10^5 per well and incubated for 16 hours. The media was replaced with 990 µl of assay medium and the cells stimulated with 10 µl of 0, 5 or 200 ng/ml RP2 FSH in 50 mM PBS + 1% BSA in triplicate wells. Total volume of liquid per well was 1 ml. All plates were then incubated in standard culture conditions. The reactions were stopped at 0, 1, 2, 4, 8 and 16 hours by removal of all media from the appropriate wells. This media was kept and stored at –20°C until assayed by for cAMP by RIA at a later date.

3.1.2 Results

Accumulation of cAMP was seen in all wells incubated for 1 or more hours, as shown in Figure 3.1. There was no significant difference ($P > 0.05$) in cAMP accumulation, as assessed by *t* test performed in SigmaPlot, between wells treated with 0 and 5 ng/ml FSH at any time point examined. However, wells treated with 200 ng/ml FSH showed a significant increase ($P < 0.05$)

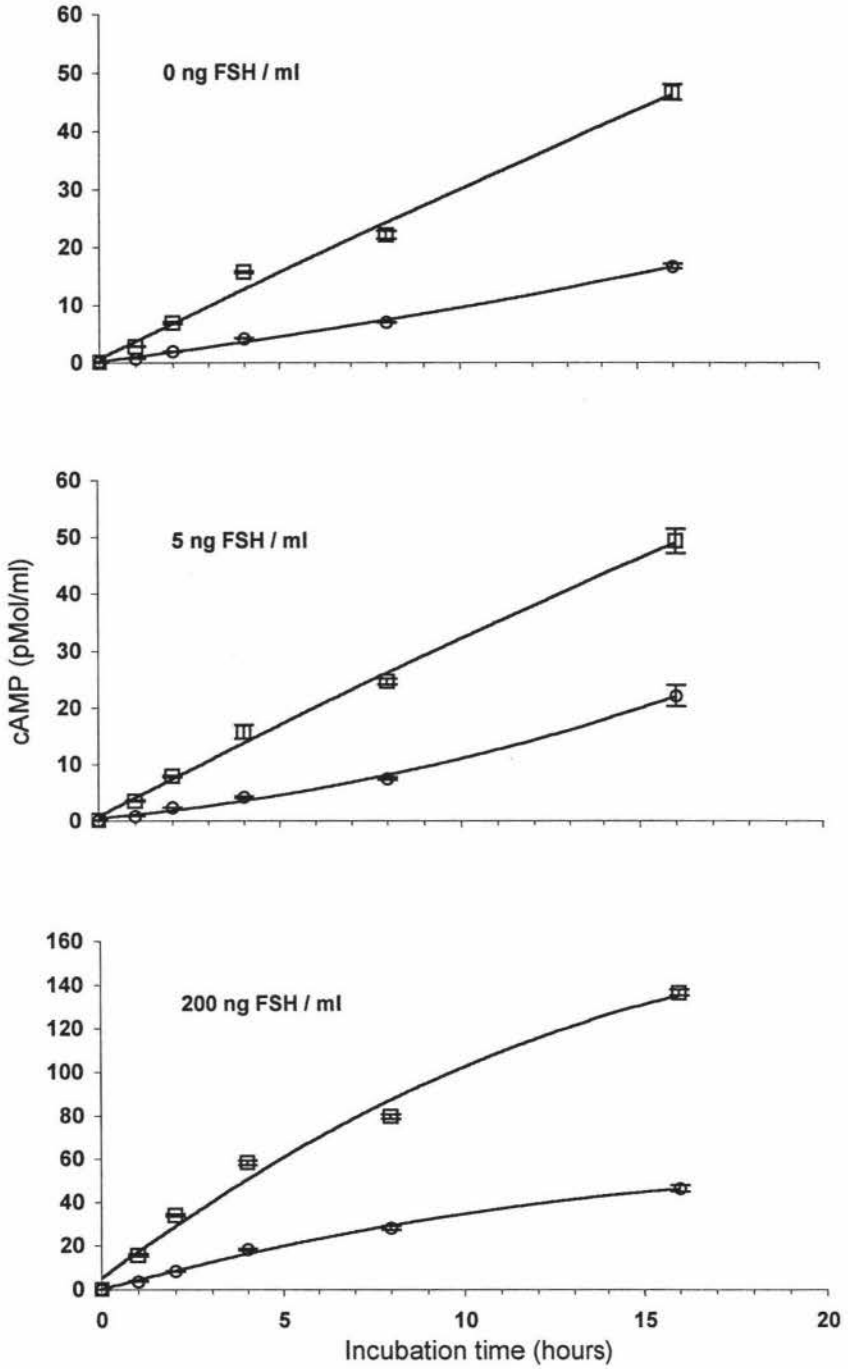


Fig 3.1 Accumulation of cAMP by CHO FSH-R cell cultures of 1 x 10^5 cells/well (O) and 5 x 10^5 cells/well (□) in response to oFSH stimulation over time. Points represent mean of triplicates and error bars represent SEM.

in cAMP accumulation after incubation for 1 or more hours. The accumulation of cAMP was significantly higher in wells with $5x10^5$ cells than those with $1x10^5$ cells/well.

From this data, an incubation time of 4 hours was chosen for further experiments.

3.2 Response to Different Forms of FSH

The cAMP response to FSH from a variety of species by the CHO FSH-R cells transfected with the human FSH receptor gene was examined in this experiment. In a previous study, a preparation of ovine FSH was found to be 10 fold less bioactive than human FSH (Albanese *et al*, 1994).

3.2.1 Experimental Method

The response of the CHO FSH-R cells to a variety of forms of FSH was examined in this experiment. A summary of the biological materials used in this experiment is listed in Table 3.1.

Except where noted, the methods and materials used were as described in section 2.2.

Costar 48 well culture plates were seeded with 1 x 10^5 cells/well and incubated for 16 hours. The media in each well was replaced with 990 µl of fresh assay medium and cells were challenged with 10 µl of different forms of FSH in 50 mM PBS + 1% BSA in triplicate wells at the concentrations detailed below:

RP2	0, 0.8, 2.1, 5, 12.8, 32, 80, 200 ng/ml
oFSH	1, 10, 100, 1000, 1^4, 5 x 10^4 ng/ml
pFSH	10, 100, 1000, 10^4, 10^5, 2 x 10^5 ng/ml
pFSH	10, 100, 1000, 10^4, 10^5, 2 x 10^5 ng/ml
hFSH	10, 100, 1000, 10^4, 10^5, 2.5 x 10^5 ng/ml
bFSH	10, 100, 1000, 10^4, 10^5, 2.5 x 10^5 ng/ml
Ovagen	100, 10000, 10^4, 10^5, 10^6, 10^7 ng/ml
Ova2	10, 100, 1000, 10^4, 10^5, 5 x 10^5 ng/ml
P2	10, 100, 1000, 2000, 10^4, 4 x 10^4 ng/ml

All plates were incubated for 4 hours in standard culture conditions, after which the media was collected from the plates and stored at -20°C until assayed for cAMP by RIA.

3.2.2 Results

All forms of FSH induced an increase in cAMP secretion from the CHO FSH-R cells, as can be seen in Figure 3.2. However, there was variation in the effectiveness of the different forms of FSH in stimulating an accumulation of cAMP. The cells were most responsive to stimulation with Ova2, which was approximately 5-fold more bioactive than the human FSH preparation tested. Ovagen, an ovine pituitary preparation used in the superovulation of sheep, and bovine FSH exhibited the least bioactivity. Of all the FSH preparations tested, there was approximately a 100-fold difference in biopotency, as measured by the difference in FSH concentrations at the point midway (inflexion point) of each curve. In an idealised four parameter logistic curve, the inflexion point represents the part of the curve that exhibits radial symetry, and is therefire the steepest part of the curve.

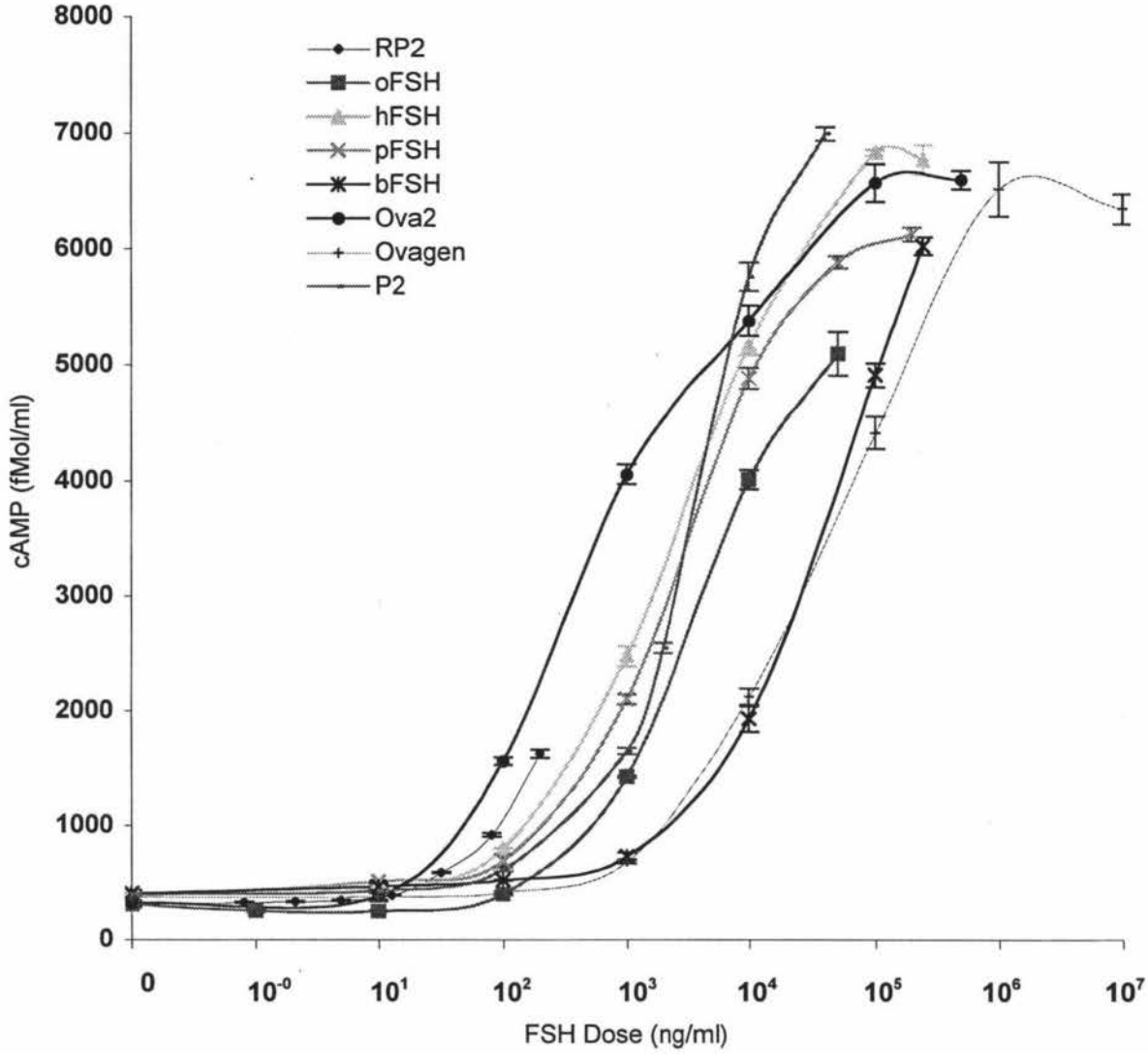


Fig 3.2 Accumulation of cAMP by CHO FSH-R cells in response to varying doses of different forms of FSH. Each point represents the mean of triplicates and error bars represent SEM.

3.3 Cross Reactivity

The specificity of the cAMP response in CHO FSH-R cells to FSH was examined by comparing the response of the cells to RP2 FSH and α-FSH, β-FSH and a number of other pituitary hormones.

3.3.1 Experimental Method

Except where noted, the methods and materials used were as described in section 2.2. A summary of the biological materials tested in this experiment is listed in Table 3.1.

Costar 48 well culture plates were seeded with 1 x 10^5 cells and incubated for 16 hours. The media was then replaced with 990 µl of assay medium and wells treated with 10 µl of various pituitary hormones in 50 mM PBS + 1% BSA in triplicate wells at the following concentrations:

RP2	0, 1, 100, 1000, 10^4, 10^5, 1.65 x 10^6 ng/ml
α-FSH	10, 100, 1000, 10^4, 10^5, 5 x 10^5 ng/ml
β-FSH	10, 100, 1000, 10^4, 10^5, 5 x 10^5 ng/ml
vasopressin	100, 1000, 10^4, 10^5, 10^6, 10^7 ng/ml
ovine LH	10, 100, 1000, 10^4, 10^5, 8.5 x 10^5 ng/ml
ovine prolactin	10, 100, 1000, 10^4, 10^5, 8.5 x 10^5 ng/ml
ovine GH	10, 100, 1000, 10^4, 10^5, 1.65 x 10^6 ng/ml
ovine TSH	10, 100, 1000, 10^4, 10^5, 1.65 x 10^6 ng/ml

All plates were incubated for 4 hours in standard culture conditions. Media was then collected from the plates and stored at -20°C for later analysis by cAMP RIA.

Table 3.1 Description of biological materials used in CHO FSH-R cell bioassay

Name	Description	Manufacturer
RP2	purified ovine FSH standard (NIH-FSH-RP2)	National Hormone and Pituitary Program of the NIH (Bethesda, MA, USA)
oFSH	ovine FSH	Bioscan (Quebec, Canada)
pFSH	porcine FSH	UCB-Bioproducts S.A. (Chemin du foriest, Belgium.)
hFSH	human FSH	UCB-Bioproducts S.A. (Chemin du foriest, Belgium.)
bFSH	bovine FSH	UCB-Bioproducts S.A. (Chemin du foriest, Belgium.)
Ovagen	crude pituitary FSH preparation for commercial use	ICP (Auckland, New Zealand)
Ova2	purified preparation of Ovagen	courtesy of Lloyd Moore, AgResearch, NZ
P2	purified possum pituitary FSH preparation	courtesy of Lloyd Moore, AgResearch, NZ
α-FSH	α sub-unit of ovine FSH	Bioscan (Quebec, Canada)
β-FSH	β sub-unit of ovine FSH	Bioscan (Quebec, Canada)
vasopressin		Sigma (St. Louis, MO, USA)
oLH	purified ovine LH standard (NIADDK-oLH-25)	NIADDK (Torrance, CA, USA)
prolactin	purifed ovine prolactin standard (NIH-P-S13)	National Hormone and Pituitary Program of the NIH (Bethesda, MA, USA)
oGH	purifed ovine GH standard (NIH-GH-S11)	National Hormone and Pituitary Program of the NIH (Bethesda, MA, USA)
oTSH	purified ovine TSH standard (NIADDK-oTSH-12)	NIADDK (Torrance, CA, USA)

3.3.2 Results

Cultured CHO FSH-R cells treated with varying doses of α-FSH, β-FSH, vasopressin, oLH, ovine prolactin, oGH, or oTSH exhibited no significant increase in cAMP accumulation compared to basal levels until a treatment dose of 1 x 10^3 ng/well (1 x 10^5 ng/ml) was reached. As expected, treatment with RP2 resulted in a dose dependent increase in cAMP accumulation starting from a dose of 1 ng/well (100 ng/ml). A comparison of the cAMP dose response curve of RP2 to that of the other hormones tested shows a cross-reactivity of < 0.1%, as measured by the difference between concentrations of the hormones at the inflexion points of each curve. These results are represented in Figure 3.3.

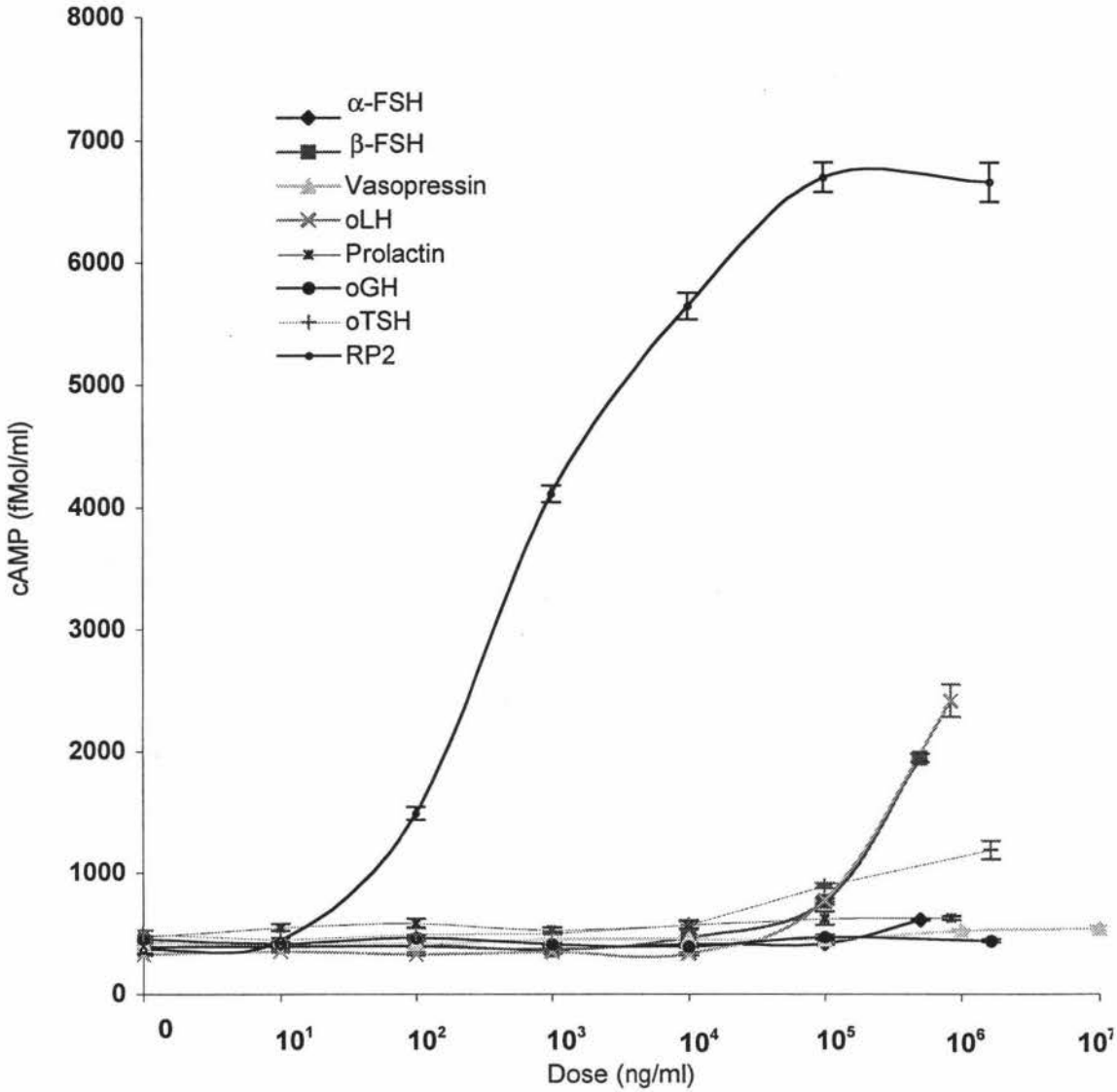


Fig 3.3 Accumulation of cAMP by CHO FSH-R cells in response to varying doses of different pituitary hormones. Each point represents the mean of triplicates and error bars represent SEM.

3.4 Serum Effects

To determine the suitability of the CHO FSH-R cells for assaying bioactive FSH in serum, the effect of serum on the cAMP response of the cells to FSH was examined in this set of experiments. The effects of varying total media volume per well and treatment with serum on cAMP secretion were also examined.

3.4.1 Experimental Method

In this experiment the effect of serum on the accumulation of extra-cellular cAMP in cultured CHO FSH-R cells was examined. Except where noted, the methods and materials used followed the protocol as described in section 2.2. Six Costar 48 well culture plates were plated with varying cell densities and assay medium volumes in the following combinations:

plate 1	1×10^5 cells/well + 470 µl assay medium
plate 2	5×10^5 cells/well + 470 µl assay medium
plate 3	1×10^5 cells/well + 220 µl assay medium
plate 4	5×10^5 cells/well + 220 µl assay medium
plate 5	2.5×10^5 cells/well + 140 µl assay medium
plate 6	5×10^5 cells/well + 140 µl assay medium

All wells in plates 1 to 4 were treated with 10 µl of RP2 in 50 mM PBS + 1% BSA at 0, 2, 5 or 200 ng/ml in triplicate wells. Plates 5 and 6 were treated with 10 µl of RP2 in 50 mM PBS + 1% BSA at 0, 0.8, 2.1, 5, 12.8, 32, 80 or 200 ng/ml in triplicates.

Plates 1 to 4 were also treated with 0, 10, 15 or 20 µl of serum from hypophysectomised sheep (hypox). Hypox was used instead of normal sheep serum, as it contained negligible amounts of FSH. Wells that had received less than 20 µl of hypox were also treated with assay buffer such that the total volume of hypox and assay buffer added equalled 20 µl.

Plates 5 and 6 were treated with 50 or 100 µl of hypox. A further 50 µl of assay buffer was added to the wells that had received 50 µl of hypox.

All plates were incubated for 4 hours in standard culture conditions. Media was then collected from the plates and stored at -20°C for later analysis by cAMP RIA.

3.4.2 Results

The effect of serum treatment and different media volume on cAMP production in CHO FSH-R cells at different cell densities is shown in Figures 3.4 to 3.6. Treatment with hypox resulted in a decrease in cAMP secretion compared with control wells at all cell densities, assay medium volumes, and doses of FSH treatment. In most cases, increasing doses of hypox resulted in greater inhibition of cAMP secretion. However, even when the levels of serum added resulted in a final serum concentration of 40% per well (Figure 3.6), there was still a significant cAMP accumulation in response to treatment with 10 µl of FSH at 32 ng/ml, which is equivalent to 0.32 ng/well of FSH.

A wide variety of conditions were tested to determine the set of conditions that resulted in the largest difference in signal transduction between stimulation with 0 and 2 ng/well RP2 FSH.

In wells that had a total media volume of 500 µl/well and a cell density of 1 x 10^5 cells/well, there was a 3.7 fold increase in cAMP accumulation between 0 and 2 ng/well RP2 with the addition of 20 µl/well of serum, and a 3.9 fold increase with 0 µl/well serum. The same conditions except with a cell density of 5 x 10^5 cells/well resulted in a 4.1 and 4.8 fold increase with 20 and 0 µl serum, respectively.

Lowering the total media volume to 250 µl/well but keeping the same cell density at 5 x 10^5 cells/well resulted in a 5.2 fold increase in cAMP secretion between 0 and 2 ng/well RP2 with 20 µl/well of serum added, and a 6.3 fold increase with 0 µl/well serum. Assay conditions of 250 µl/well media and 1 x 10^5 cells/well resulted in a 4.8 and 6.6 fold increase with the addition of 20 and 0 µl/well serum, respectively.

In the last set of conditions tested, the effect of large volume of serum (50 and 100 µl/well) was examined in plates wells containing 250 µl/well total media volume (after addition of standards and serum) and 5 x 10^5 or 2.5 x 10^5 cells/well. In wells with 5 x 10^5 cells/well, the addition of 100 and 50 µl/well of serum resulted in a 4.4 and 4.8 fold increase in cAMP accumulation between treatment with 0 and 2 ng/well FSH, respectively. Wells that had 2.5 x 10^5 cells/well showed a 4.5 and 5.4 fold increase after the addition of 100 and 50 µl/well of serum, respectively.

In summary, the maximum fold increase in cAMP accumulation following stimulation with RP2 FSH was found in wells containing 250 µl of assay medium per well. Increasing doses of serum did not greatly affect the maximum fold response of the cells, although absolute levels of cAMP were reduced. Therefore, in further experiments with serum an assay volume of 100 µl of sample or standard and a total media volume of 250 µl per well was chosen to maximise sensitivity.

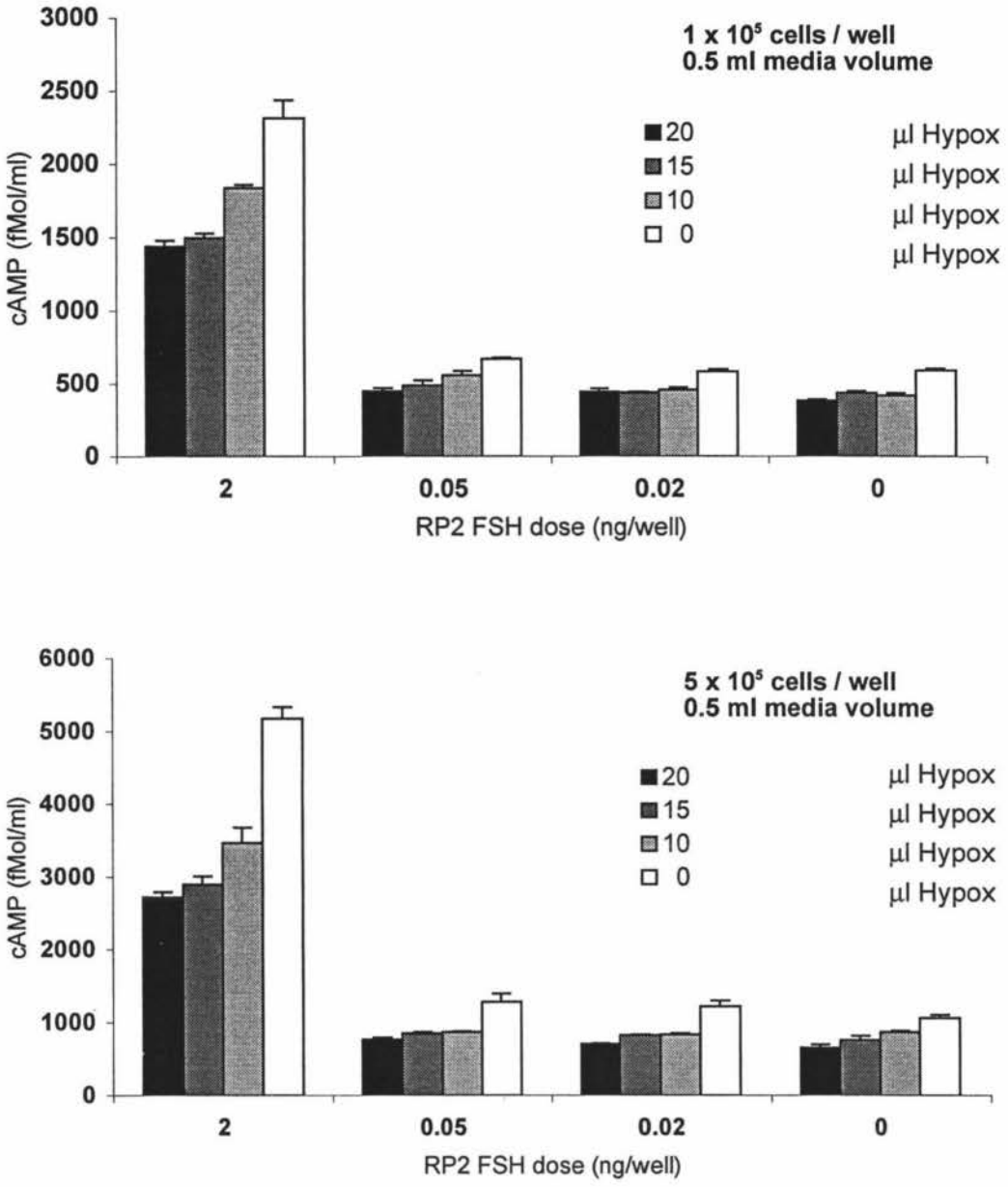


Fig 3.4 Accumulation of cAMP by CHO FSH-R cells in response to treatment with RP2 FSH and serum from hypophysectomised sheep (hypox). Each bar represents the mean of triplicates and error bars represent standard error of the mean.

