Intra-ovarian Modification of FSH Action in the Human

(Intra-ovariële modificatie van FSH werking in de mens)

PROEFSCHRIFT

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Contents

List of abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase/isomerase
AD	androstenedione
AMH	anti-Müllerian hormone
AUC	area under the curve
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'- monophosphate
CHO	Chinese hamster ovary
CRE	cAMP response element
CREB	CRE binding protein
CREM	CRE modulating protein
CT	choleratoxin
DNA	deoxyribonucleic acid
E_2	17β-oestradiol
ED_{50}	effective dose for half maximal stimulation
EGF	epidermal growth factor
ER	E_2 receptor
FCS	fetal calf serum
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
FSH_{max}	maximum serum FSH concentration
GDF9	growth differentiation factor-9
GnRH	gonadotrophin-releasing hormone
hCG	human chorionic gonadotrophin
HS	hypogonadotrophic human serum
IGF	insulin-like growth factor
IGFBP	IGF binding protein
IRMA	immunoradiometric assay
IU	international units
IVF	in vitro fertilisation
Kd	dissociation constant
kDa	kilo Dalton
LH	luteinizing hormone
mRNA	messenger ribonucleic acid
P	progesterone
P450C17	cytochrome P450 17α-hydroxylase/17-20 desmolase
P450SCC	cytochrome P450 side chain cleavage
PBS	phosphate-buffered saline
PCOS	polycystic ovary syndrome
PKA	protein kinase A
recFSH	recombinant human FSH
RIA	radioimmunoassay
SD	standard deviation
SEM	standard error of the mean
SRY	sex-determining region of the Y chromosome gene
T	testosterone
TGF	transforming growth factor
TSH	thyroid-stimulating hormone
TVS	transvaginal ultrasound scans
VEGF	vascular endothelial growth factor

Chapter I: Introduction and objectives

1.1 General introduction

In the female genital tract, the ovaries fulfill a dual function. First, they form the storage for female germ cells, the oocytes, and provide the environment in which oocytes can mature and are selected for ovulation and possible fertilization. In addition, the ovaries form an important endocrine organ which secretes several steroid hormones: androgens, oestrogens and progestins. These steroid hormones are involved in the differentiation and stimulation of various tissues and organs, and are therefore important determinants of reproductive function as a whole. The first known anatomical description of ovarian follicles is attributed to Andreas Vasalius (1514 - 1564) in *De humani corporis fabrica.* While the cystic structures in these so-called "female testicles" had been described before by various authors (Vasalius, Fallopia, Bartholin, Wharton and others), the term ova and ovaries was first suggested by Stensen (1667) and Van Horne (1668). It was Reinier de Graaff (1641 - 1673) who first described in *De Mulierum* (1672) the central importance of the ovarian follicles or "eggs" to reproduction in the female. He was the first to indicate that these organs were effectively ovaries, and in his honour preovulatory follicles are still named Graafian follicles (Setchell, 1974). Not until the second half of the 19th century, after the establishment of modern physiology and endocrinology by Claude Bernard (1818 - 1878) and Charles Brown-Séquard (1817 - 1894) the first observations regarding the hormonal regulation by the reproductive organs were reported. In that period the hypothesis of the internal secretion by the testis was proposed by Berthold in 1849 and later for the ovaries by Knauer in 1896 (Holinka, 1997). Another crucial milestone in medical science, in the first decades of the present century, was the application of radioisotopes as a diagnostic and analytical tool, leading to the discovery and development of radio-immunoassays in the late 1950's to early 1970's (Franchimont, 1975; Yalow, 1978), thus providing a most important tool for studies and diagnosis in reproductive medicine.