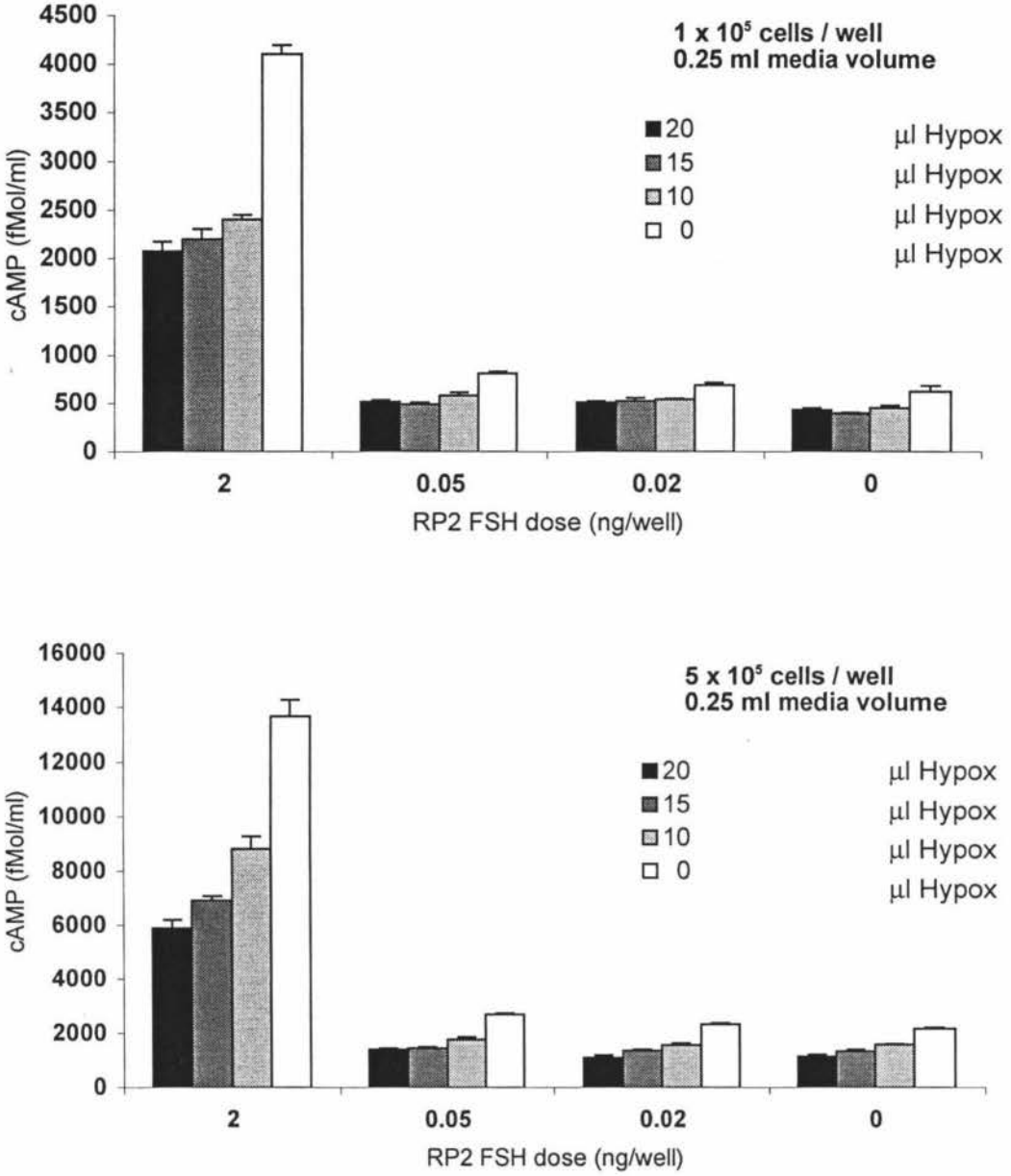


Fig 3.5 Accumulation of cAMP by CHO FSH-R cells in response to treatment with RP2 FSH and serum from hypophysectomised sheep (hypox). Each bar represents the mean of triplicates and error bars represent standard error of the means.

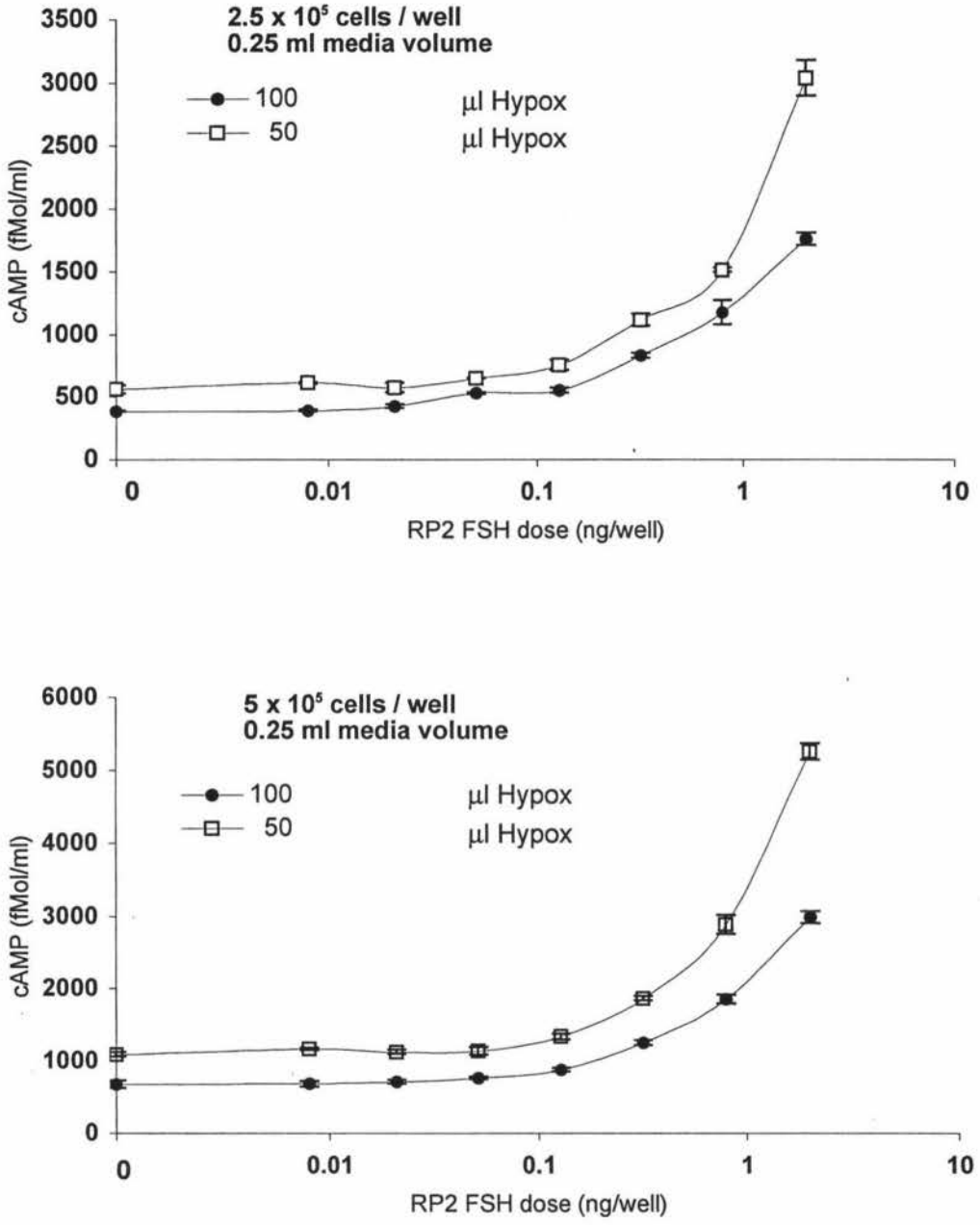


Fig 3.6 Accumulation of cAMP by CHO FSH-R cells in response to treatmen with RP2 FSH and serum from hypophysectomised sheep (hypox). Each poin represents the mean of triplicates and error bars represent standard error of th means.

3.5 Discussion

The results of these preliminary experiments indicated that the CHO FSH-R cells were capable of responding to ovine FSH in a dose dependent manner under a variety of assay conditions, such as varying culture times, cell densities, and total media volumes (Figures 3.1, 3.4, 3.5 and 3.6). The accumulation of cAMP in the receptor cell line bioassay is highly specific to FSH (Figure 3.3), with the cross-reactivity of non-FSH hormone preparations probably accounted for by contamination with FSH (Albanese *et al*, 1994).

From the data obtained by the time course evaluation (Figure 3.1), an incubation time of 4 hours was chosen for all subsequent assays as this represented the most logistically practical time period to use where the increase in cAMP compared to 0 hours was deemed sufficient.

It is interesting to note that the ovine FSH preparation, Ova2, exhibited the highest biopotency in the bioassay, rather than the human FSH preparation tested (Figure 3.2). This is in contrast to the results obtained by Albanese *et al* (1994) for whom preparations of rat and human FSH had 10-fold higher biopotency than the ovine FSH standard (S14) tested. This, data suggests that the biopotency of a preparation of FSH is more a function of its purity rather than its species of origin. The biopotency of a preparation of possum FSH reported by Moore *et al* was shown to be 16% that of the ovine FSH tested (Moore *et al*, 1997). A purified version of the same preparation of possum FSH (P2) was shown in this study to have a biopotency greater than that of Bioscan ovine FSH.

In the experience of the Reproductive Physiology Group, the addition of serum leads to a reduction in sensitivity of the rat Sertoli cell FSH bioassay as described by Padmanabhan, *et al* (1987). The reasons for this effect are not completely understood due to the complex composition of serum, but probably stem from the presence of various enzyme inhibitors and binding proteins.

Serum clearly had an inhibitory effect on the FSH stimulated and basal secretion of cAMP by the CHO FSH-R cells (section 3.3.2). Furthermore, the addition of serum also causes a reduction of the signal increase between a treatment of 0 and 2 ng/well of RP2 FSH, at all cell densities and

media volumes tested. However, was no discernible difference in effect between the addition of a small (20 µl) and large (100 µl) volume of serum.

From this data (section 3.3.2), an assay volume of 100 µl was selected for the assay of serum samples, where possible. There was also no obvious difference in the rate of increase in cAMP secretion with the treatment of 0 and 2 ng/well RP2 in wells with 1×10^5 or 5×10^5 cells/well. Therefore, a cell density of 2.5×10^5 cells/well was selected for subsequent assays. A total media volume of 250 µl/well was chosen for subsequent assays to lessen the effect of sample dilution in the assay.

Chapter Four

FSH IN SERUM

4 FSH in Serum

The receptor cell line bioassay was used to assay a variety of serum samples from several different sources. Where possible, bioactive FSH results were compared to immunoactive FSH results obtained by RIA.

Serum samples from intact sheep were provided by Dr. Keith Henderson, AgResearch, NZ. Serum samples taken from ewes around the time of hypophysectomy were provided by Norma Hudson, AgReasearch, NZ. Serum samples from ovarectomised ewes were provided by Stan Lun, AgResearch. Serum samples from ovine hypophyseal venous bleeds were kindly provided by Dr. Iain Clarke, Monash University, Melbourne, Australia.

4.1 Parallelism

The receptor cell line FSH bioassay for ovine serum was validated by comparing parrallelism of serially diluted pools of sheep serum with RP2 FSH reference standard (NIH-FSH-RP2).

4.1.1 Experimental Method

Parallelism of serum samples in the FSH bioassay was determined using two pools of serum from ovariectomised ewes diluted with hypox. Ovariectomised ewe serum was assayed at 100, 75, 50 and 25 µl with 6 replicates. Hypox was added to wells that had received less than 100 µl of serum so that the volume of sample and hypox equaled 100 µl. All plates used were seeded with 2.5×10^5 cells/well and contained 150 µl assay medium per well. Controls and standards (0, 0.8, 2.1, 5.1, 12.8, 32, 80, 200 and 1000 ng/ml in hypox) were added to the appropriate wells at a volume of 100 µl in triplicates. After incubation, media was collected from the plates and assayed for cAMP.

4.1.2 Results

The cAMP dose response curves obtained with diluted ovariectomised ewe sera were parallel to the standard curve obtained with RP2 (Figure 4.1), as indicated by *t* test for non-parallelism ($P > 0.05$) performed in SigmaPlot. The bioactive FSH concentrations of the diluted sera samples are shown on Table 4.1. A very close correlation between the observed data and values predicted by linear regression were observed ($R^2 = 0.9967$ and 0.9936). Intra- and interassay coefficients of variation were 15% ± 2.5% SEM and 15.8% ± 1.7% SEM, respectively (n=3). The conclusion drawn from these results is that the CHO FSH-R cells are suitable for use in measuring bioactive FSH in serum, and that RP2 FSH is an appropriate standard for generating a standard curve.

4.2 Assay Characteristics

Intra- and interassay coefficients of variation and sensitivity of the bioassay for serum samples were calculated from internal control in assays performed in sections 4.3, 4.4 and 4.5.

4.2.1 Sensitivity

The sensitivity of an assay is a measurement used to determine the minimum detectable concentration of the substance being assayed, also known as the detection limit. For each assay the standard deviation of the cAMP concentrations of the blank FSH standard (0 ng/ml FSH) was calculated. Sensitivity at the 95% confidence interval of each assay was then determined by calculating the FSH value represented by the mean cAMP concentration plus 2 standard deviations of the blank FSH standard. The mean sensitivity of the FSH bioassay for each type of serum assayed is shown in Tables 4.2, 4.3 and 4.4. Assays of serum samples from intact sheep had a mean sensitivity of 1.16 ng/ml RP2 FSH ± 0.19 SEM ($n = 9$, $P < 0.05$). Assays of serum samples from ovariectomised ewes before and after hypophysectomy had a mean sensitivity of 0.85 ng/ml RP2 FSH ± 0.10 SEM ($n = 9$, $P < 0.05$). The sensitivity of the bioassay for hypophyseal bleed samples was 1.42 ng/ml RP2 FSH ± 0.20 SEM ($n = 16$, $P < 0.05$)

Table 4.1 Mean bioactive FSH concentrations in serially diluted pooled serum from ovariectomised ewes.

	µl Serum	µl Hypox	Mean B-FSH (ng/ml)	%CV	Slope	R^2
Pool 1	100	0	**8.9**	18.1	0.4175	0.9967
	75	25	**7.2**	22.1		
	50	50	**4.9**	11.9		
	25	75	**2.8**	26.1		
Pool 2	100	0	**8.5**	10.0	0.4210	0.9936
	75	25	**5.9**	9.3		
	50	50	**4.1**	10.0		
	25	75	**2.1**	16.7		

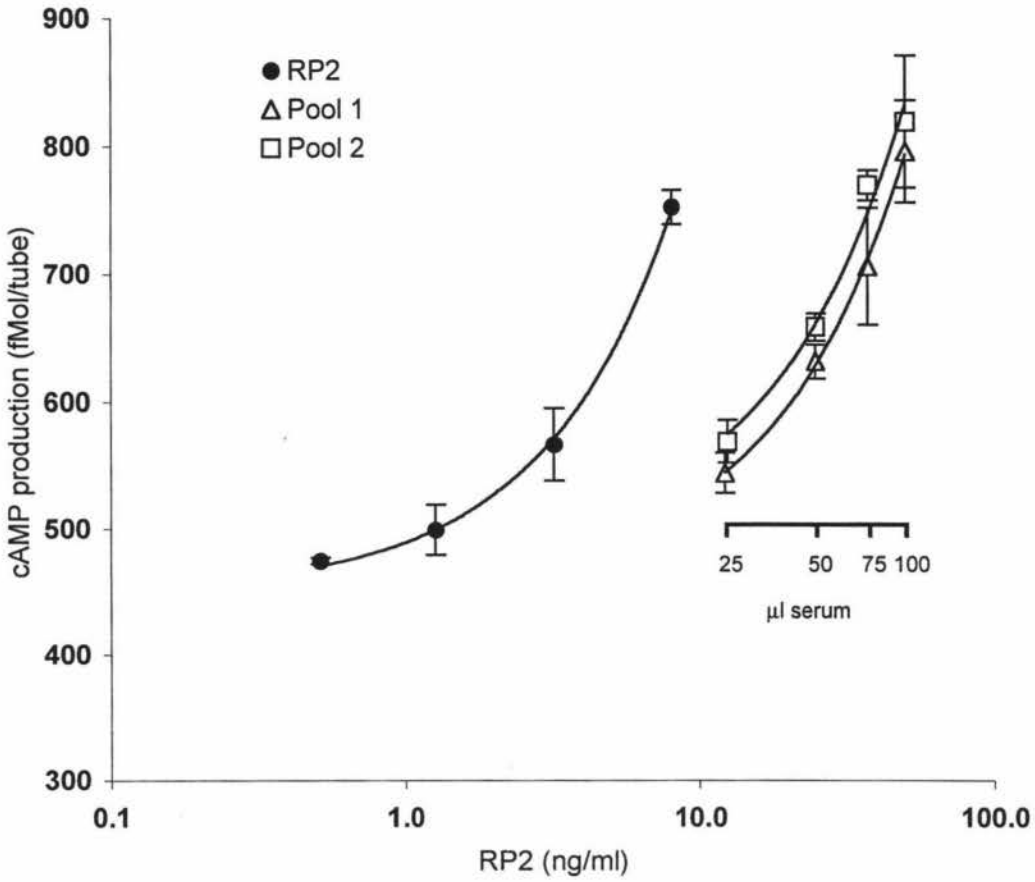


Fig 4.1 Dose dependent stimulation of cAMP by RP2 (●) and pooled ovariectomised ewe serum assayed at different volumes and supplemented with hypox (□ and Δ). Data represents the mean of replicates (n=6) and error bars represent SEM. The slope of RP2 = 0.3974, OOP #117 = 0.4175, and OOP #245 = 0.4210

Table 4.2 FSH bioassay sensitivity for serum samples from intact sheep at 95% and 99% confidence.

Blank	mean cAMP fMol/ml	std dev (n=3)	Sensivity (ng/ml) 95%	99%
Assay 1	2111.33	7.64	1.29	1.58
Assay 2	2002.67	19.07	0.46	0.87
Assay 3	717.03	20.58	0.46	0.78
Assay 4	720.91	50.62	1.41	2.11
Assay 5	547.21	16.56	0.68	0.87
Assay 6	581.77	26.21	1.34	1.68
Assay 7	727.54	37.30	1.13	1.65
Assay 8	119.18	16.83	2.46	3.86
Assay 9	122.09	7.59	1.22	1.82

Mean assay sensitivity at 95 % confidence (+2SD) = **1.16** ng/ml ± 0.19 SEM

Mean assay sensitivity at 99 % confidence (+3SD) = **1.69** ng/ml ± 0.30 SEM

Table 4.3 FSH bioassay sensitivity for serum samples from ovariectomised ewes taken before and after the time of hypophysectomy at 95% and 99% confidence.

Blank	mean cAMP fMol/ml	std dev (n=3)	Sensivity (ng/ml) 95%	99%
Assay 1	291.51	47.22	1.18	1.73
Assay 2	325.65	30.75	0.53	0.78
Assay 3	344.42	48.53	1.18	1.77
Assay 4	635.16	61.88	0.98	1.49
Assay 5	680.28	56.49	0.66	1.06
Assay 6	563.77	32.46	0.34	0.53
Assay 7	526.19	53.42	0.80	1.26
Assay 8	591.02	48.81	1.28	1.71
Assay 9	670.14	51.68	0.66	1.01

Mean assay sensitivity at 95 % confidence (+2SD) = **0.85** ng/ml ± 0.10 SEM

Mean assay sensitivity at 99 % confidence (+3SD) = **1.26** ng/ml ± 0.14 SEM

Table 4.4 FSH bioassay sensitivity for sheep hypophyseal blood serum samples at 95% and 99% confidence.

Blank	mean cAMP fMol/ml	std dev (n=3)	Sensivity (ng/ml) 95%	99%
Assay 1	869.03	7.13	1.32	1.58
Assay 2	666.73	27.66	0.45	2.14
Assay 3	659.94	70.75	3.09	5.25
Assay 4	608.42	38.44	0.39	1.13
Assay 5	672.34	27.34	2.15	3.03
Assay 6	974.23	76.00	2.23	3.88
Assay 7	936.38	16.76	1.73	2.08
Assay 8	927.44	12.98	0.76	0.97
Assay 9	1056.33	25.71	0.67	1.01
Assay 10	1042.20	40.24	0.87	1.44
Assay 11	1092.04	60.95	2.25	3.34
Assay 12	223.83	22.32	1.22	1.81
Assay 13	757.78	29.75	1.43	2.08
Assay 14	641.90	36.29	2.55	3.94
Assay 15	848.03	18.00	0.83	1.18
Assay 16	846.48	20.50	0.73	1.07

Mean Assay Sensitivity at 95 % confidence (+2SD) = **1.42** ng/ml ± 0.20 SEM

Mean Assay Sensitivity at 99 % confidence (+3SD) = **2.25** ng/ml ± 0.31 SEM

4.2.2 Assay Variation

Intra- and interassay coefficients of variation of the FSH bioassay for ovine serum samples were determined using internal control samples consisting of FSH free serum with varying concentrations of RP2 FSH added. All controls were assayed in triplicate and distributed in random wells throughout the assays.

The intra-assay coeffiecient of variation of the FSH bioassay for all serum samples, calculated by averaging the coefficient of variation of the internal controls above the detection limit within each assay, was 15.4% ± 1.4 SEM (n=34).

The interassay coefficient of variation for all serum samples (Table 4.5) for the internal standards above the detection limit at 1.2, 3.0 and 8.0 ng/ml RP2 FSH was 22%, 21.5% and 14.3%, respectively (calculated from a sample of 10 assays).

4.3 FSH in Sheep Serum

The measurement of FSH in serum from intact sheep requires an assay that can detect 1-10 ng/ml of FSH. Therefore, serum samples from intact sheep were assayed for FSH by CHO FSH-R cell bioassay to assess the suitability of the assay for this purpose. Samples from ram and ewes were measured for bioactive FSH

4.3.1 Experimental Method

Except where noted, the methods and materials used were as described in section 2.2.

Cultured CHO FSH-R cells were dispensed into Costar 48 well culture plates at 2.5 x 10^5 cells/well in 150 µl of assay medium. Standard wells were treated with 100 µl RP2 in hypox at 0, 0.8, 2.1, 5.1, 12.8, 32, 80, 200 and 1000 ng/ml in triplicate wells. Samples and internal controls (0.5, 1.2, 3.0 and 8.0 ng/ml of RP2 in hypox). were added to remaining wells in triplicate at the same volume. The media was removed after incubation for 4 hours in standard culture conditions and assayed for cAMP.

Table 4.5 Interassay coeffiecent of variation (%CV) of serum control samples in FSH bioassay.

	Internal control 0.5 ng/ml			**Internal control 1.2 ng/ml**		
	replicates (ng/ml)	mean	$x-\bar{x}$	replicates (ng/ml)	mean	$x-\bar{x}$
Assay 1	0.298	-0.02	0.32	1.359	1.52	-0.16
	-0.026		0.00	1.603		0.08
	-0.338		-0.32	1.595		0.08
Assay 2	0.056	0.49	-0.43	1.156	1.16	0.00
	1.032		0.54	1.483		0.33
	0.377		-0.11	0.827		-0.33
Assay 3	1.072	0.92	0.16	1.855	1.71	0.15
	0.912		0.00	1.448		-0.26
	0.763		-0.15	1.814		0.11
Assay 4	-0.420	0.46	-0.88	1.555	1.31	0.25
	0.662		0.20	1.269		-0.04
	1.143		0.68	1.106		-0.20
Assay 5	-1.788	-0.20	-1.59	1.555	1.31	0.25
	0.281		0.48	1.269		-0.04
	0.913		1.11	1.106		-0.20
Assay 6	-0.067	0.47	-0.53	2.013	1.41	0.61
	0.555		0.09	1.131		-0.28
	0.916		0.45	1.077		-0.33
Assay 7	0.958	0.80	0.16	1.217	1.36	-0.15
	0.726		-0.07	1.453		0.09
	0.709		-0.09	1.418		0.06
Assay 8	0.275	0.56	-0.29	1.335	1.15	0.19
	0.568		0.01	1.009		-0.14
	0.842		0.28	1.102		-0.05
Assay 9	0.923	0.77	0.15	0.766	1.13	-0.36
	0.783		0.01	1.117		-0.01
	0.601		-0.17	1.495		0.37
Assay 10	0.299	0.35	-0.05	1.112	1.18	-0.07
	0.336		-0.01	1.309		0.13
	0.411		0.06	1.112		-0.07
mean (ng/ml)	**0.46**			**1.32**		
%CV	**130.0**			**22.2**		

Table 4.5 continued

	Internal control 3.0 ng/ml			Intrnal control 8 ng/ml		
	replicates (ng/ml)	mean	$x-\bar{x}$	replicates (ng/ml)	mean	$x-\bar{x}$
Assay 1	2.431	3.05	-0.62	6.879	7.53	-0.65
	2.948		-0.10	7.461		-0.07
	3.767		0.72	8.263		0.73
Assay 2	1.891	2.98	-1.09	7.690	8.01	-0.32
	3.520		0.54	7.657		-0.35
	3.524		0.55	8.685		0.67
Assay 3	3.797	3.50	0.30	8.212	7.39	0.82
	3.137		-0.36	7.164		-0.23
	3.569		0.07	6.804		-0.59
Assay 4	2.891	3.31	-0.42	7.991	8.33	-0.34
	3.520		0.21	7.972		-0.36
	3.524		0.21	9.038		0.70
Assay 5	4.204	3.36	0.84	7.434	7.56	-0.12
	3.226		-0.14	7.072		-0.48
	2.664		-0.70	8.165		0.61
Assay 6	3.851	3.96	-0.10	8.647	8.63	0.02
	3.404		-0.55	8.138		-0.49
	4.613		0.66	9.101		0.47
Assay 7	2.356	3.28	-0.92	10.854	8.77	2.09
	4.862		1.59	9.530		0.76
	2.613		-0.66	5.922		-2.85
Assay 8	4.447	3.27	1.18	6.254	7.55	-1.29
	2.303		-0.97	7.232		-0.32
	3.056		-0.21	9.159		1.61
Assay 9	3.849	3.31	0.54	9.592	8.28	1.31
	3.122		-0.18	8.141		-0.14
	2.946		-0.36	7.113		-1.17
Assay 10	3.206	2.95	0.26	7.164	8.71	-1.54
	2.441		-0.51	8.613		-0.09
	3.195		0.25	10.344		1.64
mean (ng/ml)	**3.30**			**8.08**		
%CV	**21.5**			**14.3**		

$$\%CV = \frac{\sqrt{\text{var } t + \text{var } diff}}{meanQC} \times 100$$

vart = variation of the mean of each triplicate
var*diff* = variation of the triplicates minus the mean
meanQC = mean of all triplicates

4.3.2 Results

The mean FSH concentrations of samples assayed in triplicate are summarised in Table 4.6. The sensitivity of the bioassay ($P < 0.05$) was 1.16 ng/ml ± 0.19 SEM (n=9). The concentrations of FSH obtained ranged from < 1 to 8.0 ng/ml RP2 FSH. The intra- and interassay coefficients of variation were 15.1% ± 0.9% SEM and 18.2% (n=9), respectively.

The sensitivity of the receptor cell line bioassay exhibited a wide range of variation. From these results, it is apparent that the sensitivity of the bioassay is not always sufficient to allow FSH to be measured in intact sheep as the mean detection limit plus three standard deviations gives a value of 2.9 ng/ml. Therefore, measuring FSH concentrations in intact sheep with less than 3 ng/ml of FSH may not always be possible.

4.4 Hypophysectomised Ewe Serum

Serum samples taken from 5 ovariectomised ewes prior to and after hypophysectomy were assayed by FSH bioassay. The level of systemic FSH in ovariectomised ewes is expected to be elevated compared to normal, due to removal of the negative feedback mechanism on FSH secretion. Then following hypophysectomy, circulating FSH concentrations are expected to drop, following removal of the source of FSH.

4.4.1 Experimental Method

Except where noted, the methods and materials used were as described in section 2.2.

Costar 48 well culture dishes were seeded with 2.5 x 10^5 cells/well and 150 µl of assay medium was dispensed into all wells. FSH standard curves were generated by the addition of 100 µl of RP2 standards in hypox at 0, 0.8, 2.1, 5.1, 12.8, 32, 80, 200 and 1000 ng/ml in triplicate wells. Remaining wells were treated with 100 µl of sample or FSH internal controls (0.5, 1.2, 3.0 and 8.0 ng/ml of RP2 in hypox) in triplicate. All culture dishes were incubated for 4 hours in standard

Table 4.6 Mean bioactive (B-) FSH concentrations in serum from intact sheep.

	Sheep	Mean B-FSH (ng/ml)	SEM (n=3)	%CV
Ewes	ET 2/4	**4.6**	0.37	14.1
	ET 89/4	**8.0**	0.19	4.1
	ET 213/4	**1.6**	0.21	21.8
	ET 249/4	**6.9**	0.31	7.7
	ET 149/4	**7.3**	0.37	8.7
	ET 243/4	**0.4**	0.06	29.4
	3220	**< det limit**		
	2201	**< det limit**		
	3135	**1.5**	0.15	17.5
	0068	**< det limit**		
	F19 / 9	**3.9**	0.20	8.9
	F19 / 17	**6.5**	0.15	4.0
	F19 / 19	**2.6**	0.23	15.5
	F19 / 37	**4.4**	0.17	6.9
	F19 / 47	**3.4**	0.38	19.3
	F11 / 9	**4.4**	0.47	18.5
	F11 / 11	**6.2**	0.44	12.3
	F11 / 27	**5.4**	0.27	8.8
	F11 / 49	**3.9**	0.17	15.4
Rams	M45 / 6	**3.1**	0.71	39.7
	M45 / 10	**2.3**	0.65	48.5
	M45 / 14	**2.7**	0.54	35.3
	M45 / 48	**2.2**	0.41	32.0
	M45 / 52	**2.4**	0.42	30.3
	M50 / 32	**1.6**	0.46	48.2
	M50 / 36	**< det limit**		
	M50 / 42	**1.8**	0.04	4.3
	M50 / 50	**3.6**	0.32	15.8
	M50 / 50	**2.8**	0.19	11.6

culture conditions after which the media was collected and assayed for cAMP. Concentrations of FSH were obtained by interpolation from the plot of FSH standards against cAMP concentrations.

The resulting bioactive FSH concentrations were plotted against immunoactive FSH concentrations obtained by RIA (provided by Norma Hudson, AgResearch). The heterologous RIA for ovine FSH was identical to that described by McNatty *et al* (1984). The closeness of fit of the observed data to values predicted by a linear regression was quantified by the correlation coefficient (R^2) of bioactive and immunoactive FSH concentrations. The value of R^2 was calculated using the following formula:

$$R^2 = 1 - \frac{\sum(y-\bar{y})^2}{(\sum y^2) - \frac{(\sum y)^2}{n}}$$

4.4.2 Results

The results of these experiments can be seen in Figures 4.2 to 4.6. The FSH profiles of all animals examined showed the general trend of declining levels of bioactive and immunoactive FSH following hypophysectomy. However, even by the last time point, all animals except ewe 208 ++ (Figure 4.4) still had detectable levels of bioactive FSH. Both the bioactive and immunoactive FSH values showed a large fluctuation in concentrations, even among adjacent samples. The pattern of bioactive and immunoactive FSH appeared very similar in all animals examined, although the absolute FSH values differed in ewe 0039 ++ (Figure 4.3)

The detection limit of the bioassay ($P < 0.05$) was 0.85 ng/ml ± 0.1 SEM (n=9). The intra- and interassay coefficients of variation for these assays were 14.3% ± 1.1% SEM and 18% (n=9), respectively.

A summary of the results is shown in Figure 4.7 in which the mean of the FSH values from all five animals are expressed as a percentage of pre-surgery FSH concentrations. This data clearly shows the decline in bioactive and immunoactive FSH, which decreased at a similar rate, following hypophysectomy.

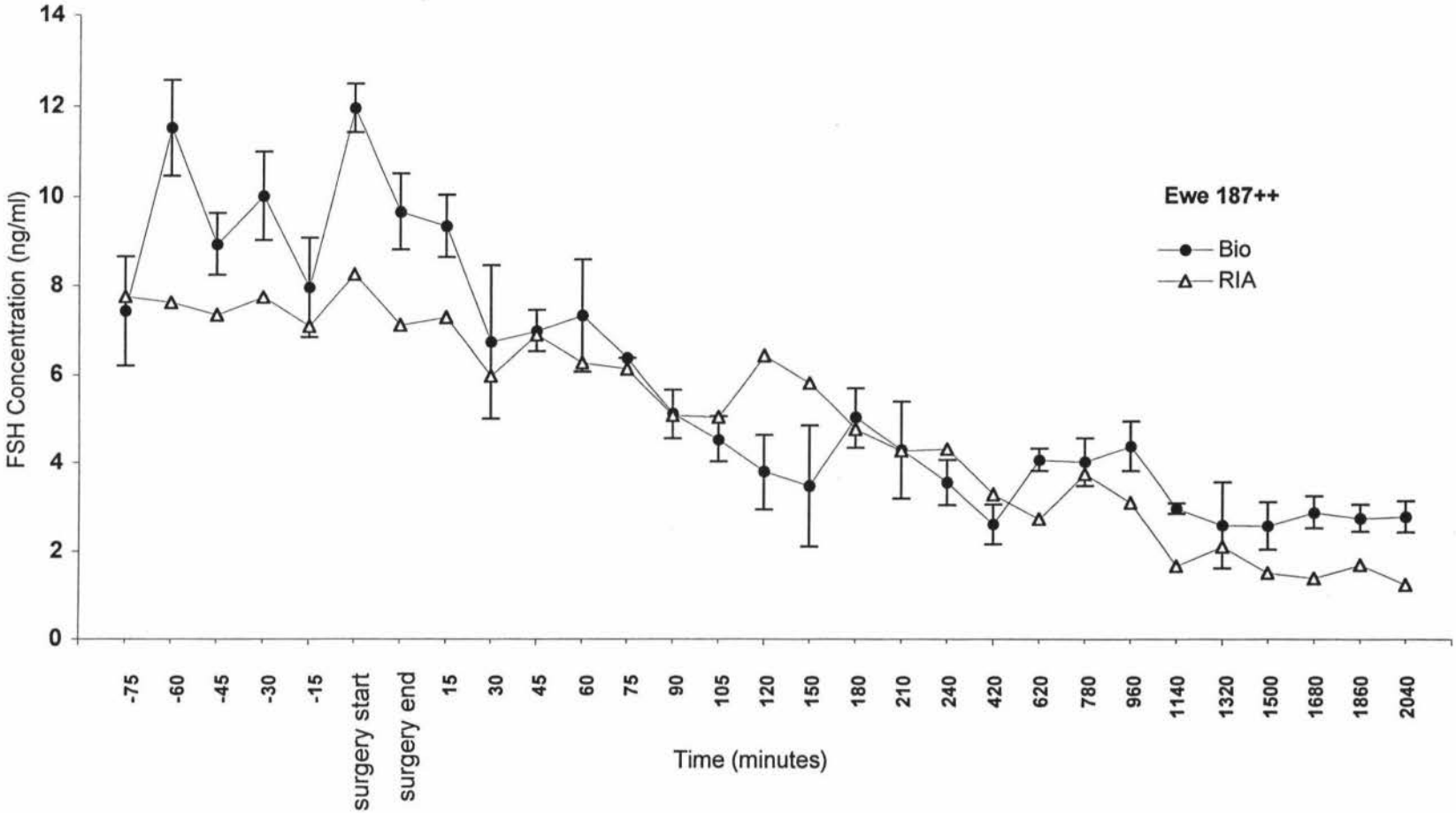


Fig 4.2 Comparison of bioactive (●) and immunoactive (Δ) FSH serum concentrations in ewe 187++ before and after hypophysectomy. Each point represents the mean of triplicates and error bars represent SEM. Surgery start and surgery end denote the beginning and end of the hypophysectomy procedure.

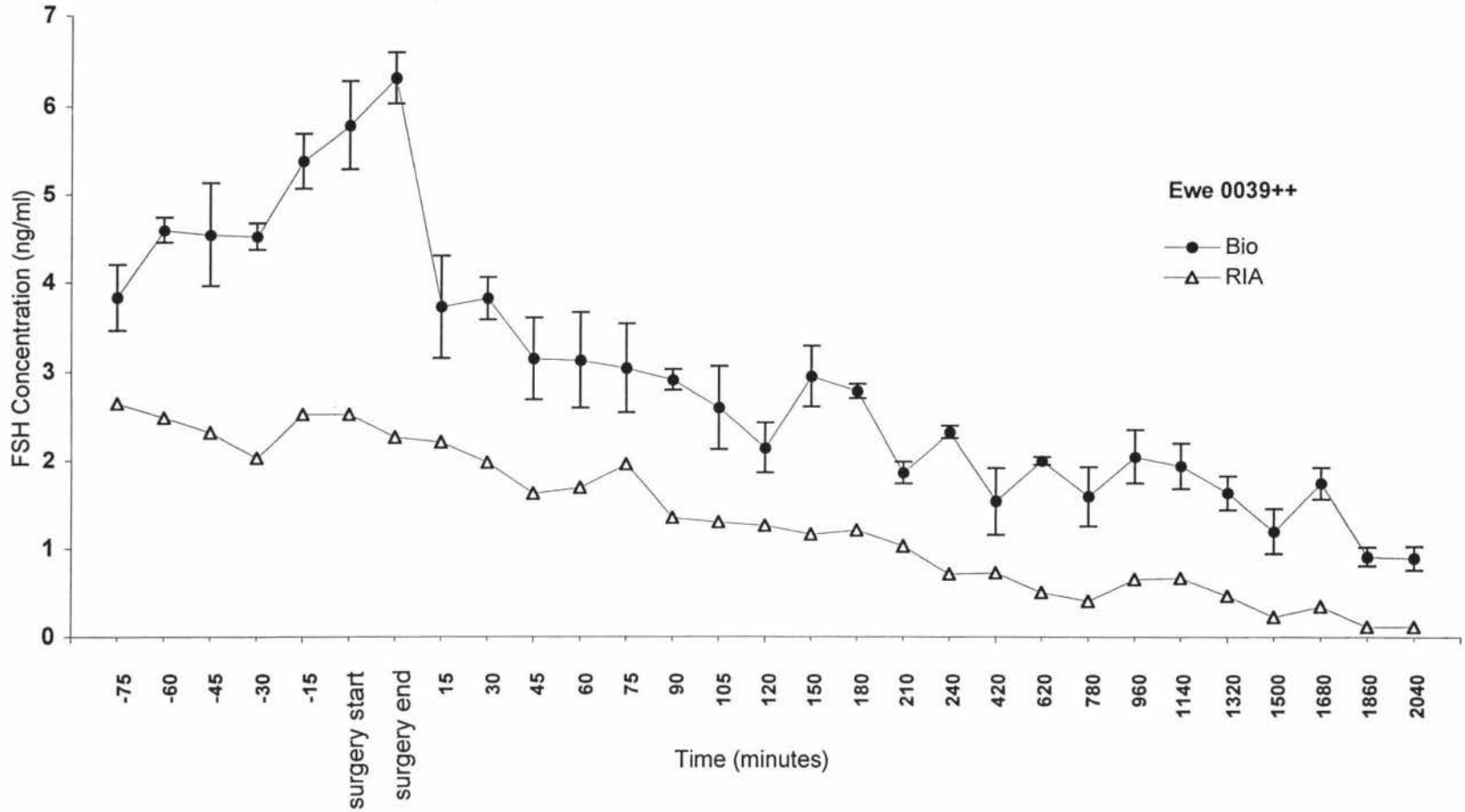


Fig 4.3 Comparison of bioactive (●) and immunoactive (Δ) FSH serum concentrations in ewe 0039++ before and after hypophysectomy. Each point represents the mean of triplicates and error bars represent SEM. Surgery start and surgery end denote the beginning and end of the hypophysectomy procedure.

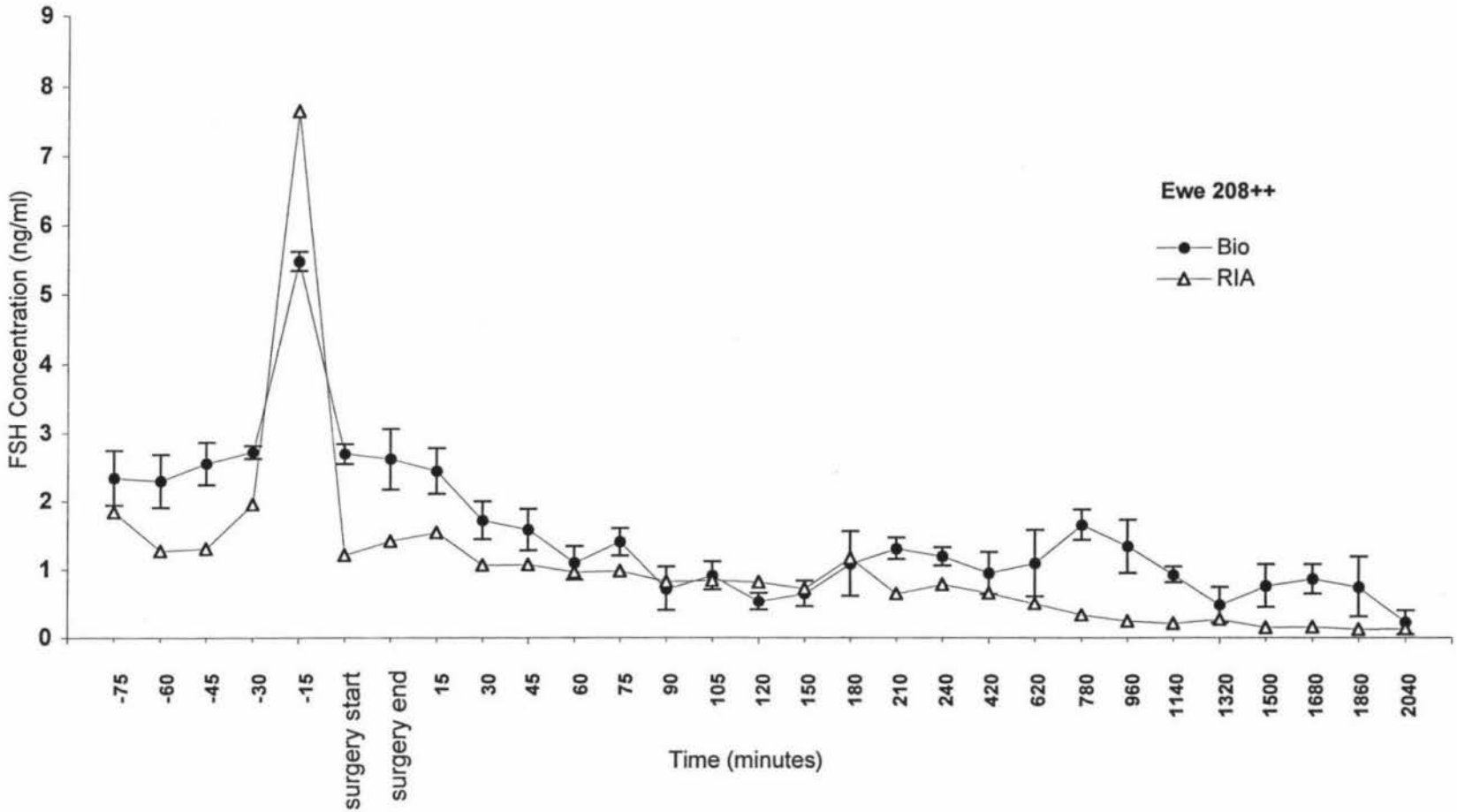


Fig 4.4 Comparison of bioactive (●) and immunoactive (Δ) FSH serum concentrations in ewe 208++ before and after hypophysectomy. Each point represents the mean of triplicates and error bars represent SEM. Surgery start and surgery end denot the beginning and end of the hypophysectomy procedure.

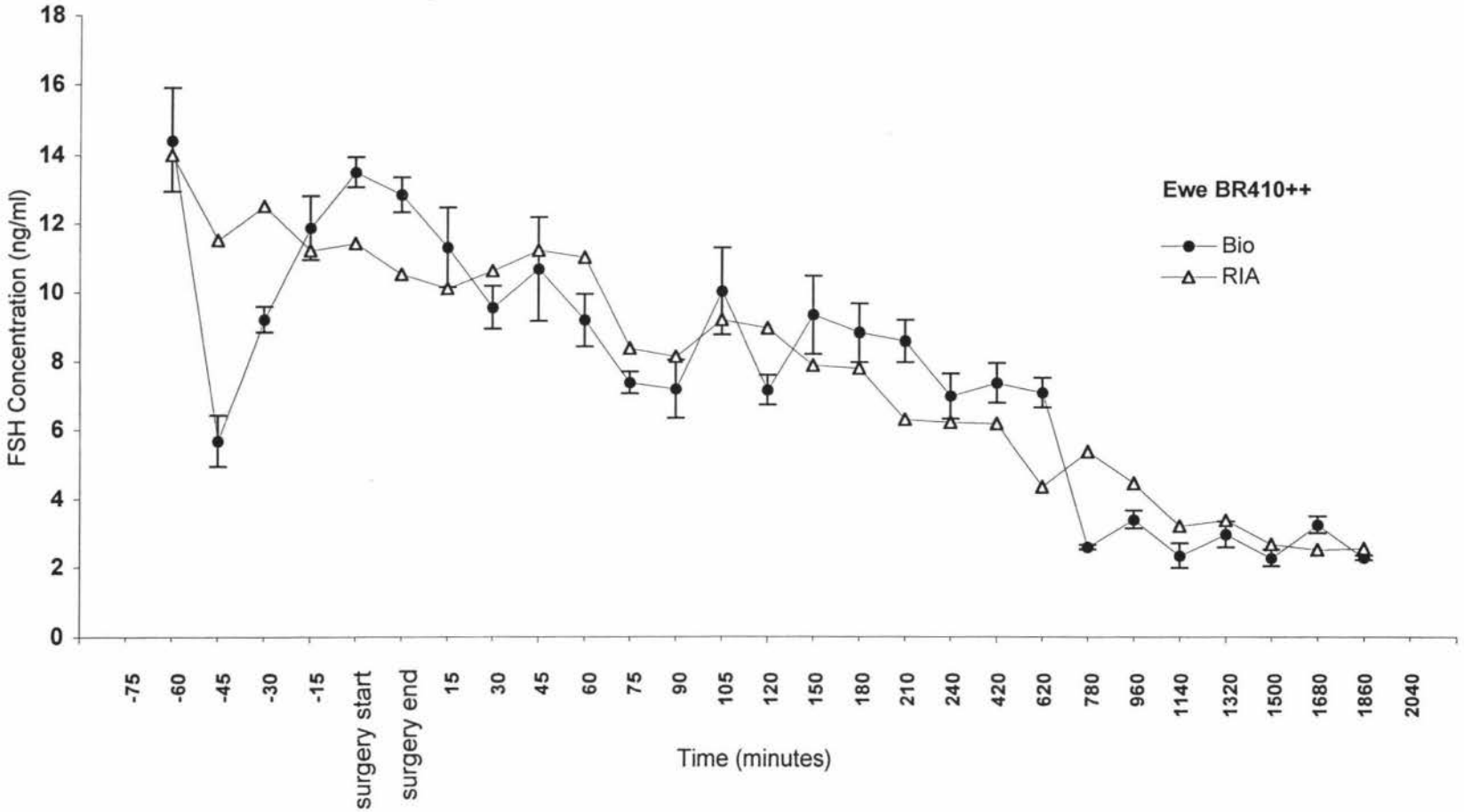


Fig 4.5 Comparison of bioactive (●) and immunoactive (Δ) FSH serum concentrations in ewe BR410++ before and after hypophysectomy. Each point represents the mean of triplicates and error bars represent SEM. Surgery start and surgery end denote the beginning and end of the hypophysectomy procedure.

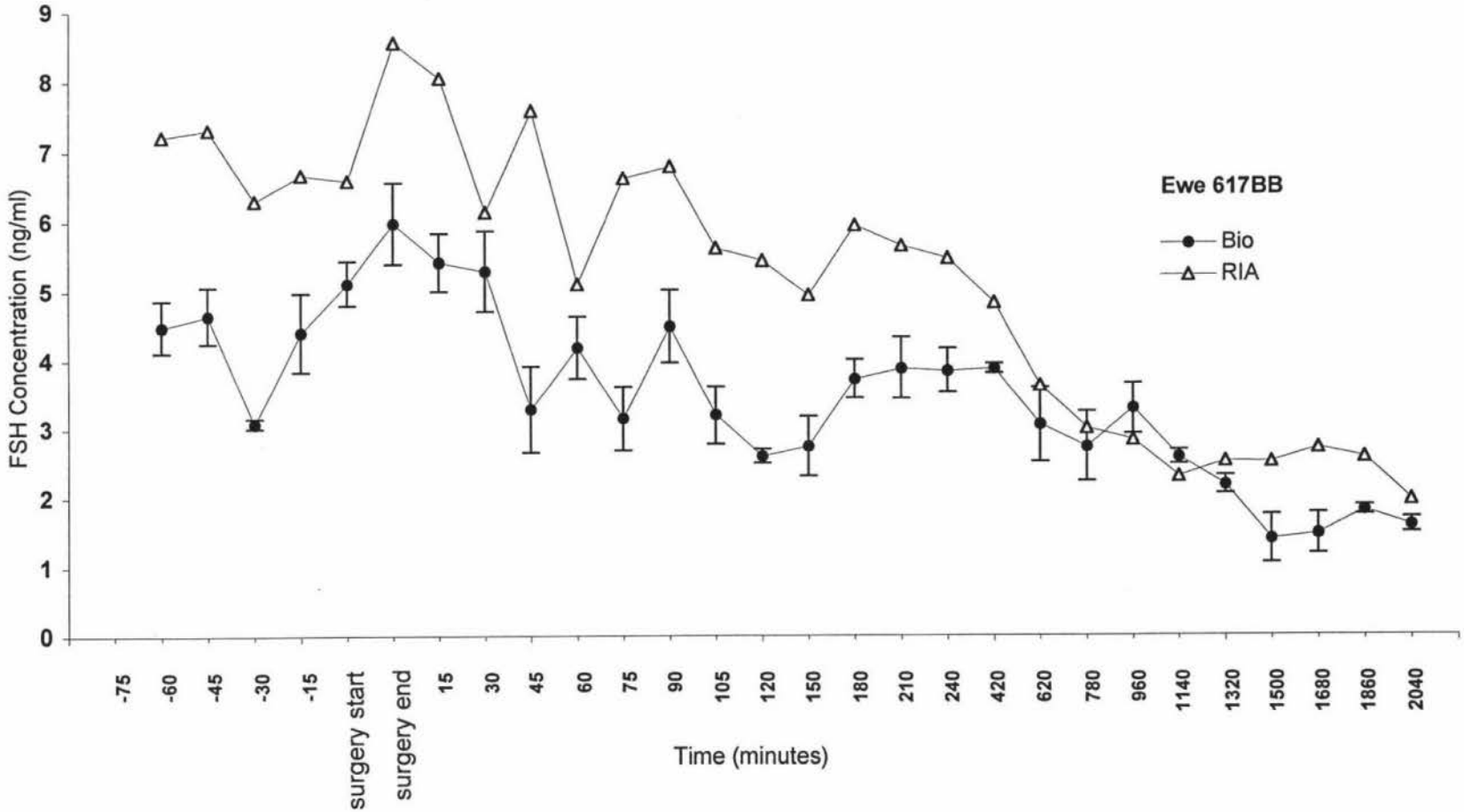


Fig 4.6 Comparison of bioactive (●) and immunoactive (Δ) FSH serum concentrations in ewe 617BB before and after hypophysectomy. Each point represents the mean of triplicates and error bars represent SEM. Surgery start and surgery end denote the beginning and end of the hypophysectomy procedure.

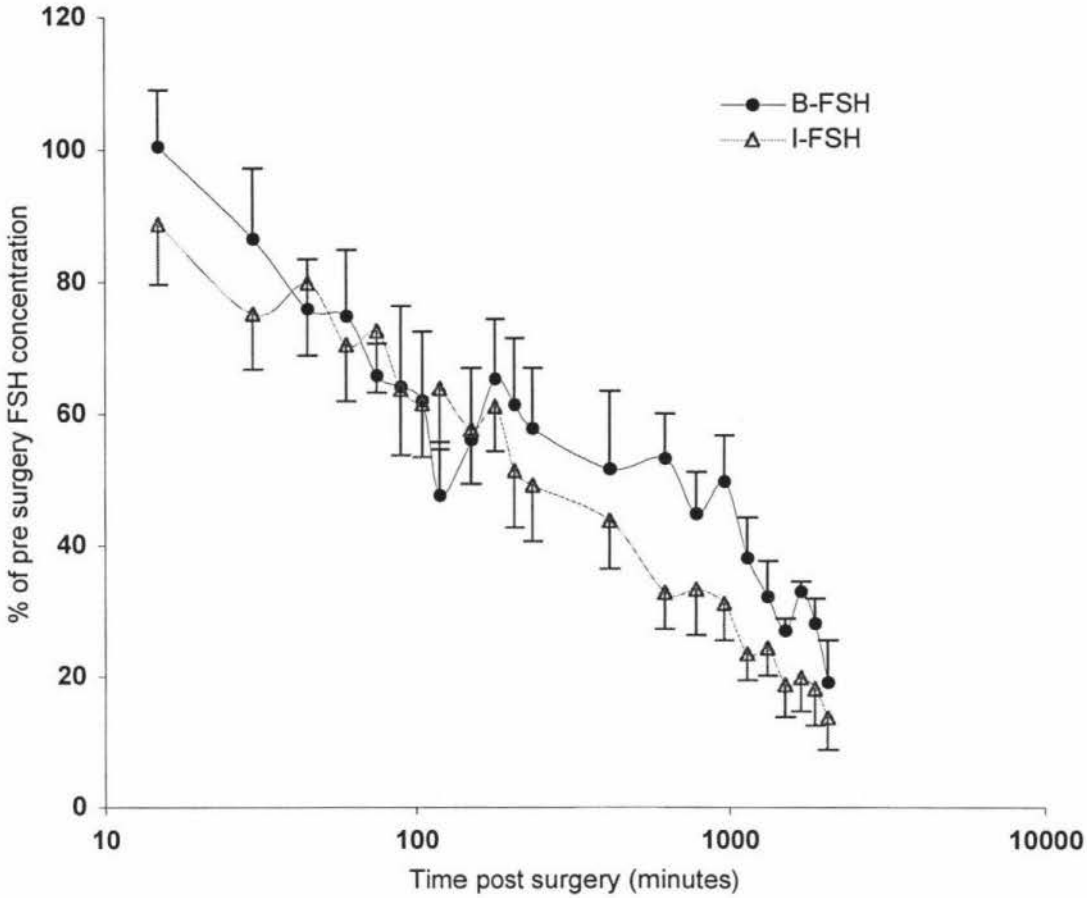


Fig 4.7 Means of post hypophysectomy bioactive FSH (●) and Immunoactive FSH (Δ) concentration values expressed as percentage of pre-surgery serum FSH concentrations in 5 ewes. Each point represents the mean of samples from 5 ewes and error bars represent SEM.

4.4.2.1 Correlation

All bioactive and immunoactive FSH concentrations in serum samples from ovarectomised ewes around the time of hypophysectomy (Figure 4.8) were plotted against each other. The correlation coefficient (R^2) and slope of the regression line were 0.7513 and 0.9224, respectively. The R^2 value represents the degree of correlation between the bioassay and RIA results, which is a measure of how closely the observed data matches the values predicted by a linear regression (where 1= perfect fit). The mean biologic:immunologic (B/I) ratio of the samples was 1.64 ± 0.083 SEM.

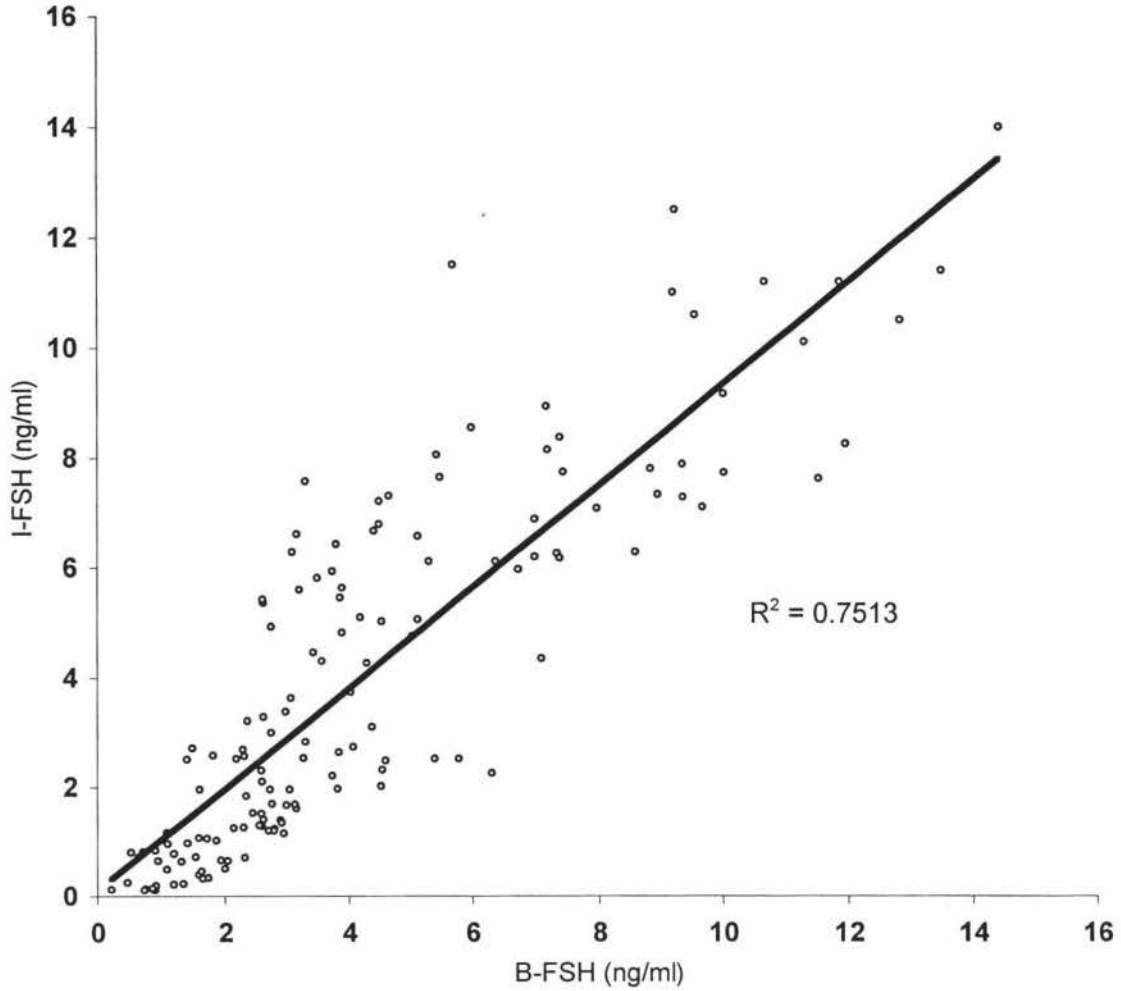


Fig 4.8 Correlation between bioactive (B-) and immunoactive (I-) FSH concentrations in serum samples from ovariectomised ewes around the time of hypophysectomy. Bioactive FSH concentrations were obtained by FSH bioassay and immunoactive FSH values were obtained by RIA. The closeness of fit of the scatterplot to it's linear regression line is indicated by R^2.

4.5 Hypophyseal Serum

Serum samples taken directly from sheep hypophyseal veins were assayed for FSH using the receptor cell line bioassay.

4.5.1 Experimental Method

Except where noted, the methods and materials used were as described in section 2.2. Samples and controls were assayed at a volume of 50 µl in triplicate wells seeded with 2.5 x 10^5 cells and containing 200 µl of assay medium. An assay volume of 50 µl was chosen for these serum samples (instead of 100 µl) due to a scarcity of sample material. Standard curves were generated using 50 µl of RP2 standards in hypox at 0, 0.8, 2.1, 5.1, 12.8, 32, 80, 200 and 1000 ng/ml added to triplicate wells. Plates were incubated for 4 hours and the media collected for assay by cAMP RIA.

The resulting bioactive FSH concentrations were compared to immunoactive FSH concentrations obtained by RIA (provided by Winny Ng Chie, AgResearch), and the correlation coefficient (R^2) between bioactive and immunoactive FSH concentrations were calculated using the formula detailed in Section 4.2.1. The heterologous RIA for ovine FSH was identical to that described by McNatty *et al* (1984).

4.5.2 Results

Bioactive FSH concentrations were plotted with immunoactive FSH levels of the samples obtained by RIA (provided by Winny Ng Chie, AgResearch). Results from these experiments are summarised in Figures 4.9 to 4.12. The time interval between samples is not known. The sensitivity of the assay ($P < 0.05$) was 1.42 ng/ml RP2 ± 0.20 SEM (n=16). Intra- and interassay coefficients of variation were 16.8% ± 1.4% SEM and 21.8% (n=16), respectively.

The bioactive FSH profiles exhibited a large fluctuation in concentrations, ranging from below the detection limit and up to 37.7 ng/ml. This pattern was not evident in the immunoactive FSH data except in sheep CAV 632 (Figure 4.12). However, the range of values was still less than the bioassay results for that animal. The majority of the bioactive FSH levels were also significantly higher than immunoactive FSH levels. This data suggests that the release of FSH from the pituitary was fairly constant over the period of time that the samples were collected, as shown by the immunoactive FSH concentrations. However, this data also suggests that there is an extremely large variation in biopotency of the FSH being released.

4.5.2.1 Correlation

All bioactive and immunoactive FSH concentrations in serum samples from sheep hypophyseal venous bleeds were plotted against each other (Figure 4.13). The R^2 value for the observed data was 0.3877 and slope of the regression line was 0.3786. The mean B/I ratio of the samples was 2.98 ± 0.065 SEM.

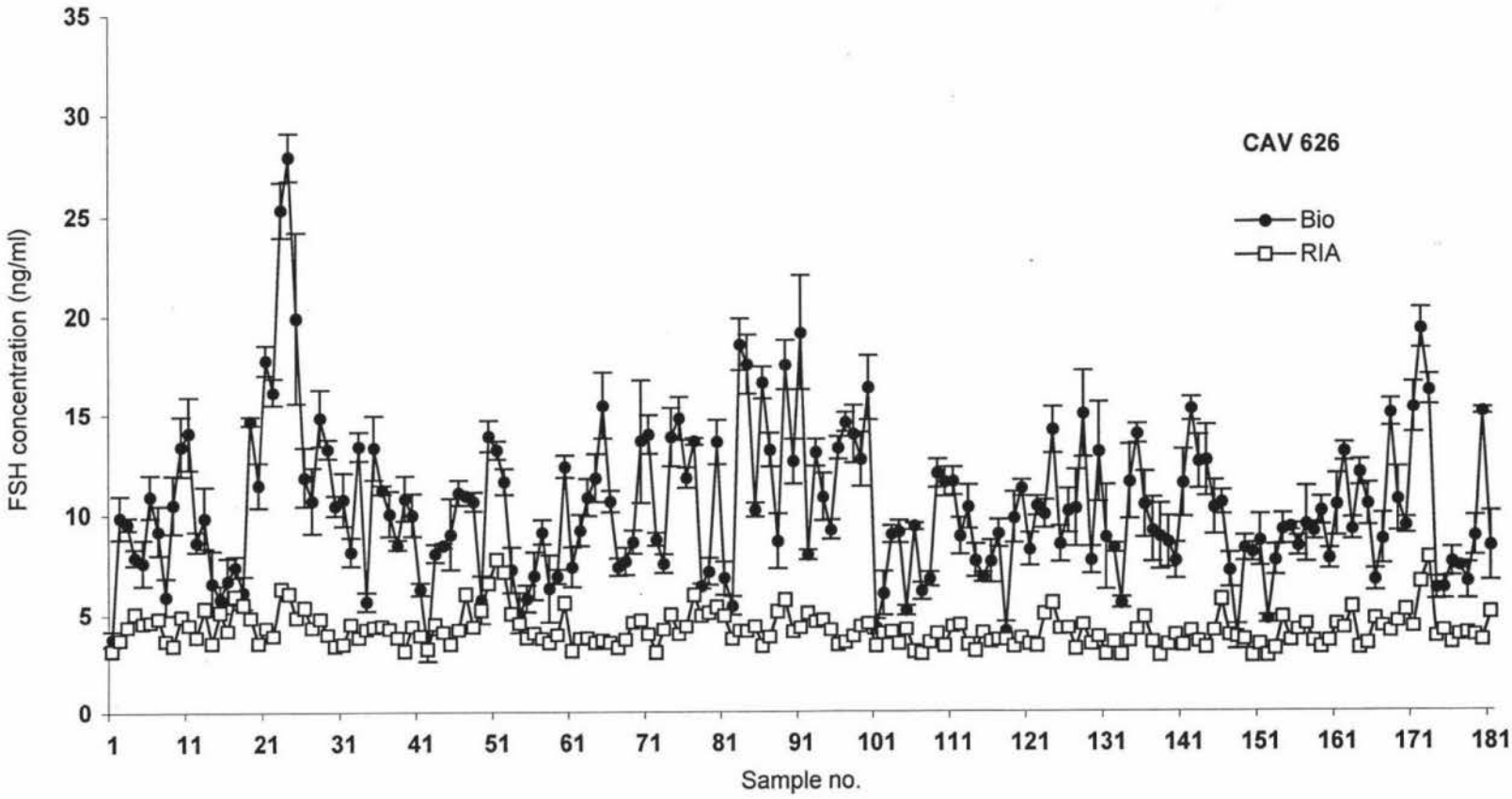


Fig 4.9 Bioactive (●) and immunoactive (□) FSH concentrations in hypophseal blood samples taken from sheep CAV 626. Points represent the mean of triplicate samples and error bars represent SEM.

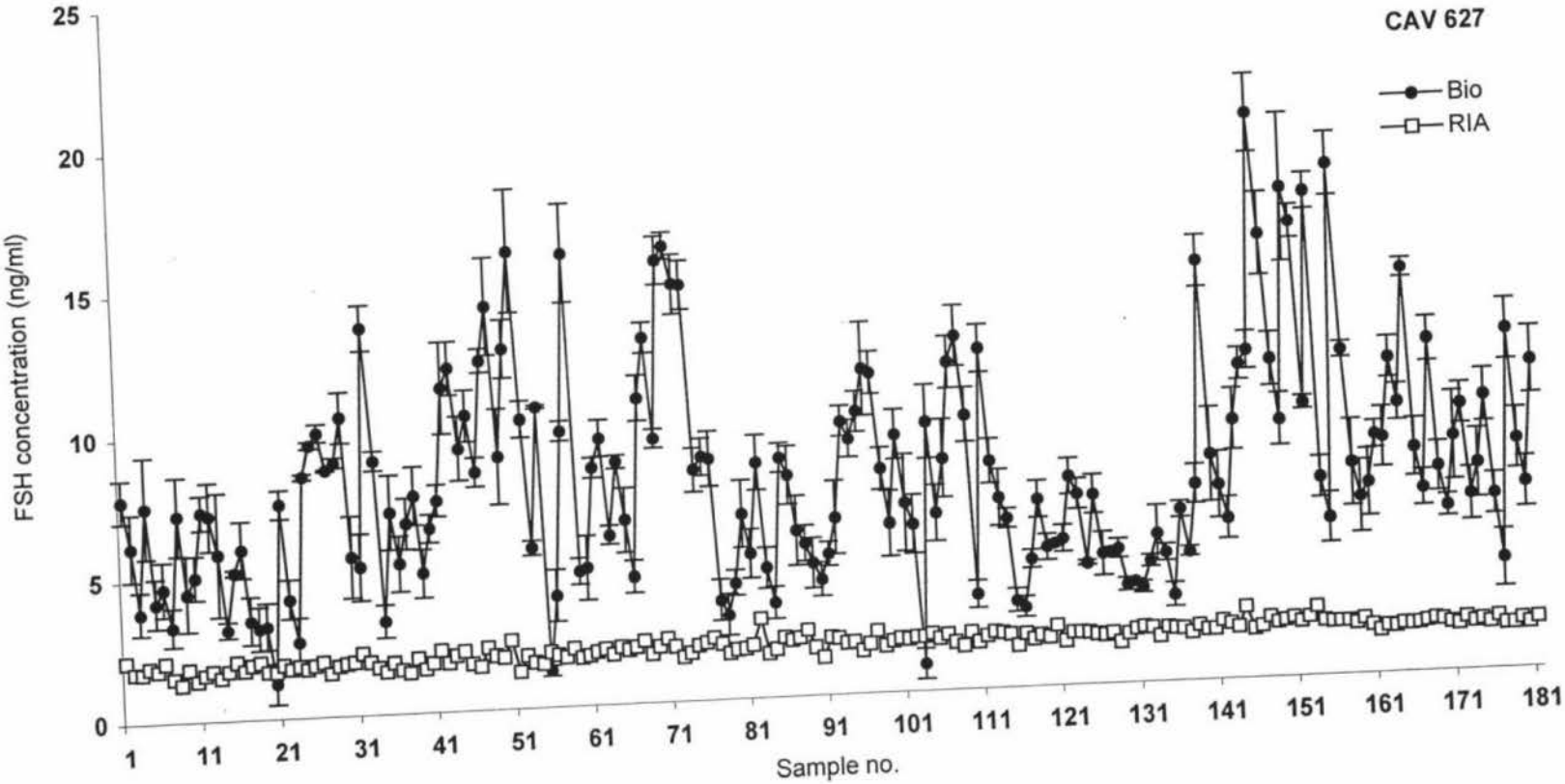


Fig 4.10 Bioactive (●) and immunoactive (□) FSH concentrations in hypophseal blood samples taken from sheep CAV 627. Points represent the mean of triplicate samples and error bars represent SEM.

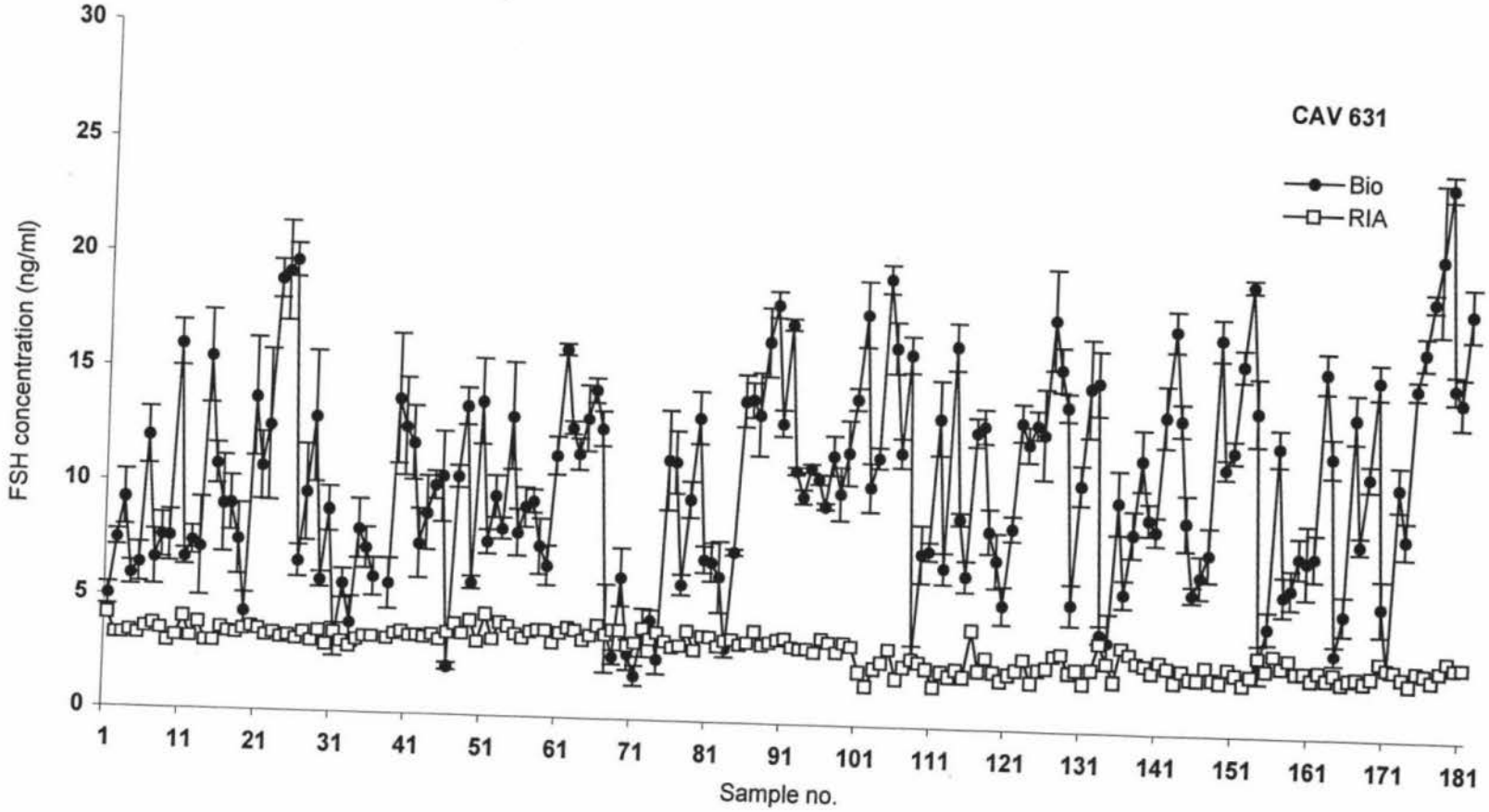


Fig 4.11 Bioactive (●) and immunoactive (□) FSH concentrations in hypophseal blood samples taken from sheep CAV 631. Points represent the mean of triplicate samples and error bars represent SEM.

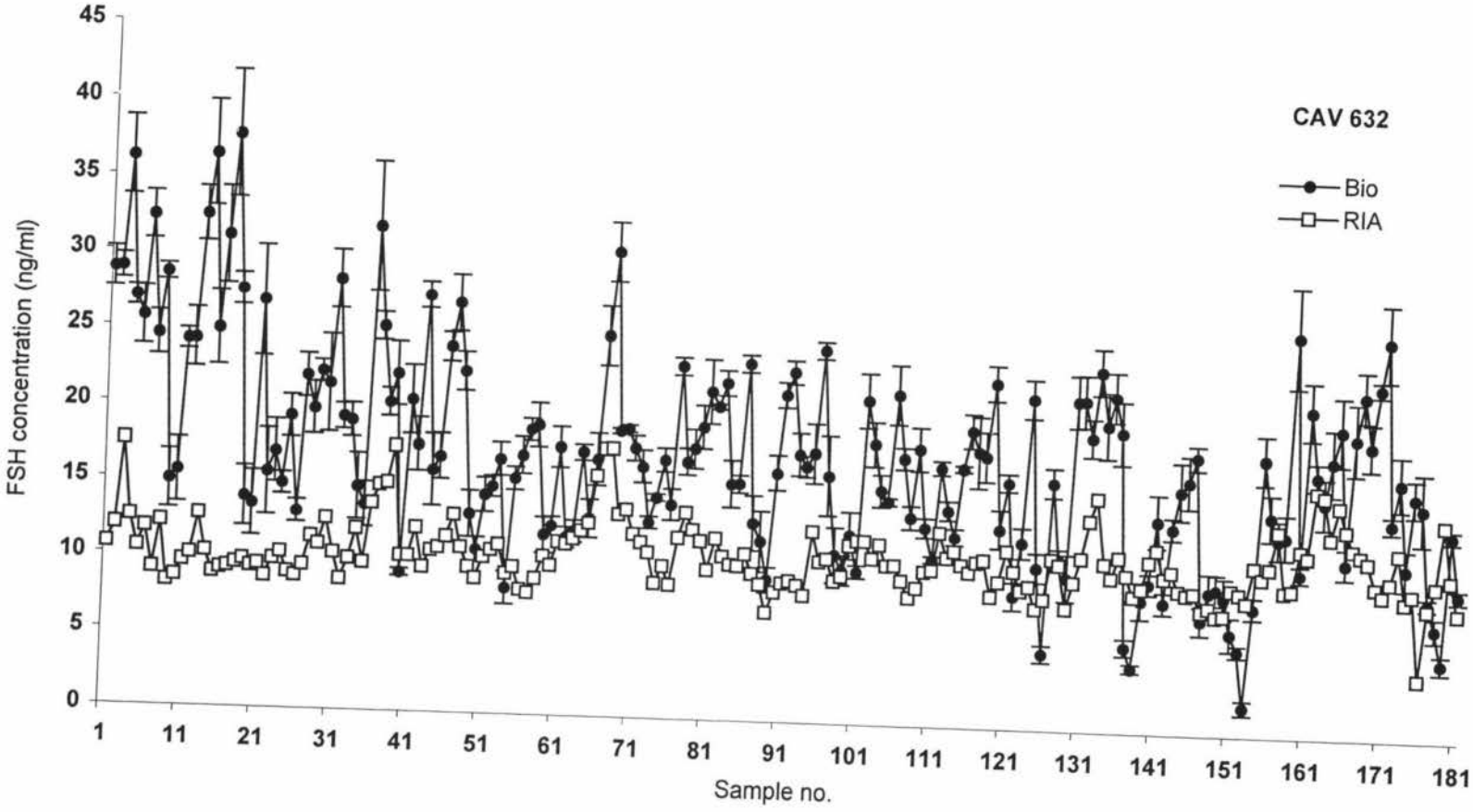


Fig 4.12 Bioactive (●) and immunoactive (□) FSH concentrations in hypophseal blood samples taken from sheep CAV 632. Points represent the mean of triplicate samples and error bars represent SEM.

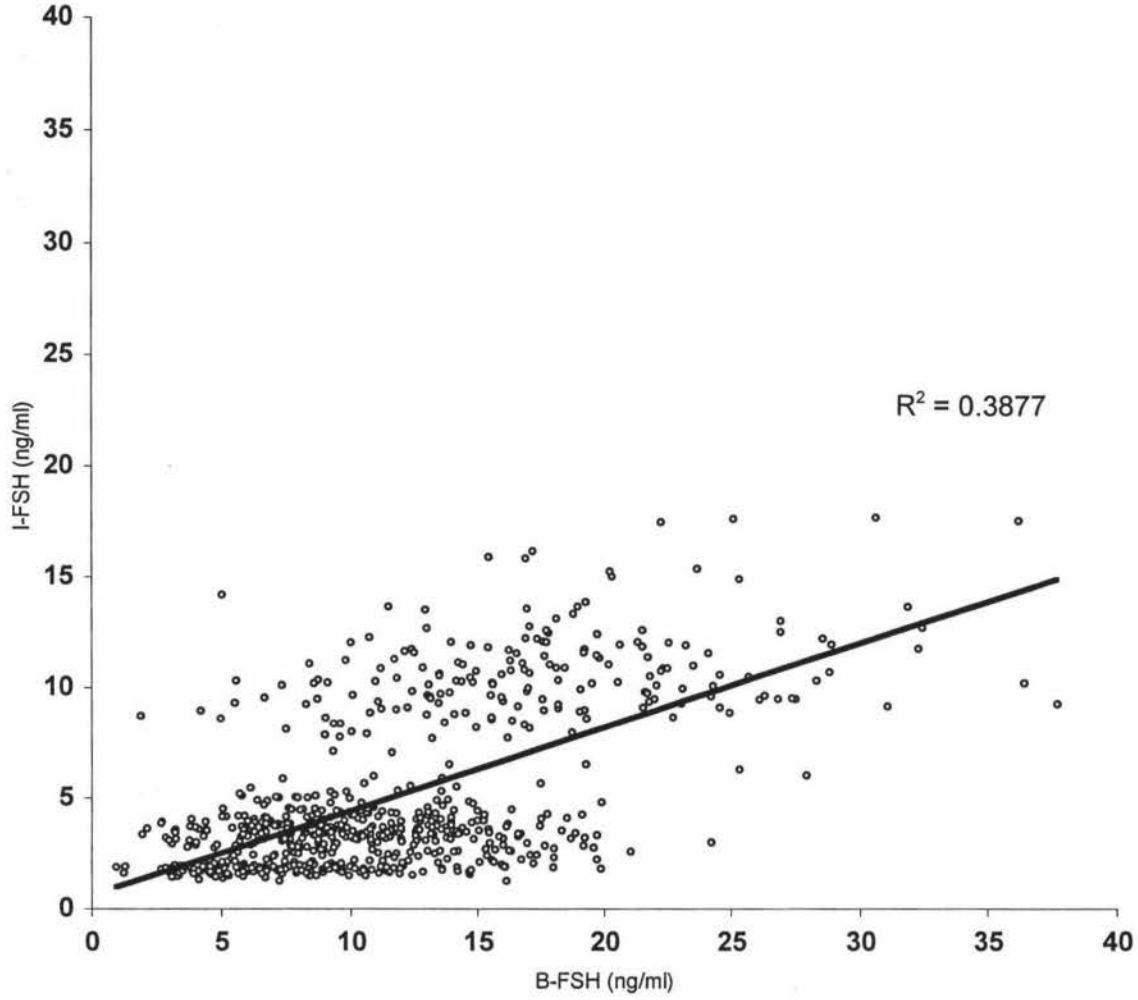


Fig 4.13 Correlation between bioactive (B-) and immunoactive (I-) FSH concentrations in ovine hypophyseal blood samples. Bioactive FSH concentrations were obtained by FSH bioassay and immunoactive FSH values were obtained by RIA. The closeness of fit of the scatterplot to it's linear regression line is indicated by R^2.

4.6 Discussion

Results from this study show that the measurement of cAMP accumulation in CHO cells transfected with the recombinant human FSH receptor gene provides a useful tool for the measurement of serum FSH bioactivity. In conclusion, reliable and reproducible FSH data was obtained for serum samples from intact and surgically altered sheep using the receptor cell line bioassay. However, the sensitivity of the bioassay showed large variation from assay to assay and therefore occasions may arise when the detection limit is insufficient to measure serum samples with low FSH values.

The detection limit of the bioassay is adequate for measuring serum samples, provided sufficient material is assayed. Where sample size is limited, the performance of the bioassay appears to be negatively affected. In assays of hypophyseal venous bleed samples, where only 50 µl of sample per well was available, the sensitivity of the receptor cell line bioassay was 1.42 ng/ml RP2. In assays where sample volume was 100 µl, the sensitivity of the assay ranged from 0.85 to 1.16 ng/ml RP2. The intra- and interassay coefficients of variation fell within acceptable limits. Serum samples from ovariectomised ewes assayed at different dilutions showed good parallelism to the RP2 standard curve, as indicated by *t* test.

4.6.1 FSH in Surgically Altered Ewes

Serum samples were taken at various time intervals from 5 ovariectomised ewes prior to and after hypophysectomy in an unrelated study to measure the decline in immunoactive FSH and change in the half-life of circulating FSH (Hudson, et al, unpublished data). These samples also were measured by bioassay to determine bioactive FSH content. The results of these experiments (Figures 4.1 to 4.6) very clearly showed a decline in bioactive and immunoactive FSH concentrations following hypophysectomy. The profiles of bioactive and immunoactive FSH followed very similar patterns, although in one animal (Figure 4.3), the levels of bioactive FSH all were obviously higher than that of immunoactive FSH. It is interesting to note that the levels of bioactive FSH are all above the detection limit of the assay even 34 hours after hypophysectomy, with only one exception (Figure 4.4). This indicates the presence of circulating bioactive forms of FSH with slow metabolic clearance rates.

The correlation between bioactive and immunoactive FSH concentrations was calculated using all pairs of assay values. The R^2 value and slope of the regression line (0.7513 and 0.9224, respectively) indicated a good correlation between bioactive and immunoactive FSH concentrations. However, on average the bioactive FSH concentrations were significantly higher than immunoactive FSH levels of the same sample, indicated by the B/I ratio of 1.64.

4.6.2 FSH in Hypophyseal Venous Samples

Serum samples taken over a period of time from the hypophyseal veins, which drains directly from the anterior pituitary, were assayed in the receptor cell line FSH bioassay. The samples collected provided a unique opportunity to examine pituitary FSH released prior to being influenced by the various metabolic clearance mechanisms that peripheral circulating FSH would have been exposed to. Although sample collection times were unknown, the procedure for taking these samples was extremely complex and labour intensive. Therefore, the sampling time for each animal was not likely to have been longer than 24 hours.

The bioactive FSH profiles from all of the animals examined showed an extremely high fluctuation in concentrations, ranging from below the detection limit of the assay to 37.7 ng/ml RP2. On the other hand, the immunoactive FSH profiles for each animal, as measured by RIA, were far more constant, with FSH concentrations primarily between 1 and 4 ng/ml RP2. In addition, the bioactive FSH value of each sample was considerable higher than the immunoactive level, with very few exceptions. This trend was demonstrated by the slope of the linear correlation line of bioactive and immunoactive concentrations (0.3786) and the extremely high B/I ratio of 2.98. There also was a very low correlation between bioactive and immunoactive concentrations, as shown by the R^2 value of 0.3877.

The discrepancy between bioactive and immunoactive FSH concentrations is difficult to explain. The assay detection limits and coefficients of variation indicate that the fluctuation in FSH concentrations observed was not due to random error. It is possible that highly biopotent FSH is released from the pituitary in a pulsatile manner, whereas the release of less potent forms occurs at a more constant rate, as indicated by the immunoactive FSH concentrations. It is also possible that these highly biopotent forms of FSH may have a very fast metabolic clearance rate, and so

are not observed in normal circulating blood samples. However, while the pulsatile release of FSH from the pituitary has been demonstrated in humans (Motta *et al*, 1991-92; Ushiroyama, *et al*, 1999), this has yet to be demonstrated in sheep. Further studies to examine this effect will require the use of sensitive FSH bioassay systems, such as the one detailed in this study.

Chapter Five

FSH IN NON-SERUM SAMPLES

5 FSH in Non-Serum Samples

The performance of the receptor cell line FSH bioassay for measuring samples in substrates other than serum was examined. Whereas the measurement of serum FSH is limited by the detection limit of the assay, samples in substrates other than serum often provide their own difficulties. Occasionally, the material to be assayed may contain levels of FSH above the detection limit of the assay and so may require dilution. In addition, the material to be assayed may even contain substances toxic to the cultured cells.

Purified ovine pituitary extract samples were provided by Dr. Lloyd Moore, AgResearch. Media from cultures of sheep pituitary cells were provided by Dr. Keith Henderson, AgResearch.

5.1 Parallelism

Serially diluted purified pituitary extracts and media from ovine pituitary cell cultures were assayed by FSH bioassay to determine parallelism with the RP2 standard curve.

5.1.1 Experimental Method

The parallelism of two purified ovine pituitary extracts to RP2 FSH was determined using serial dilutions made with 20 mM tris-HCl, pH 8.6 at the following ranges:

pituitary LM1	1, 1/5, 1/10, 1/40
pituitary LM2	1/100, 1/200, 1/400, 1/800

Parallelism in media from six cultured sheep pituitaries was tested by serially diluting the media with DMEM + 10% hypox v/v at the dilutions listed below:

pituitary 130A	1, 1/5, 1/10, 1/100, 1/1000
pituitary 130B	1, 1/2, 1/5, 1/10, 1/50
pituitary 212A	1, 1/5, 1/10, 1/100, 1/1000, 1/5000
pituitary 212B	1, 1/2, 1/5, 1/10, 1/50
pituitary 73BB	1, 1/5, 1/10, 1/100, 1/1000, 1/5000
pituitary 23++	1, 1/5, 1/10, 1/100, 1/1000

Samples were assayed with 6 replicates at 10 µl per well. Plates were seeded with 2.5 x 10^5 cells per well and contained 240 µl of assay medium per well. RP2 standards of 0, 0.8, 2.1, 5.1, 12.8, 32, 80, 200 and 1000 ng/ml made in the appropriate medium, was added to standard wells in triplicate at a volume of 10 µl. Internal controls were added to the appropriate wells at 10 µl in triplicate. After incubation, media was collected from the plates and assayed for cAMP.

5.1.2 Results

The slopes of the cAMP response curves of serially diluted pituitary extracts and serially pituitary culture media were both parallel with that of RP2 (Figure 5.1 and 5.2), as evaluated by *t* test for non-parallelism. The *t* value for the comparison of slopes between RP2 and diluted pituitary extracts was –0.2034 ($P < 0.05$). A comparison of the slopes of RP2 and diluted pituitary culture media gave a *t* value of –0.0113 ($P < 0.05$). These results indicate that CHO FSH-R cells are suitable for use in measuring bioactive FSH in pituitary culture media and, and that RP2 FSH is an appropriate standard for generating a standard curve.

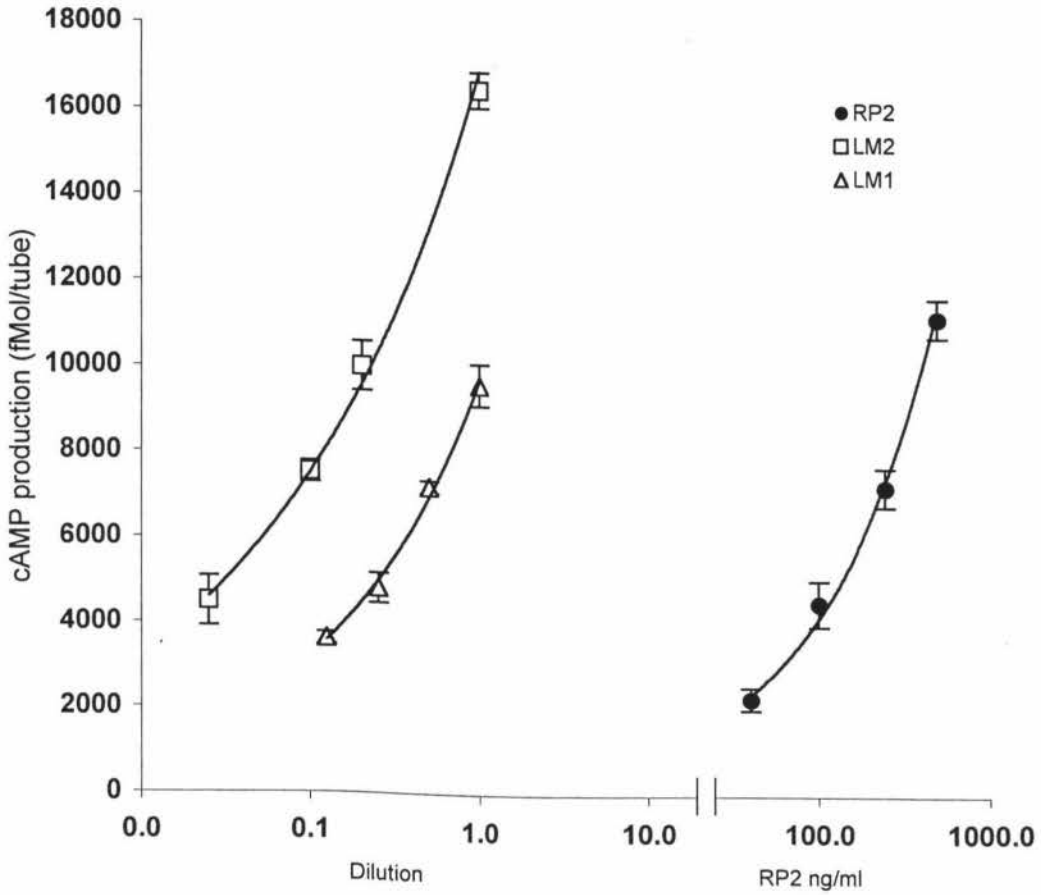
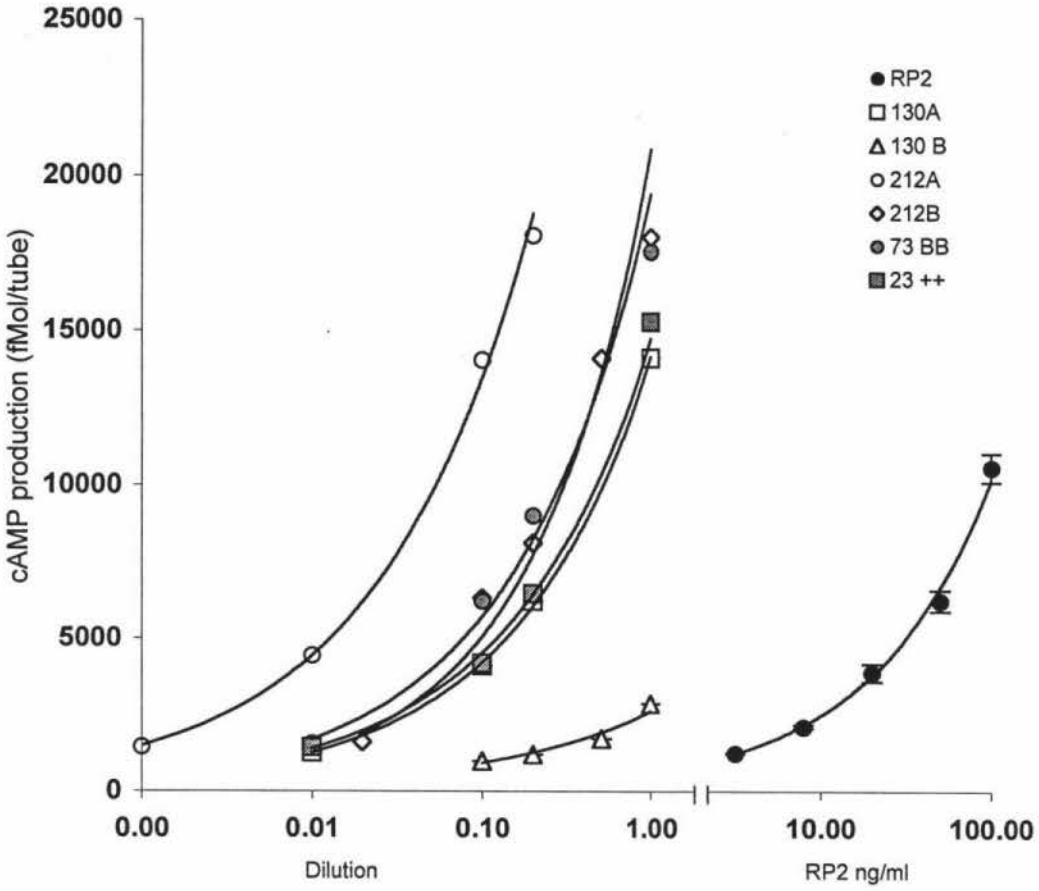


Fig 5.1 Dose dependent stimulation of cAMP by RP2 (●) and purified ovine pituitary extracts, LM1 and LM2, assayed at different dilutions (□ and Δ). Data represents the mean of replicates (n=6) and error bars represent SEM. The slope of RP2 = 0.7172, LM1 = 0.6546, and LM2 = 0.7022. The R^2 value of RP2 = 0.9470, LM1 = 0.9126, and LM2 = 0.9679



	R^2	Slope
RP2 standard	0.986	0.805
130A	0.958	0.841
130B	0.996	0.724
212A	0.932	0.787
212B	0.901	0.962
73BB	0.909	0.803
23++	0.968	0.848

Fig 5.2 Dose dependent stimulation of cAMP by RP2 (●) and media from 6 cultures of ovine pituitary cells, assayed at different dilutions (✧,●, ■, O, □ and Δ). Data represents the mean of replicates (n=6).

5.2 Assay Characteristics

Intra- and interassay coefficients of variation and sensitivity of the bioassay for purifed pituitary extract samples were calculated from internal control in assays performed in sections 5.1 and 5.3

4.2.1 Sensitivity

The mean sensitivity of the FSH bioassay for purified pituitary extract samples, measured in section 5.3, was 1.12 ng/ml RP2 FSH ± 0.15 SEM (n = 18, $P < 0.05$), as shown in Table 5.1. Assays of pituitary culture media had a mean detection limit of 1.14 ng/ml RP2 FSH ± 0.13 SEM (n = 7, $P < 0.05$), as shown in Table 5.2.

4.2.2 Assay Variation

Intra- and interassay coefficients of variation of the FSH bioassay for ovine pituitary culture media and purified pituitary extracts were determined using internal control samples. All controls were assayed in triplicate and distributed in random wells throughout the assays.

The intra-assay coeffiecient of variation of the FSH bioassay for purified pituitary extracts, calculated by averaging the coefficient of variation of the internal controls above the detection limit within each assay, was 13.8% ± 1.2% SEM (n=18). The coefficient of variation within assays for pituitary culture media was 15.4% ± 2.2% SEM (n=7).

The interassay coefficient of variation for purified pituitary extract samples (Table 5.3) for the internal controls at 2.5, 6.0, 16.0 and 40.0 ng/ml RP2 FSH was 23%, 15.8%, 14.3% and 14.5%, respectively (calculated from a sample of 10 assays). For assays of pituitary culture media, the interassay coefficients of variation of internal controls at 2.5, 6.0 and 16 ng/ml RP2 FSH was 26.3%, 17.7% and 15.7%, respectively (n=7), as shown in Table 5.4.

Table 5.1 FSH bioassay sensitivity for purified pituitary extract samples at 95% and 99% confidence.

Blank	mean cAMP fMol/ml	std dev (n=3)	Sensivity (ng/ml) 95%	99%
Assay 1	710.88	57.63	0.75	1.29
Assay 2	851.72	60.76	1.00	1.45
Assay 3	798.92	34.26	0.69	0.93
Assay 4	890.87	25.03	1.08	1.41
Assay 5	757.53	34.56	2.04	2.57
Assay 6	859.24	39.98	1.05	1.5
Assay 7	893.58	53.93	1.93	2.89
Assay 8	1016.53	91.49	0.72	1.19
Assay 9	1122.47	25.01	0.72	0.86
Assay 10	1033.96	69.98	0.72	1.17
Assay 11	1060.50	41.62	1.49	2.36
Assay 12	1076.83	113.29	2.8	4.01
Assay 13	1134.17	97.34	1.47	2.13
Assay 14	1039.26	53.12	0.36	0.65
Assay 15	973.78	49.84	0.19	0.36
Assay 16	1944.77	53.18	1.76	2.17
Assay 17	1770.37	49.53	0.84	1.12
Assay 18	1680.80	62.27	0.52	0.83

Mean assay sensitivity at 95 % confidence (+2SD) = **1.12** ng/ml ± 0.15 SEM

Mean assay Sensitivity at 99 % confidence (+3SD) = **1.61** ng/ml ± 0.21 SEM

Table 5.2 FSH bioassay sensitivity for pituitary cell culture media samples at 95% and 99% confidence.

Blank	mean cAMP fMol/ml	Std dev (n=3)	Sensivity 95%	99%
Assay 1	1576.7	39.67	0.64	2.04
Assay 2	1508.3	11.20	1.03	1.43
Assay 3	1451.0	16.54	1.07	2.47
Assay 4	1514.3	38.42	1.12	2.57
Assay 5	1689.8	25.24	1.13	2.1
Assay 6	1547.7	33.40	1.86	3.24
Assay 7	2214.5	52.33	1.32	2.89

Mean Assay Sensitivity at 95 % confidence (+2SD) = **1.14** ng/ml ± 0.13 SEM

Mean Assay Sensitivity at 99 % confidence (+3SD) = **2.22** ng/ml ± 0.21 SEM

Table 5.3 Interassay coeffiecent of variation (%CV) of purified pituitary extract control samples in FSH bioassay.

	Internal control 2.5 ng/ml			**Internal control 6.0 ng/ml**		
	replicates (ng/ml)	mean	$x-\bar{x}$	replic	mean	$x-\bar{x}$
Assay 1	12.859	12.61	0.25	31.558	39.65	-8.09
	11.737		-0.88	34.989		-4.66
	13.244		0.63	52.412		12.76
Assay 2	19.903	17.74	2.16	47.497	37.99	9.50
	17.145		-0.59	30.600		-7.39
	16.167		-1.57	35.887		-2.11
Assay 3	16.111	16.53	-0.42	38.289	39.21	-0.92
	16.305		-0.22	36.628		-2.59
	17.172		0.64	42.724		3.51
Assay 4	13.433	14.42	-0.99	36.952	39.74	-2.79
	14.729		0.31	39.133		-0.61
	15.096		0.68	43.134		3.39
Assay 5	11.303	15.77	-4.47	45.577	44.40	1.18
	17.059		1.29	52.336		7.94
	18.950		3.18	35.279		-9.12
Assay 6	15.222	17.04	-1.82	39.323	42.91	-3.59
	17.113		0.07	42.916		0.01
	18.792		1.75	46.495		3.58
Assay 7	14.650	16.20	-1.55	44.778	37.79	6.99
	16.053		-0.15	32.770		-5.02
	17.901		1.70	35.819		-1.97
Assay 8	15.665	16.84	-1.18	36.771	40.06	-3.29
	14.345		-2.50	39.497		-0.56
	20.518		3.68	43.905		3.85
Assay 9	19.173	17.66	1.51	49.202	39.90	9.31
	16.896		-0.77	34.505		-5.39
	16.918		-0.74	35.982		-3.91
Assay 10	16.492	15.48	1.01	41.134	40.70	0.44
	15.643		0.16	43.330		2.63
	14.312		-1.17	37.622		-3.07
mean (ng/ml)	**16.03**			**40.23**		
%CV	**14.3**			**14.5**		

Table 5.3 continued

	Internal Control 16.0 ng/ml			Internal Control 40 ng/ml		
	replicates (ng/ml)	mean	$x-\bar{x}$	replic	mean	$x-\bar{x}$
Assay 1	2.872	2.92	-0.04	6.847	6.48	0.37
	3.003		0.09	6.316		-0.16
	2.871		-0.04	6.278		-0.20
Assay 2	2.670	2.30	0.37	5.479	5.39	0.09
	2.017		-0.28	4.856		-0.53
	2.204		-0.09	5.822		0.44
Assay 3	3.111	2.85	0.26	6.138	5.99	0.14
	2.746		-0.10	6.198		0.20
	2.694		-0.16	5.645		-0.35
Assay 4	1.505	1.77	-0.27	5.005	6.39	-1.38
	1.321		-0.45	7.876		1.49
	2.496		0.72	6.276		-0.11
Assay 5	1.994	2.47	-0.47	5.561	5.99	-0.43
	2.425		-0.04	6.255		0.27
	2.987		0.52	6.153		0.16
Assay 6	2.847	2.32	0.53	5.743	5.16	0.58
	1.413		-0.91	4.404		-0.76
	2.695		0.38	5.347		0.18
Assay 7	2.017	2.53	-0.51	7.368	6.38	0.99
	1.855		-0.67	4.979		-1.40
	3.703		1.18	6.791		0.41
Assay 8	1.592	2.12	-0.53	4.388	6.21	-1.83
	2.495		0.37	6.089		-0.13
	2.287		0.16	8.167		1.95
Assay 9	2.736	2.72	0.01	5.586	5.33	0.25
	2.594		-0.13	5.608		0.27
	2.841		0.12	4.806		-0.53
Assay 10	2.186	2.28	-0.10	6.675	6.67	0.00
	2.165		-0.12	6.022		-0.65
	2.496		0.21	7.317		0.65
mean (ng/ml)	**2.43**			**6.00**		
%CV	**23.0**			**15.8**		

$$\%CV = \frac{\sqrt{\text{var } t + \text{var } diff}}{meanQC} \times 100$$

var*t* = variation of the mean of each triplicate
var*diff* = variation of the triplicates minus the mean
*mean*QC = mean of all triplicates

Table 5.4 Interassay coeffiecent of variation (%CV) of pituitary cell culture media control samples.

	Internal control 2.5 ng/ml			**Internal control 6.0 ng/ml**			**Internal control 16.0 ng/ml**		
	replicates (ng/ml)	mean	$x-\bar{x}$	replicates (ng/ml)	mean	$x-\bar{x}$	replicates (ng/ml)	mean	$x-\bar{x}$
Assay 1	3.048	2.86	0.19	7.865	6.35	1.52	14.296	15.35	-1.06
	2.808		-0.05	5.565		-0.78	12.200		-3.15
	2.731		-0.13	5.606		-0.74	19.559		4.21
Assay 2	2.363	3.16	-0.79	7.405	6.33	1.08	14.847	15.93	-1.08
	3.363		0.21	5.443		-0.88	16.266		0.34
	3.747		0.59	6.132		-0.19	16.669		0.74
Assay 3	3.478	3.16	0.32	7.509	6.72	0.79	12.134	15.83	-3.69
	2.708		-0.45	6.121		-0.60	16.514		0.69
	3.291		0.13	6.520		-0.20	18.827		3.00
Assay 4	1.472	2.50	-1.03	7.691	6.89	0.80	14.536	16.05	-1.51
	2.254		-0.24	7.915		1.03	15.506		-0.54
	3.768		1.27	5.052		-1.83	18.097		2.05
Assay 5	1.952	2.20	-0.24	5.507	6.07	-0.56	16.953	19.34	-2.38
	2.587		0.39	5.725		-0.35	17.867		-1.47
	2.050		-0.15	6.978		0.91	23.191		3.85
Assay 6	3.248	2.39	0.86	8.583	6.39	2.19	15.317	17.39	-2.07
	2.169		-0.22	6.273		-0.12	16.889		-0.50
	1.747		-0.64	4.327		-2.07	19.958		2.57
Assay 7	1.701	2.22	-0.52	7.385	6.17	1.22	14.886	16.57	-1.69
	2.175		-0.05	5.613		-0.55	16.848		0.27
	2.795		0.57	5.504		-0.66	17.988		1.41
mean (ng/ml)	**2.64**			**6.42**			**16.64**		
%CV	**26.3**			**17.7**			**15.7**		

5.3 FSH in Pituitary Extracts

Ovine pituitary extracts were purified by Lloyd Moore, AgResearch, using hydrophobic interaction chromatography to separate FSH from LH. The different FSH isoforms in the FSH containing fraction were then separated based on charge and pH using high performance liquid chromatography (HPLC) and 2.5 ml fractions were collected. The FSH in these fractions, in 20 mM tris-HCl, pH 8.6, were characterised by RIA, radio-receptor binding assay (RRA), amino acid analysis, and, as described in this study, by CHO FSH-R cell bioassay.

5.3.1 Experimental Method

Triplicate 10 µl samples and controls were assayed in Costar 48 well plates containing 2.5 x 10^5 cells/well and 240 µl assay medium/well. Standard curves were generated by the addition of 10 µl of RP2 standard in 20 mM tris-HCl, pH 8.6, at 0, 0.8, 2.1, 5.1, 12.8, 32, 80, 200 and 1000 ng/ml in triplicate wells. Plates were incubated for 4 hours and the media collected to determine extra-cellular cAMP content.

Bioactive FSH concentrations were compared to FSH levels obtained by radio-receptor binding assay (RRA) and RIA (RIA and RRA results provided by Dr. Lloyd Moore, AgResearch). The heterologous RIA for ovine FSH was identical to that described by McNatty *et al* (1984). Concentrations of FSH determined by RRA (Cheng, 1975) were obtained using membrane preparations prepared from bovine testes and ^{125}I-ovine FSH as the label. A comparison of the results obtained by bioassay, RIA and RRA was performed by calculating the total FSH eluted from the HPLC as determined by the individual assay method. The fraction by which 50% of the total FSH had been eluted (HP50) was then calculated for each assay system.

5.3.2 Results

For each pituitary the bioactive FSH concentrations of the samples obtained were plotted next to immunoactive FSH concentrations obtained by RIA and receptor binding affinity concentrations by RRA. The FSH concentrations for each individual pituitary are shown in Figures 5.3 to 5.14 Fig. The bioassay, RIA and RRA profiles for most pituitaries appeared very similar, although there were striking differences in several sheep. The RRA profile for pituitary EFP 41 (Figure 5.4) was considerably higher than bioassay and RIA profiles, while sheep EFP 72 (Figure 5.5) exhibited different profiles for bioassay, RIA and RRA.

In all extracts assayed, the concentrations of FSH were near or below the detection limit of the assay in fractions 1 – 10. In most cases, levels of bioactive and immunoactive FSH rose sharply at about fraction 15, reaching a peak between fractions 28 and 34, and then declined to levels near or below the detection limit between fractions 50 and 60. The mean fraction by which 50% of the total FSH had been eluted (HP50) as measured by FSH bioassay was 29.6 ± 0.79 SEM. The HP50 as measured by RIA and RRA was 30.8 ± 9 SEM and 31.0 ± 0.99 SEM, respectively. No significant difference was seen between HP50 values measured by RIA and RRA. However a significant difference ($P < 0.05$) was seen in the HP50 value measured by bioassay and the RIA and RRA HP50 values, as determined by *t* test.

The bioactive and immunoactive FSH levels of each pituitary extract are summarised in Figure 5.15. The results obtained from all the pituitary extracts by bioassay, RIA, and RRA are represented as the mean of the percentage of total FSH eluted at each HPLC fraction, and from this plot, a good agreement between bioactive and immunoactive results can be seen.

The intra- and interassay coefficients of variation were 13.8% ± 1.2% SEM (n=18) and 16.9%, respectively (n=10). The detection limit of the bioassay ($P < 0.05$) was 1.12 ng/ml RP2 ± 0.15 SEM (n=18).

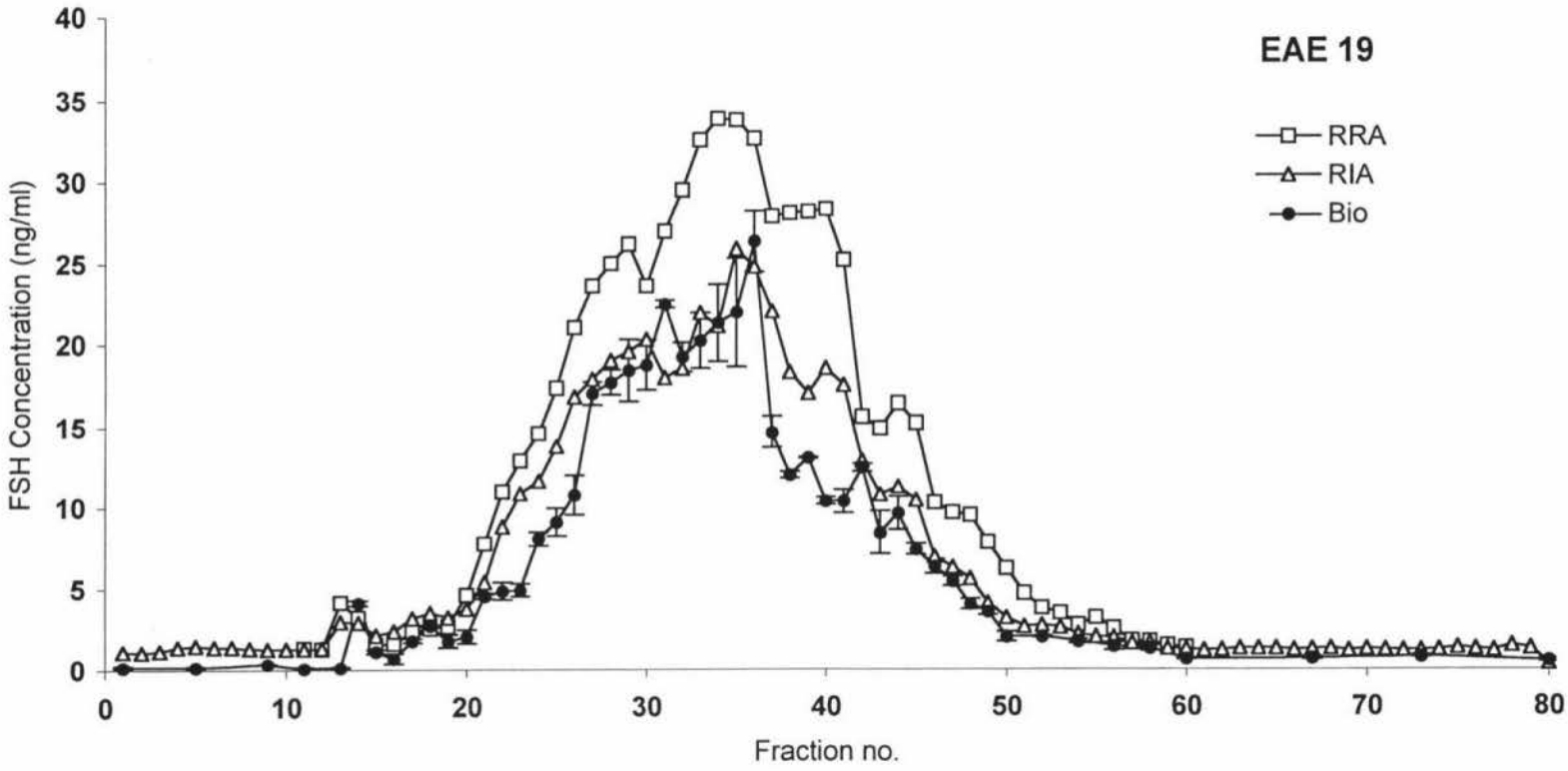


Fig 5.3 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep EAE19. Each point represents the mean of triplicates and error bars represent SEM.

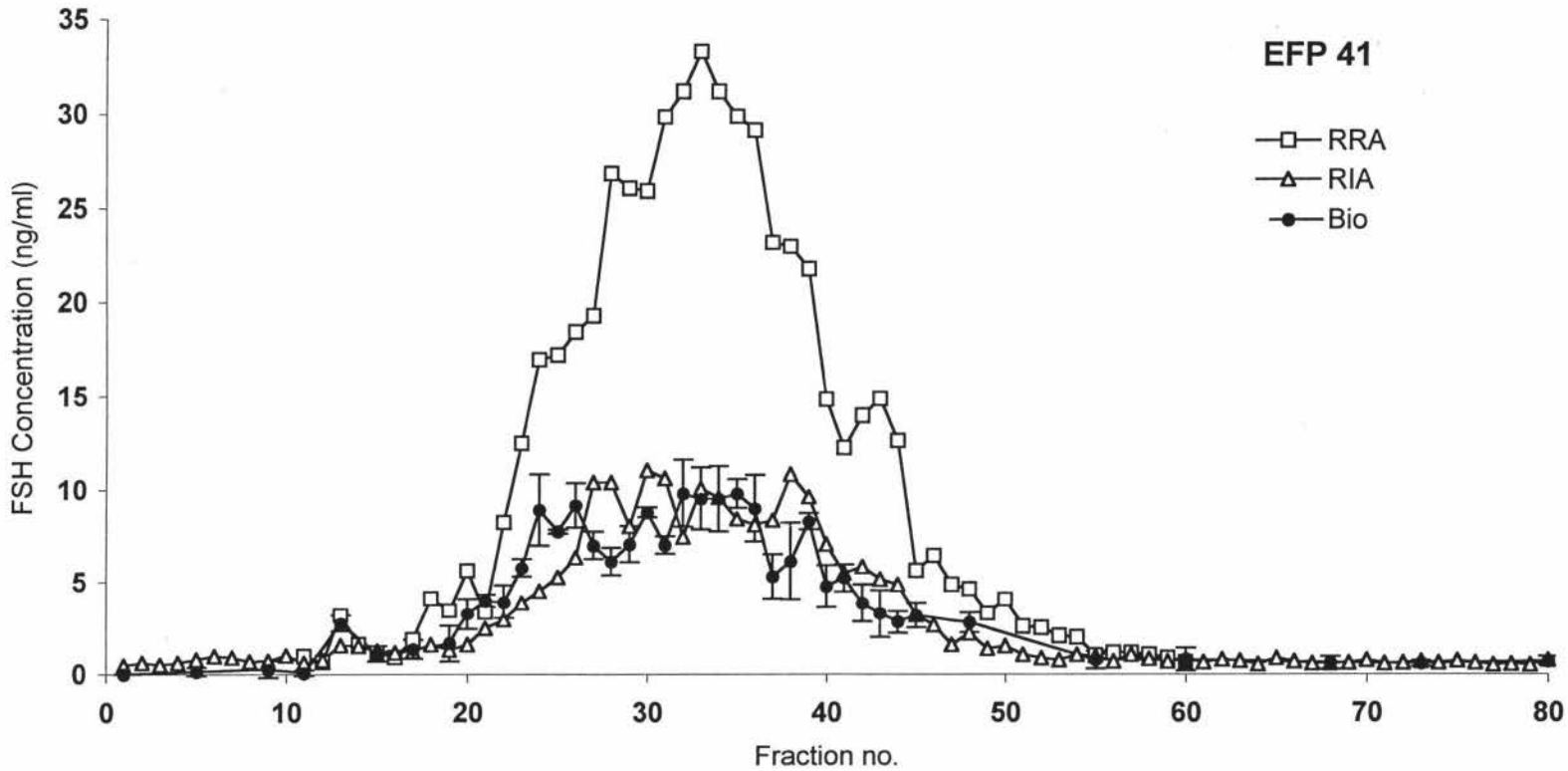


Fig 5.4 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep EFP41. Each point represents the mean of triplicates and error bars represent SEM.

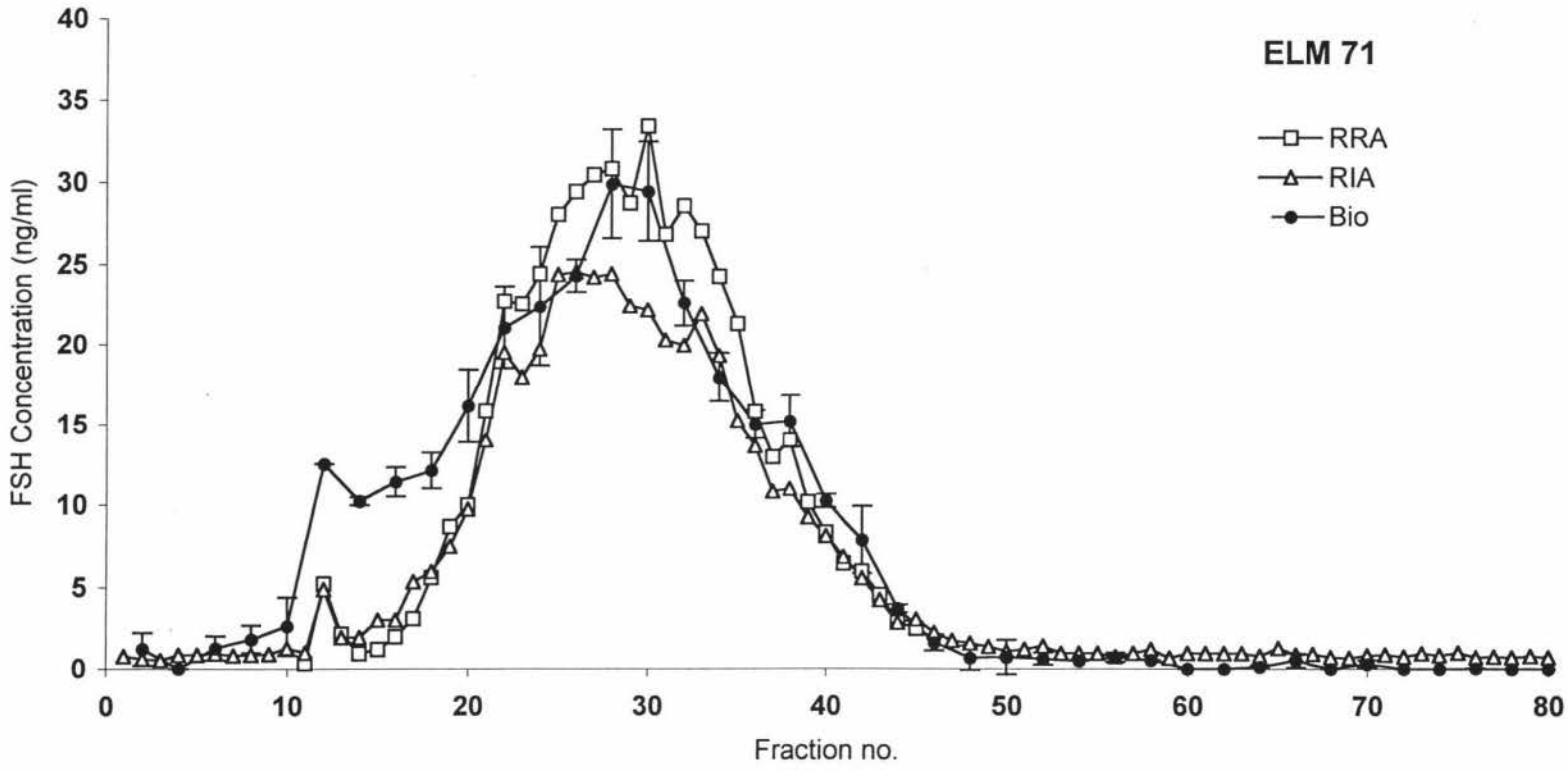


Fig 5.5 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep ELM71. Each point represents the mean of triplicates and error bars reperesent SEM.

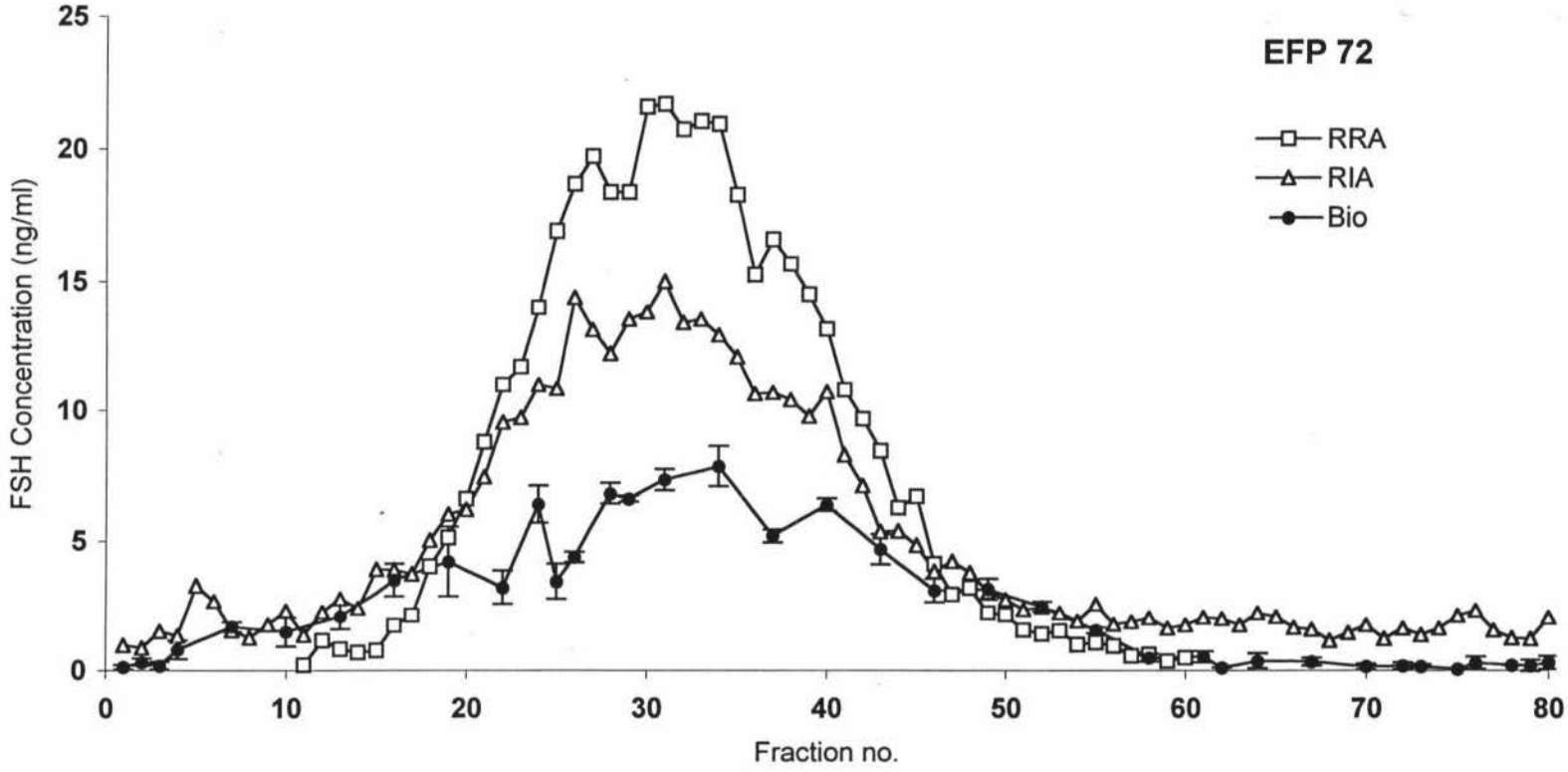


Fig 5.6 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep EFP72. Each point represents the mean of triplicates and error bars represent SEM.

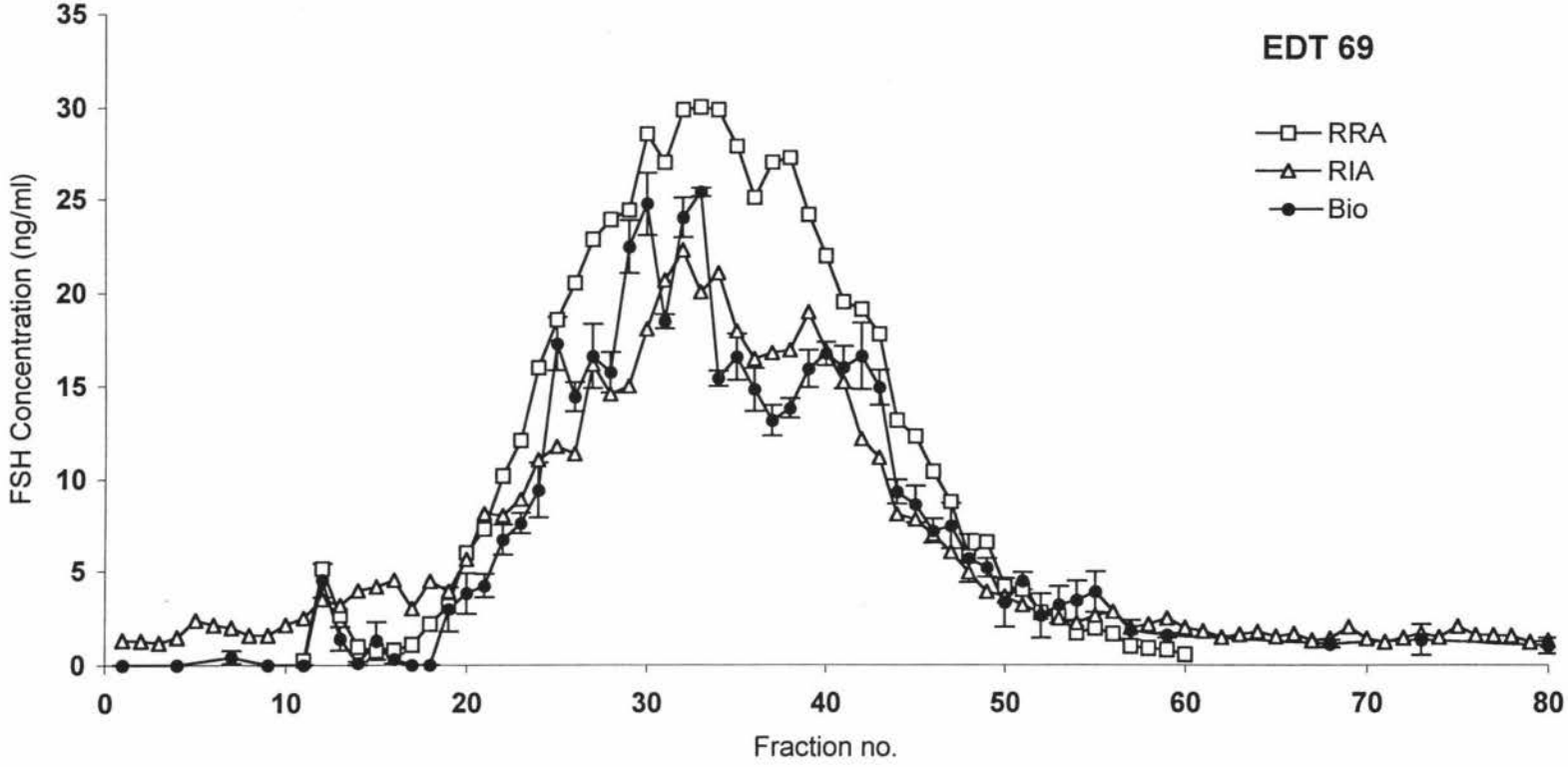


Fig 5.7 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep EDT69. Each point represents the mean of triplicates and error bars represent SEM.

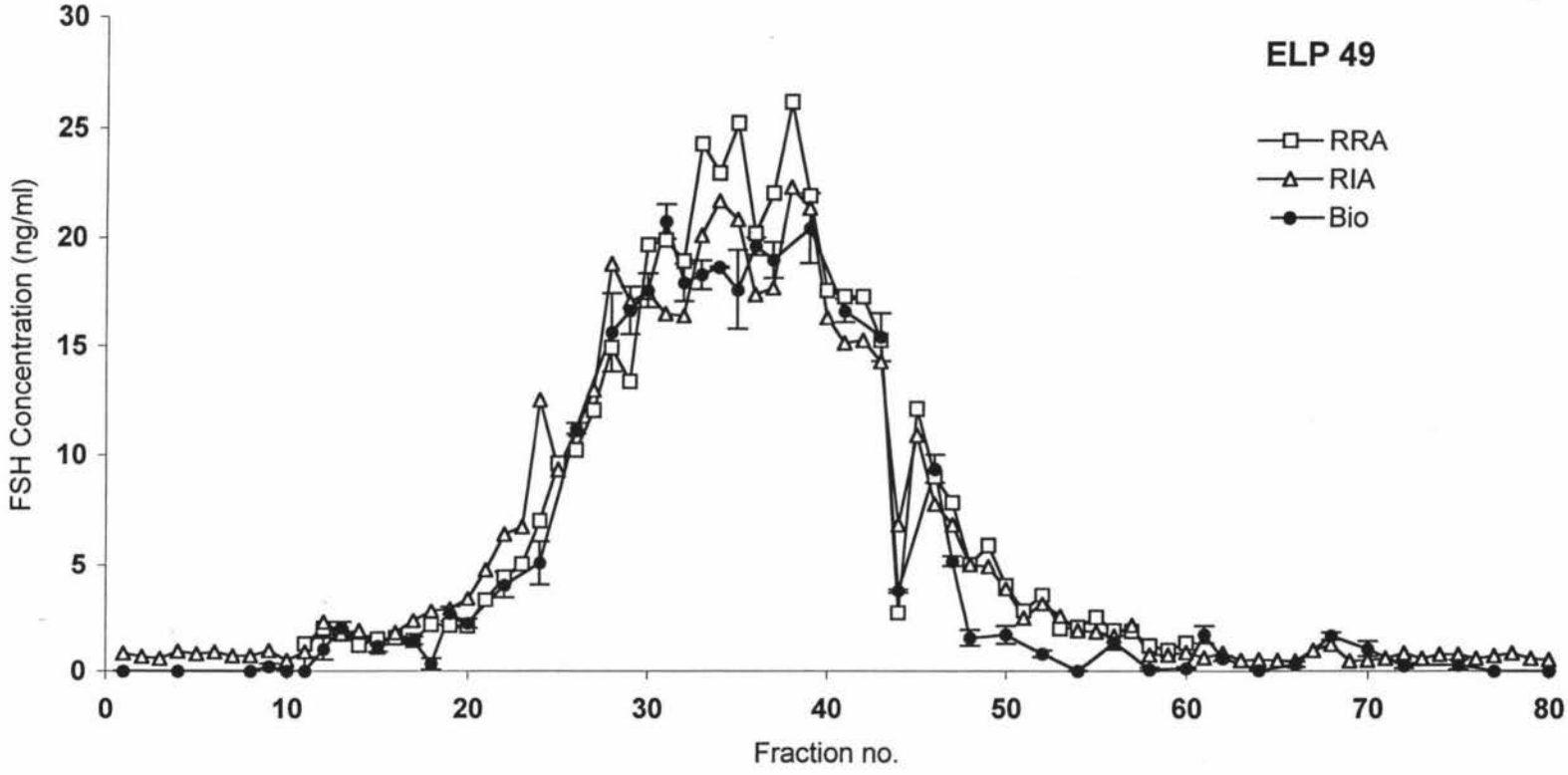


Fig 5.8 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep ELP49. Each point represents the mean of triplicates and error bars represent SEM.

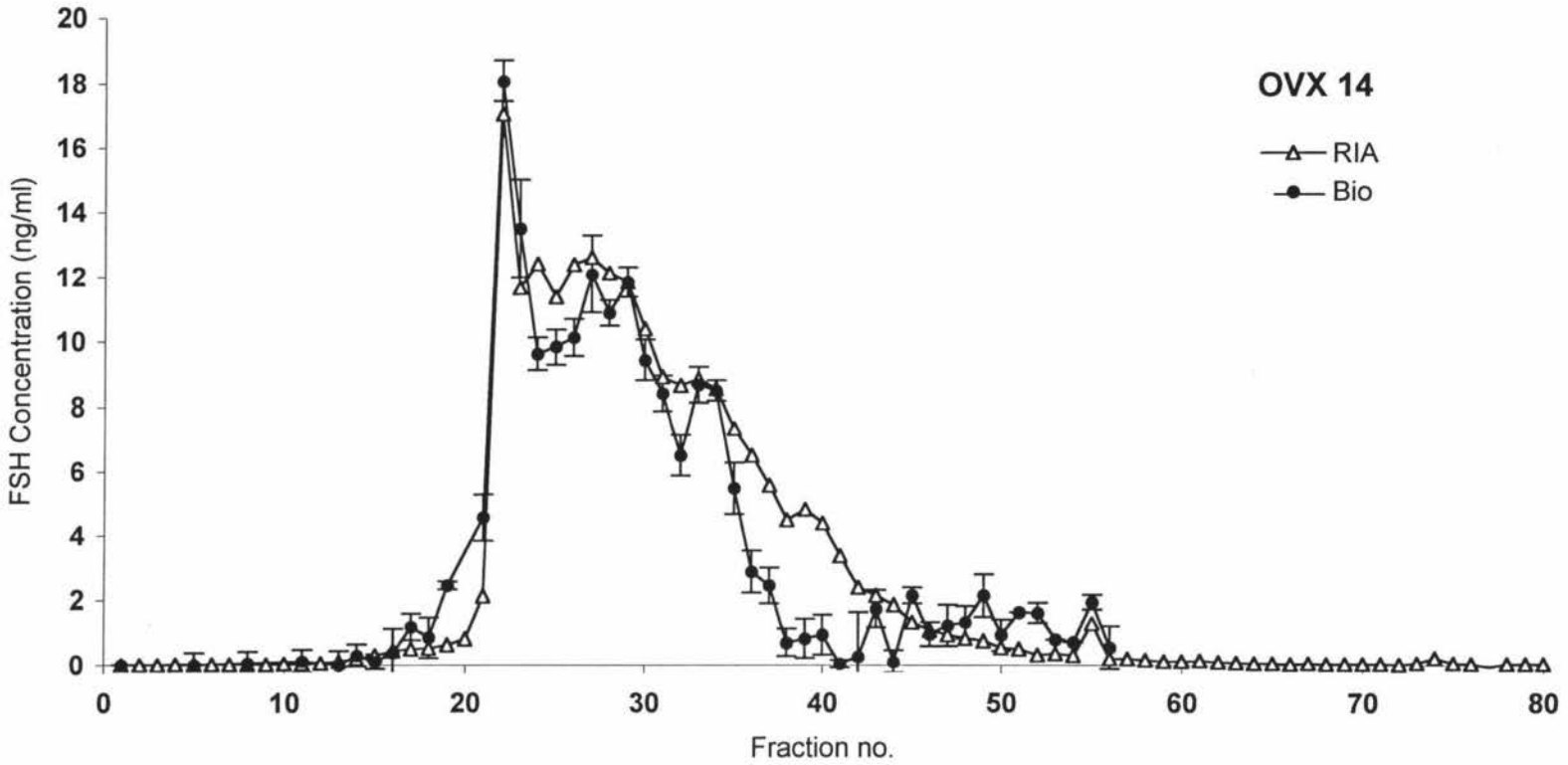


Fig 5.9 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and immunoactive FSH concentrations obtained by radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep OVX14. Each point represents the mean of triplicates and error bars represent SEM.

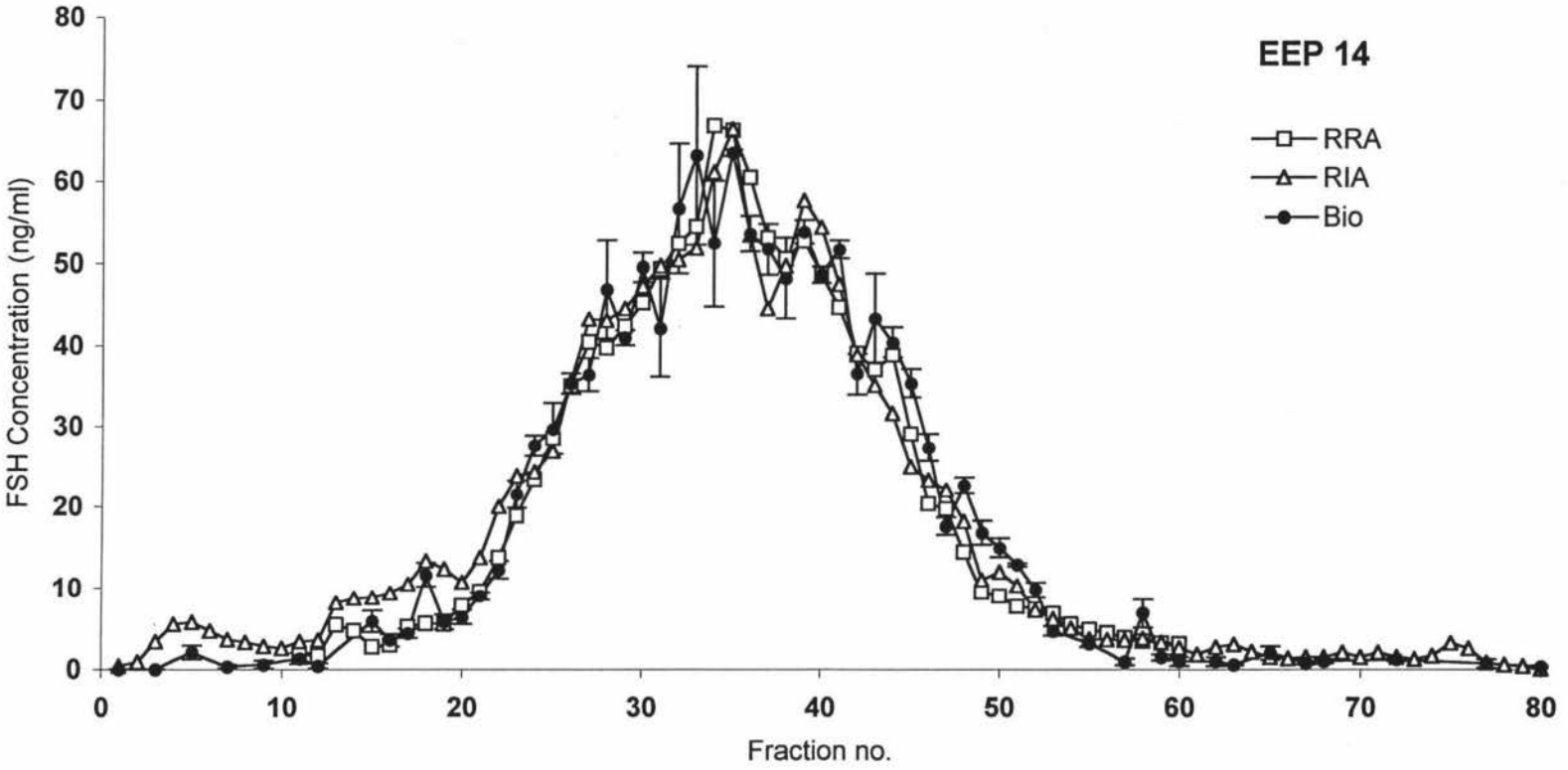


Fig 5.10 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep EEP 14. Each point represents the mean of triplicates and error bars represent SEM.

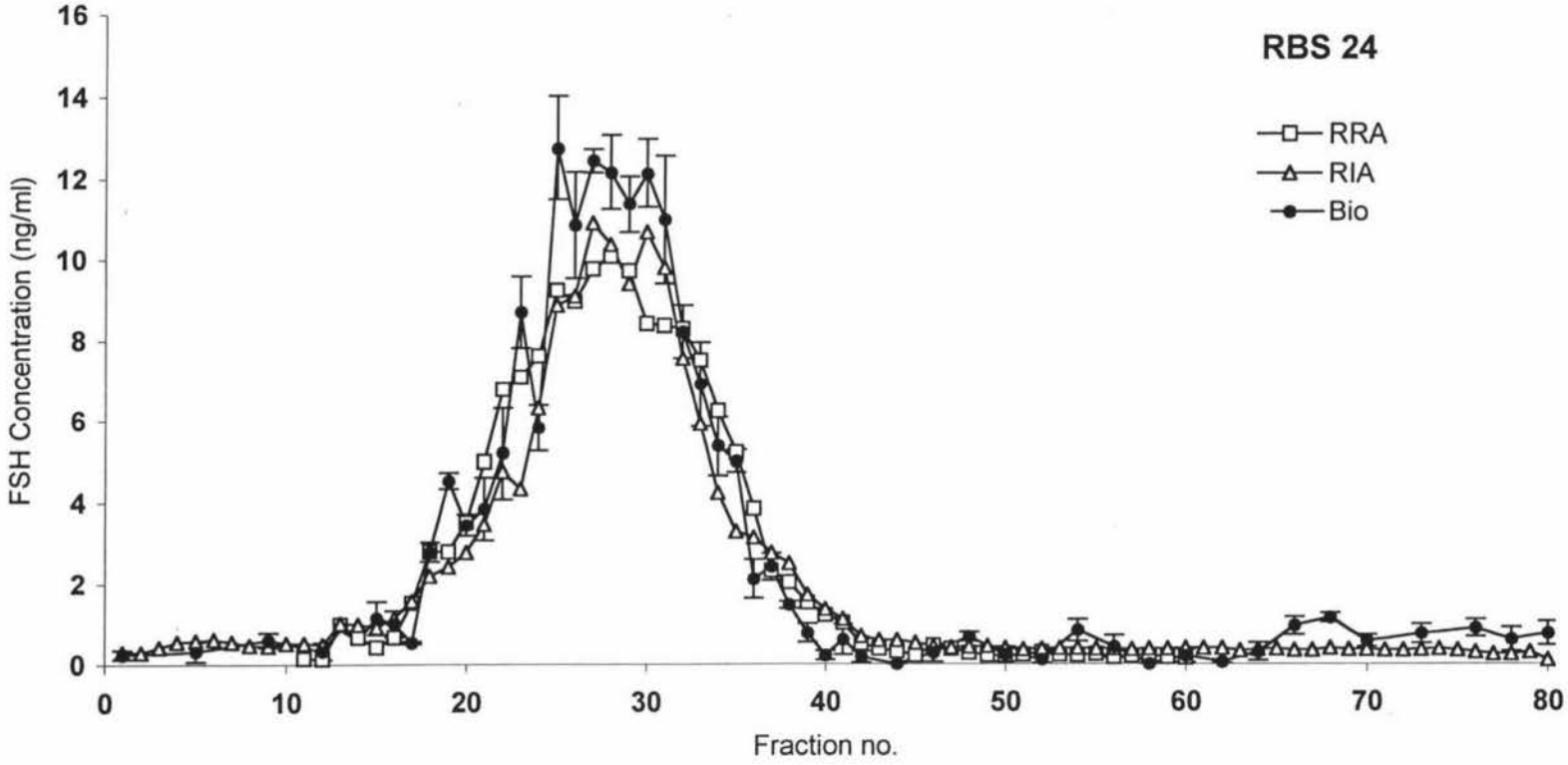


Fig 5.11 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep RBS 24. Each point represents the mean of triplicates and error bars represent SEM.

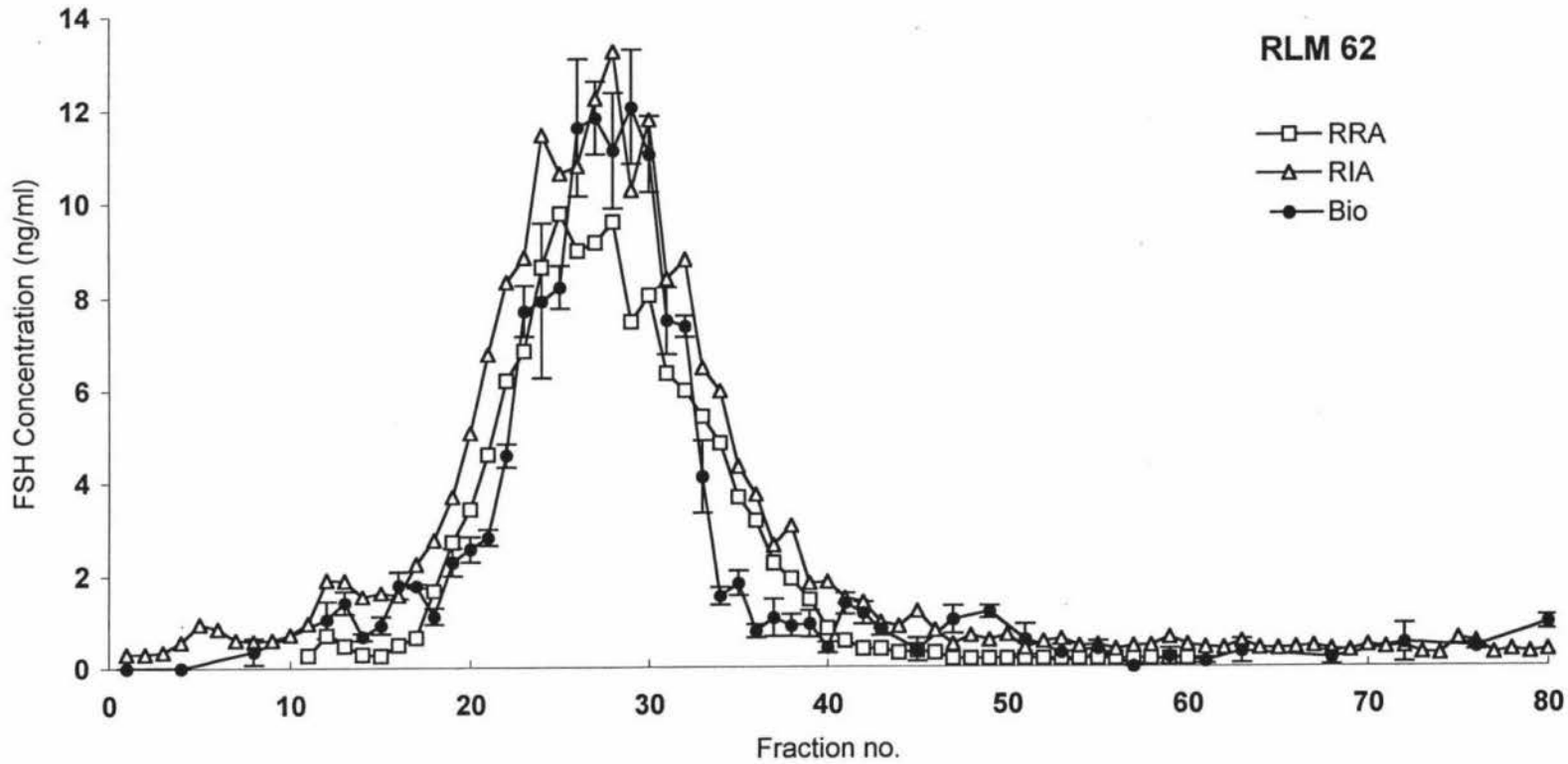


Fig 5.12 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep RLM 62. Each point represents the mean of triplicates and error bars represent SEM.

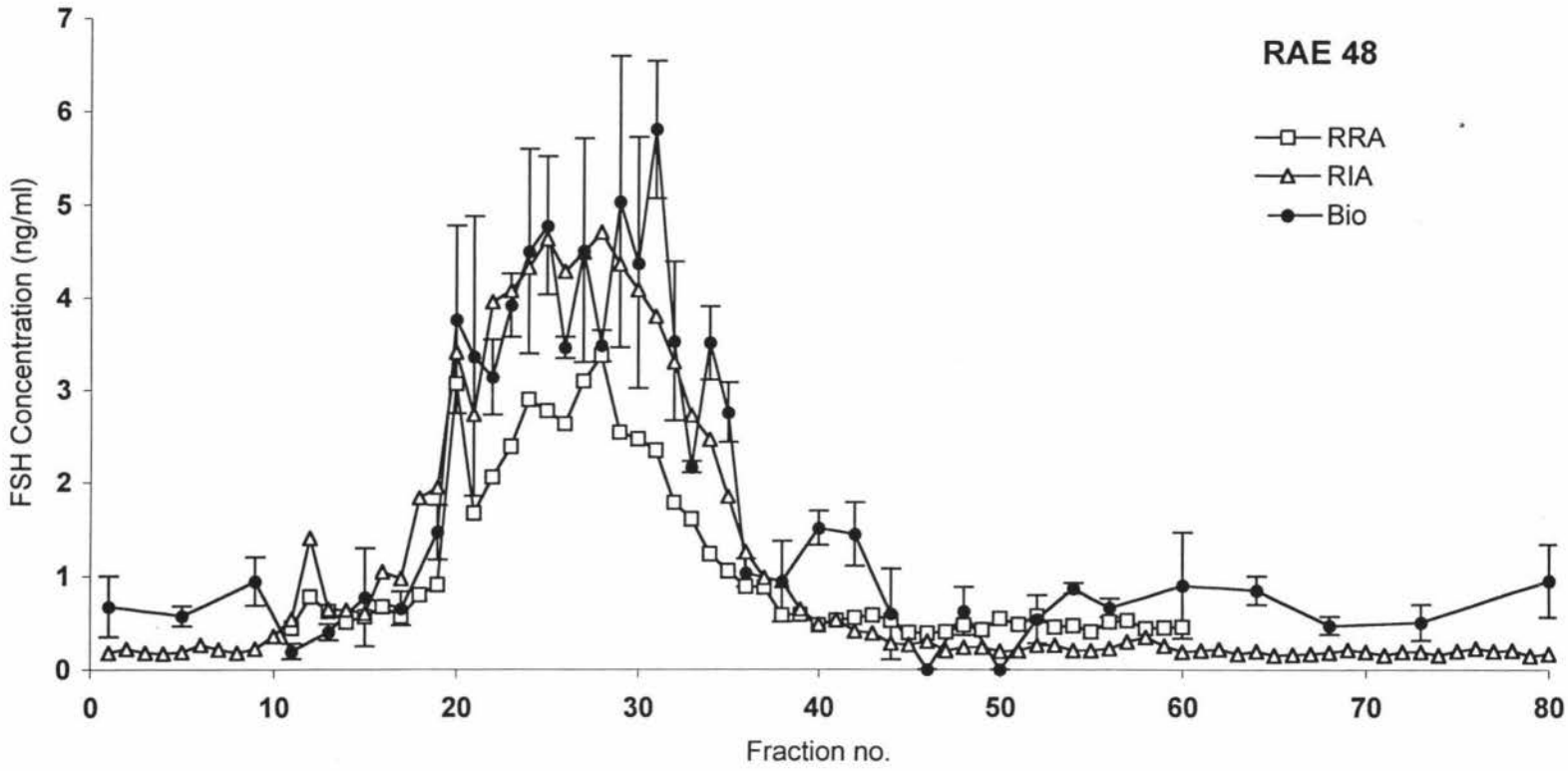


Fig 5.13 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep RAE 48. Each point represents the mean of triplicates and error bars represent SEM.

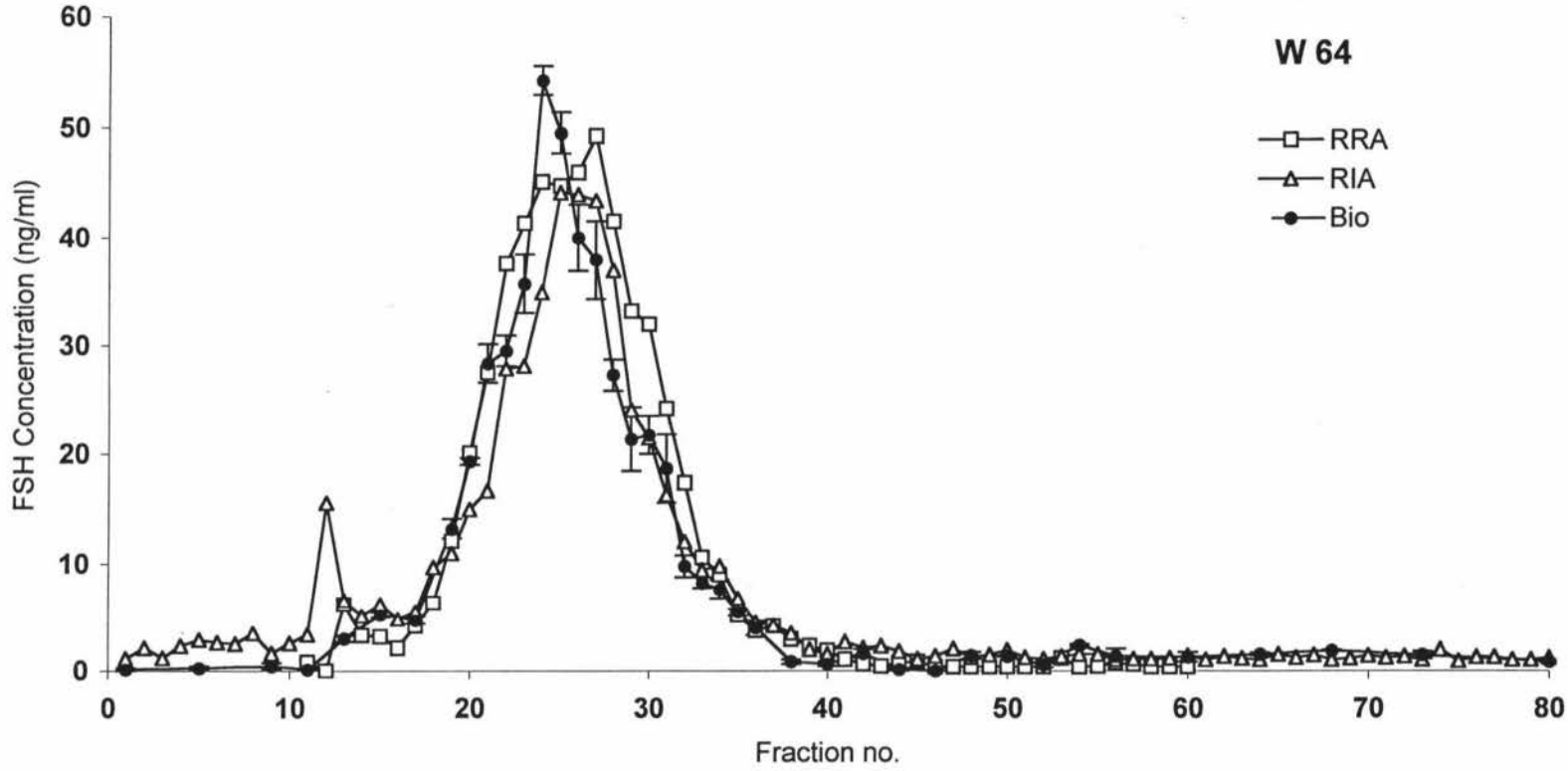


Fig 5.14 Comparison of bioactive FSH concentrations obtained by CHO FSHR-R cell bioassay (●) and FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ) in HPLC fractions of purified pituitary extract from sheep W 64. Each point represents the mean of triplicates and error bars represent SEM.

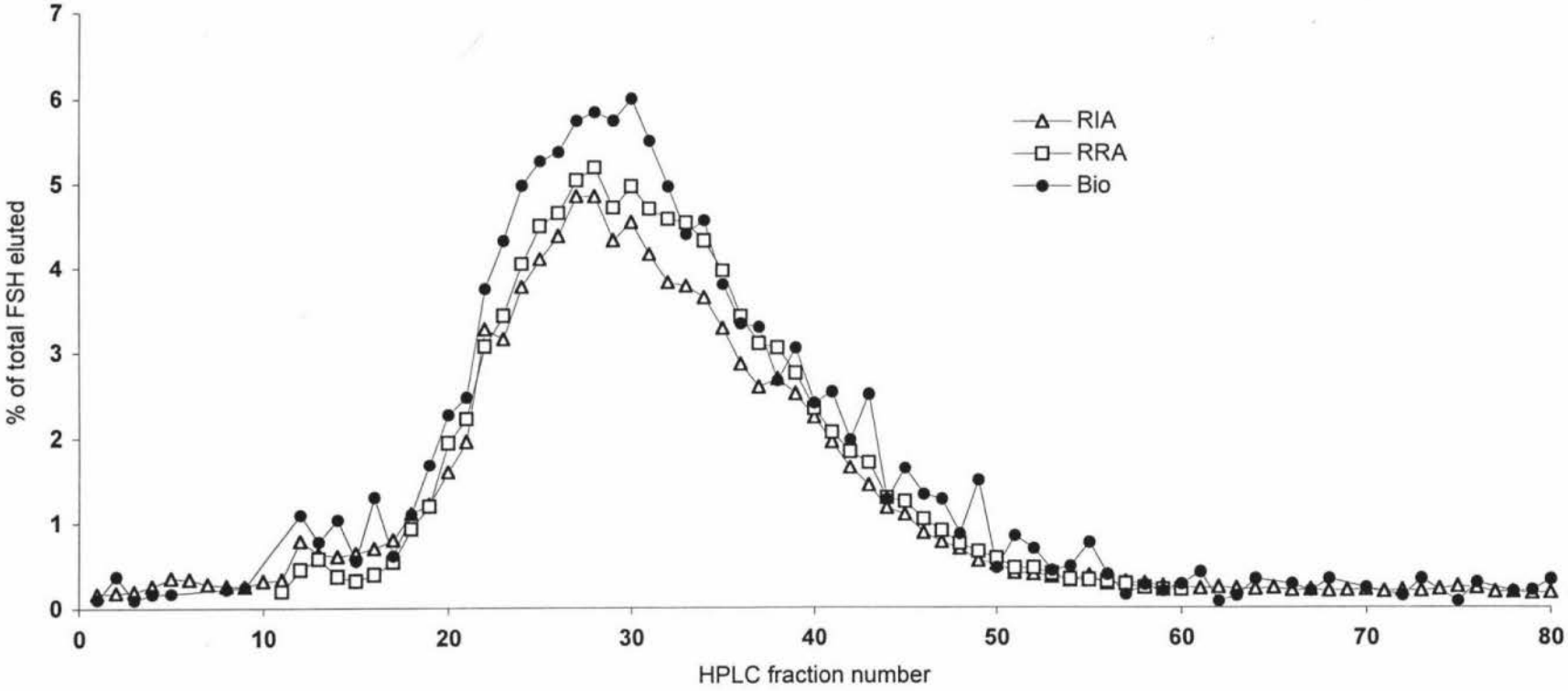


Fig 5.15 Comparison of the mean percentage of the total bioactive and immunoactive FSH eluted in each HPLC fraction of 12 individual sheep pituitary extracts. Bioactive FSH concentrations were obtained by CHO FSH-R cell bioassay (●) and immunoactive FSH concentrations obtained by radio receptor assay (□) and radioimmunoassay (Δ).

5.3.2.1 Correlation

The bioassay and RIA data pairs for all pituitary extract samples were plotted against each other (Figure 5.16). The R^2 value for the observed data was 0.9328, and the slope of the linear regression line fitted to the data was 0.93. The mean B/I ratio of the samples was 1.02 ± 0.036 SEM.

The correlation between FSH concentrations obtained by bioassay and RRA are shown in Figure 5.17. The R^2 value for the observed data and slope of the linear regression line were 0.8039 and 0.99, respectively. The mean biologic: receptor binding activity (B/RBA) ratio was 1.18 ± 0.113 SEM.

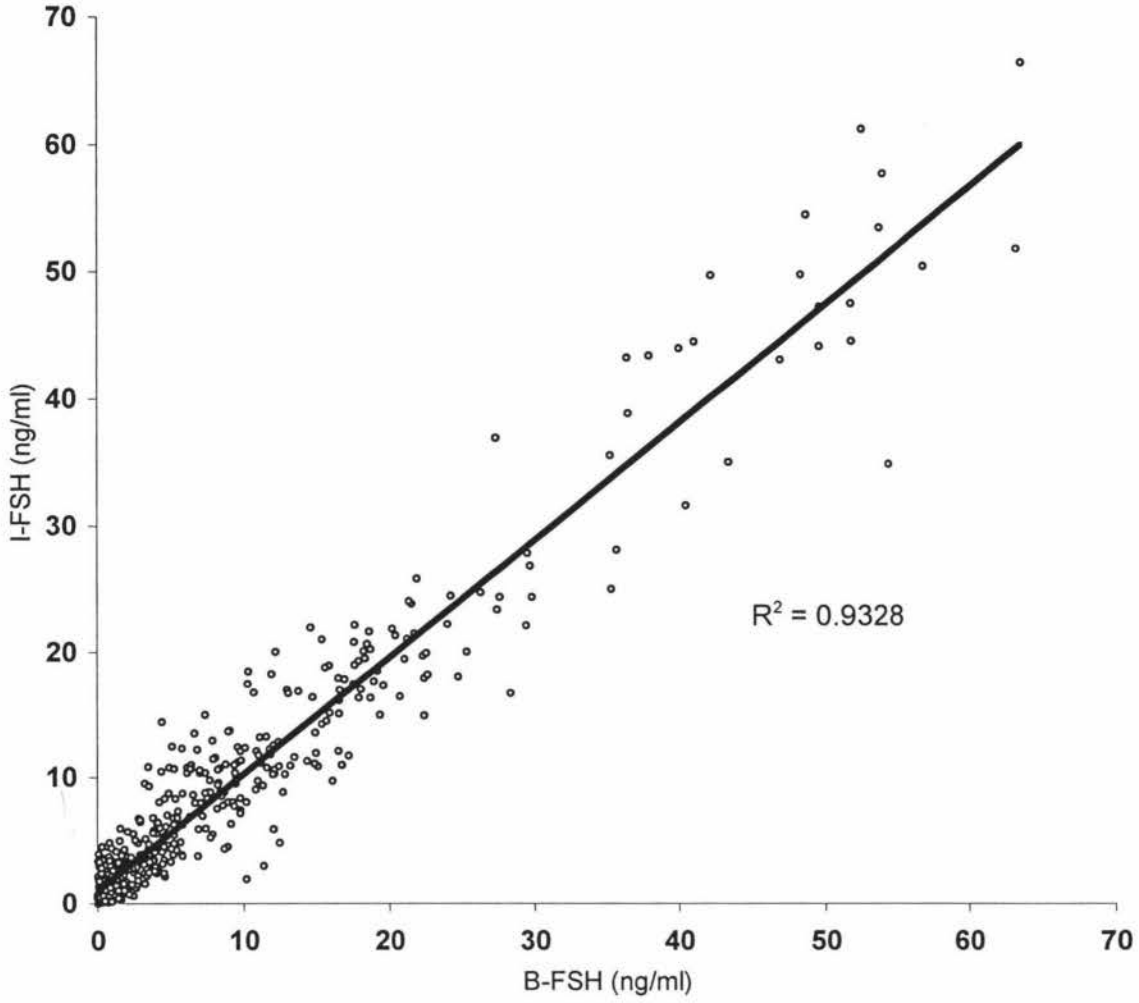


Fig 5.16 Correlation between bioactive (B-) and immunoactive (I-) FSH concentrations in purified pituitary extract samples. Bioactive FSH concentrations were obtained by FSH bioassay and immunoactive FSH values were obtained by RIA. The closeness of fit of the scatterplot to it's linear regression line is indicated by R^2. The slope of the regression line is 0.93.

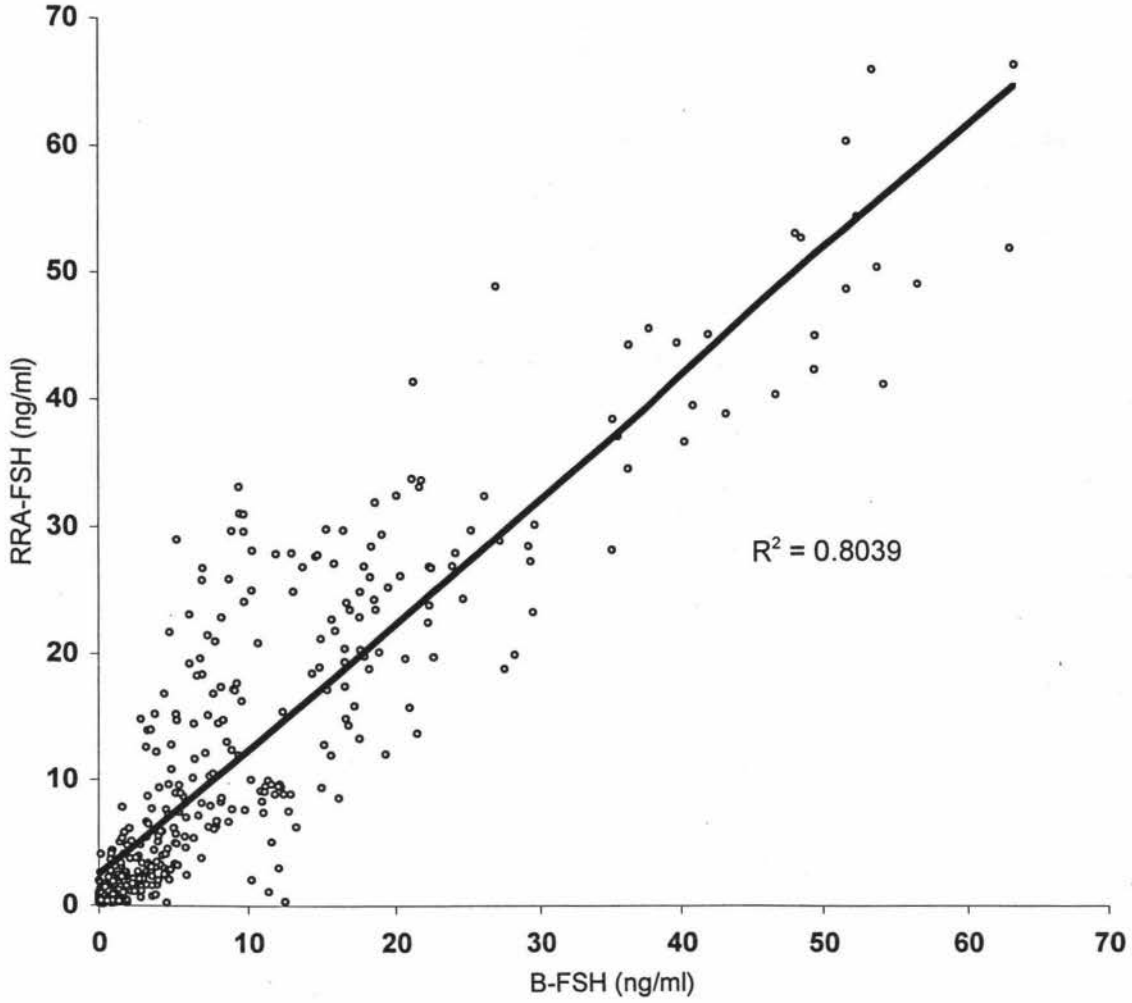


Fig 5.17 Correlation between bioassay (B-) and radio receptor assay (RRA-) FSH concentrations in purified pituitary extract samples. The closeness of fit of the scatterplot to it's linear regression line is indicated by R^2. The slope of the regression line is 0.99.

5.4 Discussion

The receptor cell line bioassay was successfully used to assay purified pituitary extract samples (20 mM tris-HCl) and pituitary culture media. The assay was validated for these samples by comparing the parallelism of serially diluted sample with RP2 standard. The slopes of the standard and samples were not found to be different.

5.4.1 Polymorphism of FSH

Follicle-stimulating hormone consists of a family of heterologous molecular isoforms that can be partially resolved by chromatographic techniques, separating different isoforms according to various physiochemical properties, such as charge and pH (Simoni *et al*, 1994). A comparison of *in vitro* bioactivity with immunoactivity in past studies has led to the belief that less acidic, less glycosylated FSH isoforms have a higher biopotency in vitro compared with the more acidic forms (Simoni *et al*, 1994). This concept has been reinforced in this study by the measurement of bioactive FSH in ovine pituitary preparations separated by chromatography, where segregation between bioactive and immunoactive FSH isoforms has been demonstrated. Although a clear-cut separation of pure molecular isoforms of pituitary FSH was not obtained, as this would require an extremely long and complex procedure, it is assumed that each HPLC fraction collected would contain isoforms with similar physiochemical properties.

Although an analysis of the total data set indicated very similar results were obtained from bioassay, RIA and RRA, the bioassay HP50 value was found to be significantly different to the HP50 values obtained by RIA and RRA. The difference observed in bioassay HP50 values compared to HP50 values for RIA and RRA indicates a slight differential separation of the bioactive and immunoactive FSH isoforms in the HPLC column. From the results obtained, it appears that the more biopotent isoforms of FSH were eluted slightly before less bioactive forms. This result suggests a possible potential for the isolation of more bioactive forms of FSH for commercial purposes.

The correlation observed between bioassay and RIA values of purified pituitary extract samples was high (R^2 = 0.9328, slope = 0.93), indicating good agreement between the data obtained from

both assay methods. Similarly, the correlation between bioassay and RRA also showed good agreement (R^2 = 0.8039, slope = 0.99). The B/I and B/RBA ratios were 1.02 and 1.18, respectively.

The FSH profiles of all pituitary extracts except OVX 14 (Figure 5.9) had a similar shape, resembling a bell-shaped distribution curve. The difference in the FSH profile exhibited by pituitary OVX 14 is noteworthy, as this sample originated from and ovariectomised ewe. All other samples were from intact sheep. This finding provides support for the presence of endocrine factors affecting the bioactivity of circulating FSH isoforms in sheep. Therefore, the availability of sensitive bioassays for FSH may provide a useful tool for investigating the heterogeneity of FSH and the role it plays in the reproductive status of various species.

Chapter Six

Discussion

6 Discussion

Follicle-stimulating hormone is commonly measured by RIA and, more recently, enzyme immunoassay (Pappa *et al*, 1999). This allows rapid measurement of large numbers of samples and has been important in the elucidation of phenomena such as pulsatile hormonal release (Motta *et al*, 1991), and furthered the understanding of the mechanisms by which endocrine organs communicate. However, RIA and enzyme immunoassay measures the immunological activity of FSH, characterised as the ability of FSH to interact with a chosen antibody. Moreover, the immunological activity of FSH may not necessarily reflect the biological activity of FSH, defined as the ability of FSH to elicit a biological response, as opposed to interacting with an antibody. In actuality, when the immunological and biological activity of FSH was measured, there was generally a good correlation between RIA and bioassay values, as indicated by results in this study. However, the B/I ratios were not always equal or close to one suggesting that there are differences between the immunological and biological characteristics of FSH. In particular, differences in the B/I ratios have been noted under various physiological conditions, suggesting the isoform production may be influenced by hormonal factors (McNatty *et al*, 1989a; McNatty *et al*, 1998).

6.1 Biological to Immunological Ratios

The introduction of technologies, such as RIA, permitted the study of changes in serum gonadotrophins after hormonal manipulations or during specific endocrine states. With the development of *in vitro* bioassays, the changes in the potency of gonadotrophins in serum could be measured. However, when samples were assayed by bioassay and RIA, the ratio of the bioactivity to immunoactivity did not always equal one. Indeed, changes in B/I ratios were observed during various endocrine states (Padmanabhan *et al*, 1992; Jakkkola et al, 1990; Reddi *et al*, 1990; Creus *et al*, 1996; McNatty *et al*, 1998). Therefore, hormonal manipulations are thought to alter the rates of production and secretion of individual isohormones. For this reason, the use of B/I ratios for observing endocrine interactions has become widespread.

B/I ratios are generated by quantifying a sample for bioactivity and immunoactivity, and serves as an index of the overall potency of FSH. RIA measures the total amount of gonadotrophin in the

sample to normalise changes in biological activity per unit of immunoactivity. Changes in B/I ratios are considered to be the result of a change in the amount of biological activity per unit of immunological activity. However, this is based on the assumption that the RIA does not discriminate between isohormones. While this has been assumed, to date it has not been proven.

The suitability of using B/I ratios as a measure of hormonal status is affected by several factors. Firstly, as the assessment of biological and immunological activity cannot discern differences in plasma FSH half-life, the validity of the B/I ratio as a marker of endocrine status is further restricted. Secondly, the choice of FSH standard is critically important. In any assay system, it is assumed that the standard, labeled hormone and the sample will behave similarly. However, the response to endocrine manipulations may not be accurately quantified when expressed in terms of a reference standard that does not contain all of the isoforms of FSH. Therefore, if variation in the relative abundance and types of isoforms exists between standards, similar studies may find differences in the B/I index, unless identical standards are used.

Despite the limitations described, the use of B/I ratios as a measure of endocrine status remains a widely employed and practical tool for the evaluation of hormonal states, and is still one of the primary applications for *in vitro* bioassays. However, recognition of the current methodological limitations provides the impetus for developing more sophisticated tools, such as that described in this study.

6.2 Comparison of FSH Bioassay Methods

The biological activity of FSH *in vivo* is characterised by its ability to stimulate follicular development in the ovary (McNatty *et al*, 1993) and spermatogenesis in the testis (Boitani *et al*, 1993). However, these are difficult end-points to measure for an assay system. There are several steps involved in FSH stimulation of gonadal development, including binding of FSH to cell surface receptors, stimulation of cAMP production and initiation of cellular events, such as induction of aromatase activity (Ortavant *et al*, 1969; McNatty *et al*, 1989b). Several *in vitro* bioassays for FSH have been based on these characteristics of FSH action (Padmanabhan *et al*, 1987; Jia and Hsueh, 1986). In contrast, *in vivo* bioassays are based on ovarian weight gain in response to treatment with FSH (Mannaerts *et al*, 1991).

The advantage offered by *in vivo* bioassays is that the half-life of FSH is taken into account, whereas this is not measurable in *in vitro* bioassays. Thus, an isoform of FSH with a fast metabolic clearance rate will behave differently from one with a long half-life in an *in vivo* bioassay, but both may behave similarly in an *in vitro* bioassay. However, *in vivo* assays are cumbersome, technically expensive and have a high ethical cost. In contrast, *in vitro* binding assays are relatively straightforward to perform, but do not measure post receptor events. Previous studies have clearly indicated that receptor binding and the ability to induce signal transduction are distinct properties of FSH (Valove *et al*, 1994). The measurement of FSH stimulated cAMP production, such as in the CHO FSH-R cell bioassay, has the advantage that it measures G protein-linked receptors, or 'functional' receptors. The disadvantage of this system is that it does not measure any subsequent intracellular events, such as with the rat Sertoli cell bioassay for FSH (Padmanabhan *et al*, 1987), where the end-point measured is aromatase activity. Therefore, it could be argued that the Sertoli cell bioassay provides a truer representation of the bioactivity of FSH. However, advantages of the receptor cell line bioassay include a technically simpler methodology and it does not utilise primary tissue culture, therefore it has a negligible ethical cost. The availability of simple and specific bioassays for FSH not only has consequences for research purposes, but may also enable more advanced quality control testing of commercially available FSH products, such as human urinary FSH preparations (Rodgers et al, 1995) or FSH products used in the superovulation of livestock (Braileanu et al, 1998).

6.3 FSH Bioassay Performance

Results of this study show that the CHO cell line that expresses human FSH receptors originally developed for measuring human FSH (Albanese *et al*, 1994) is also suitable for measuring ovine FSH. Therefore, the development of an assay that measures bioactive ovine FSH with a high degree of sensitivity and specificity is described.

Important features of this cell line include elimination of primary tissue culture, and long-term culture stability in terms of both cell viability and FSH receptor expression. The CHO FSH-R cell line has been validated for use in the FSH bioassay based upon a number of important properties. Firstly, the cell line has been demonstrated to be stable in culture and does not exhibit variation in hormone responsiveness, even after 24 months of continuous passage. Secondly, the

CHO FSH-R cells have been shown to respond to different forms of FSH, originating from a variety of mammalian species, in a dose dependent fashion. Thirdly, the cell line does not respond in a dose dependent manner to other glycoprotein hormones at physiological concentrations, and only minimally at supraphysiological concentrations. Similarly, the cAMP assay clearly shows that the cell line does not respond to α- or β-FSH except at supraphysiological concentrations. It is possible that at such high concentrations of pituitary hormone, the response of the CHO FSH-R cells is due to FSH contamination, rather than non-specific binding to the receptors.

The sensitivity of the bioassay is high enough to allow accurate measurements of FSH at levels that might be expected *in vivo* (1-10 ng/ml). Furthermore, amounts of bioactive FSH measured by the receptor cell line appear to correlates well with that measured by FSH RIA, the only exception being where FSH was measured in hypophyseal venous blood samples. This study has also shown that the CHO FSH-R cell line still responds to FSH in a dose dependent manner when challenged with unextracted sheep serum. In addition, the bioassay has exhibited a high degree of parallelism between the RP2 FSH standard and serum, pituitary extract, and culture media samples. These samples represent types of materials that are commonly examined in reproductive physiological studies. Urinary samples were not examined with the receptor cell line bioassay, although they may also be used to provide useful physiological information (Oosterhuis, *et al*, 1998).

During the course of evaluating the receptor cell line bioassay for measuring ovine FSH, several interesting sets of data had been generated. The measurement of bioactive FSH in ovariectomised ewes following hypophysectomy has revealed the presence of bioactive isoforms of FSH detectable in serum even 34 hours after surgery. This indicates the presence of FSH isoforms with extremely slow metabolic clearance rates. In addition, the good level of correlation observed between bioactive and immunoactive concentrations reinforced confidence in the accuracy of the bioassay system. However, clear differences in bioassay and immunoassay results indicate that the two assay systems are indeed sensitive to different components in serum.

Release of gonadotrophins from the pituitary is modulated by the pulsatile secretion of GnRH from the hypothalamus (Southworth *et al*, 1991; Weiss *et al*, 1992). Although pulsatile GnRH stimulation is required for the normal maintenance of both LH and FSH release from the pituitary, only LH has been shown to be secreted in a pulsatile manner in sheep (Porter *et al*,

1997). While the pulsatile release of FSH has been documented in humans (Motta *et al*, 1991), this phenomena has not been demonstrated in sheep to date. Indeed, *in vitro* studies have shown a significant difference in GnRH stimulated secretion patterns of LH and FSH in cultured pituitary cells (Henderson *et al*, 1989; Jakubowiak *et al*, 1992).

In this study, the measurement of bioactive FSH in ovine hypophyseal venous blood samples, which contains FSH prior to entry into the peripheral circulation, may provide evidence for the pulsatile release of FSH from the anterior pituitary. Levels of bioactive FSH in these samples fluctuated markedly, whereas immunoactive and receptor binding concentrations were comparatively more stable. A possible explanation for this result is that total concentrations of FSH released from the pituitary were relatively constant, but there was large variation in the bioreactivity of the isoforms present. Another possibility is that levels of FSH released were not constant, but this change was not detected by RIA or RRA. However, because the time interval between samples is not known, the nature of FSH pulses cannot be verified from these experiments.

The measurement of bioactive FSH in purified ovine pituitary extracts has helped to provide information on the nature of the heterogeneity of FSH. The profile of bioactive FSH in fractions that were separated based on physiochemical properties showed a good correlation with immunological and receptor binding activity profiles. However, differences in HP50 values indicate a small but significant shift in the biopotency of FSH towards fractions eluted earlier.

In summary, there is excellent correlation between RIA, RRA and bioassay results, thus indicating that the immunological characteristics of FSH generally mimic its biological characteristics. However, there were instances when the B/I ratio differed markedly from one, indicating a discrepancy between these properties of FSH.

6.3.1 Coefficient of Variation

As with all assay methods, the CHO FSH-R cell bioassay exhibited some variability in the evaluation of samples both within and between assays. The source of this variability stems from a number of factors. These may include operator errors, differences in the concentrations of stock solutions and variation in environmental conditions, such as fluctuations in temperature and

humidity within the incubator. Variation may occur depending on the position of the well in relation to the entire culture dish. To minimise any bias caused by this possibility, the samples were placed in random wells. In addition, the receptor cell line FSH bioassay is essentially composed of two assays; the cAMP RIA and the cell culture element of the assay. Therefore, the variability in the data generated could be compounded by errors in either component.

Intra-assay and inter-assay coefficients of variation of the cAMP RIA fell within normal limits experienced by operators in the Reproductive Physiology Group laboratories, as did the minimum detectable concentration and ED_{50}. Coefficients of variation for the bioassay were higher than those reported by other laboratories using the CHO FSH-R cells to measure human FSH (Albanese *et al*, 1994; Tano *et al*, 1995). However, in those studies, the concentration of the control samples used to calculate the variability was not reported. As the concentration of the controls selected has a large influence on the coefficient of variation generated, the variability of this bioassay cannot be directly compared without knowing that information. Furthermore, differences in the standards used by different laboratories make direct comparisons difficult, as the variation in values of samples assayed is dependent on the detection limit of the assay.

6.3.2 Sensitivity

Estimates of the smallest concentration of RP2 FSH that gives significantly greater ($P < 0.05$) cAMP accumulation than that of the zero standard in the receptor cell line bioassay varied depending on the material being assayed. Values ranged from 0.85 ng/ml in serum samples from recently hypophysectomised sheep to 1.42 ng/ml in serum from hypophyseal venous blood samples. The high detection limit of the bioassay with regard to hypophyseal blood samples most likely stemmed from the reduction in sample volume used as compared to other serum assays performed in this study. A higher assay volume used in serum samples is desirable to counteract the reduction in cAMP accumulation due to serum effects by challenging the cells with a relatively higher total amount of FSH per well. In assays of non-serum samples examined in this study, a small assay volume of 10 µl yielded an adequate sensitivity of < 1.5 ng/ml RP2 FSH, considering the high concentration of FSH found in many of those samples.

The detection limit of the receptor cell line assay was higher than that of the Sertoli cell FSH bioassay reported by Padmanabhan *et al* (1987). However, operators in the Reproductive Physiology Group have found the performance of the Sertoli cell bioassay to be highly variable, as it is technically more demanding than the receptor cell line bioassay. In addition, the absolute sensitivity of the receptor cell line bioassay is highly dependent on the activity of the FSH standard used, thus the sensitivities of assays can only be directly compared if the relative activities of the standards used are known. Nevertheless, the results generated in this study using the receptor cell line bioassay have been more reliable than those obtained from the Sertoli cell bioassay by this operator. Indeed, assays performed using the CHO FSH-R bioassay in studies examining possum FSH had exhibited improved assay characteristics with a sensitivity of 0.72 ng/ml RP2 FSH and an intra-assay coefficient of variation of 11.8% (Moore *et al*, 1997). Nonetheless, the sensitivity of the bioassay occasionally may not be sufficient for measuring FSH in serum from intact sheep. Ideally, a five-fold increase in assay sensitivity would be desirable for measuring low levels of FSH in serum. It is possible that additional modifications in culture or assay parameters will help to improve the sensitivity of the assay. Under present conditions, the receptor cell line bioassay is suitable for measuring FSH in surgically altered sheep or hypophyseal blood concentrations of FSH where levels are generally higher than those in the peripheral circulation. In addition to blood samples, the bioassay is also excellent for monitoring FSH activity in pituitary extracts and in media from tissue culture.

6.3.3 Standard Curve Function

The ED_{50} for cAMP stimulation required 40-50 ng/ml of RP2 FSH for assays of serum and culture media, which is comparable to results obtained in other studies (Albanese *et al*, 1994; Moore *et al*, 1997). However, the ED_{50} for assays of purified pituitary extract assays was 167 ng/ml of RP2 FSH, which is considerably higher than that of other assays. Reasons for this discrepancy are unclear, although a possibility is that the high pH of tris-HCl buffer (pH 8.6) may have a harmful effect on the CHO FSH-R cells.

The pattern of increase in cAMP accumulation in response to increasing FSH stimulation appears to follow the standard sigmoid shaped curve seen in many FSH bioassays (Albanese *et al*, 1994; Padmanabhan *et al*, 1987; Tano *et al*, 1995). Therefore, the four parameter logistic curve-fitting algorithm was used to generate the FSH standard curves in this study. Alternatively, a five

parameter logistic curve fit, which requires the curve to have rotational symmetry about the inflexion point, could have also been used. The increase in signal transduction seen from basal cAMP concentrations to a maximum cAMP stimulation at 100 μg/ml RP2 FSH was 18 fold. This large range from minimum to maximum stimulation enabled the *in vitro* bioassay to measure a wide range of FSH concentrations. However, the extremely high dose of FSH required to stimulate a maximal cAMP response is less than optimal as the majority of samples measured will fall towards the bottom end of the curve. This region of the curve is much flatter than near the ED_{50} region, which results in a far lower sensitivity.

6.4 Future Directions

From a practical point of view, an assay sensitivity ≤ 0.5 ng/ml RP2 would be desirable to provide greater confidence in the measurement of FSH in serum from intact sheep. The possibility of increasing sensitivity by the assay of sample sizes greater than 100 μl was not examined due to limitations in sample material. However, this avenue for improving sensitivity in measuring serum FSH remains open.

At present, the receptor cell line bioassay is performed using 48 well culture dishes. The transfer of the culture to 96 well microtitre plates was examined in preliminary studies, but for the purposes of measuring FSH, these proved unsuitable due to insufficient sensitivity. However, the use of 96 well plates would be useful as a larger number of samples could be assayed per plate. Proposed optimisations of the bioassay methodology could be directed towards this possibility.

Further optimisation of the cAMP assay component of the FSH bioassay would greatly enhance the practicality of the bioassay. Although the sensitivity of the cAMP RIA is adequate for the purposes of measuring the cAMP response of the CHO FSH-R cells to FSH, the assay methodology requires the handling of radioactivity and large numbers of test tubes. Development of specific and highly sensitive enzyme-immunoassays (EIA) would simplify the measurement of cAMP by eliminating the need for radioactivity and enabling the process to be carried out using 96 well plates instead of test tubes. Development of a specific cAMP EIA is currently under development in the Reproductive Physiology Group at AgRsearch, Wallaceville (Henderson, K., unpublished data).

In addition to the expression of the recombinant human FSH receptor gene, the CHO FSH-R cell construct has also been transfected with a cAMP responsive luciferase reporter gene, as described by Albanese *et al* (1994). By utilising a cAMP responsive promoter, transcription of the luciferase reporter gene occurs following stimulation of the cells with FSH. Therefore, the CHO FSH-R cell line can also be used to measure FSH using a rapid luciferase assay, in which the activity of luciferase on a substrate can be detected by chemi-luminescence. However, the use of this pathway was not chosen in this study as the reported increase in the signal transduction potential averaged 3.8 fold in the CHO FSH-R cells (Albanese *et al*, 1994). In this study, maximal stimulation with FSH resulted in a greater than 15-fold increase in cAMP compared to basal levels. This difference in end-point resolution may reflect a poor coupling of the receptor to intracellular signaling pathways necessary for activation of the luciferase gene. However, the use of the luciferase system provides important advantages over the measurement of cAMP. The luciferase assay eliminates the need to use radioactivity. In addition, the bioassay methodology, as described by Albanese *et al* (1994), is completed within two days. Possible future refinements aimed at improving the efficiency of the luciferase-signaling pathway would contribute toward significant improvements of the FSH bioassay methodology.

6.5 Conclusions

The measurement of cAMP accumulation in CHO cells transfected with the recombinant human FSH receptor has been demonstrated in this study. Use of the FSH receptor cell line in a bioassay for ovine FSH has been validated using serum samples from intact and surgically altered sheep, purified sheep pituitary extracts and culture media from sheep pituitary cell cultures. The minimum detectable concentration of FSH ($P > 0.05$) was 0.85 ng/ml of NIH-FSH-RP2 ovine FSH standard. The results obtained show that the sensitivity, specificity and reproducibility of the receptor cell line FSH bioassay was adequate for the measurement of ovine FSH in a variety of biological materials. This assay should therefore be a useful tool for investigating the heterogeneity of FSH and the role it may play in different physiological and pathophysiological conditions.

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