The gonadotrophins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were already recognized more than 60 years ago to be the main regulators of ovarian function and oocyte maturation (Smith and Engle, 1927; Zondek, 1930; Fevold *et al.*, 1933). Initial experiments in treating anovulatory patients with gonadotrophins (purified from pregnant mare serum) began in the mid 1940's but appeared unsatisfactory. Successful treatment of anovulation with human pituitary gonadotrophins was first described in the late 1950's (Gemzell *et al.*, 1958). Important improvements in the treatment of anovulatory women with gonadotrophins have arisen from advancements in the monitoring

of ovarian function (ultrasonography and hormone assays) and hormone purification. In addition, developments in recombinant DNA technology have resulted in the production of pure human gonadotrophin preparations. The cornerstone of ovulation induction treatment remains the establishment of a temporary increase in gonadotrophin levels exceeding the so-called "ovarian FSH threshold level", as has been initially proposed by Brown in the late 1970's (Brown, 1978). Apart from failure to respond despite increasing administration of gonadotrophins, an undesired complication of this treatment modality is overstimulation of the ovaries and the occurrence of multiple pregnancies (van Santbrink, 1998).

Over the past 20 years much attention has been focussed especially on the role of intra-ovarian factors in reproductive physiology. These factors modulate the actions of hormones at the level of target cells. Various growth factors (acting in an autocrine or paracrine fashion) have been shown to be produced and active in the (human) ovary through their own receptors. In addition, several factors have been described to exert inhibitory or stimulatory effects on gonadotrophin receptor binding and/or activation. Therefore, the FSH threshold, or sensitivity to FSH stimulation of a given follicle may be dependent on the activity of these local factors. The local regulation of FSH action may represent the decisive mechanism by which follicle development and dominant follicle selection is controlled. More insight in the mechanisms by which local factors regulate ovarian function and oocyte maturation may provide more effective and efficient methods for infertility treatment, as well as birth control.

1.2 Physiology of ovarian function

1.2.1 Ovarian development and physiology

During early embryo development, the ovaries derive from the coelomic epithelium and underlying mesenchymal tissue, together with the primitive germ cells derived from the primitive endoderm. Around the 5th to 6th week of embryo development the indifferent gonads are established structurally (Rabinovici and Jaffe, 1990). The primitive gonads differentiate into testes under the influence of testes-determining factor (TDF), a gene product encoded by a Y chromosomal gene, the SRY (sex-determining region of the Y chromosome gene). In the absence of the SRY the gonads differentiate into ovaries, first recognizable as such around the 8th week (Speroff *et al.*, 1994). Primordial germ cells arise before gonad differentiation takes place, and undergo rapid mitotic multiplication, resulting in a pool of primary oocytes reaching a maximum number of 6 to 7 million around the 16th to 20th week of embryo development. Until birth, this number diminishes to 2 million under the influence of factors yet unknown (Baker, 1963). The decrease continues

throughout the woman's life, leaving only about 400,000 oocytes at menarche. Eventually only 0.1 % of this oocyte pool will fully mature and ovulate during reproductive life. The follicle pool becomes completely depleted around the age of menopause (Faddy and Gosden, 1996).

In the human adult ovary, primordial follicles consist of oocytes arrested in the prophase of the first meiotic division, which are surrounded by a single layer of flattened pre-granulosa cells. These primordial follicles continuously leave the resting pool. It is unknown how the initiation of follicle growth and early follicular development is regulated, but it has been suggested to be independent of gonadotrophin stimulation (Gougeon, 1996; Braw-Tal and Yoseffi, 1997). The first signs of follicular growth are the transformation of the pre-granulosa cells into cuboidal cells of the granulosa cell layer(s) and proliferation and formation of theca cells surrounding the follicle. When the follicle diameter has increased from 50 µm in the primordial stage to around 300 µm in the early antral stage, inter-cellular cavities start to develop, leading to antrum formation. The final stages of development prior to ovulation last about 2 weeks, where follicle size increases from 5 mm to a preovulatory size of around 20 mm. The entire period from primary until fully maturated, preovulatory follicle is estimated to be at least 85 days (Gougeon, 1986; Gougeon, 1996). During the final 2 weeks, one follicle from the cohort of small growing follicles is selected to gain dominance and subsequently to ovulate. Eventually, most follicles leaving the pool of resting follicles will not reach full maturation but will go into atresia. The process of follicle atresia involves programmed cell death (apoptosis) and appears to be confined to preantral and small antral follicles (< 10 mm) (Hsueh *et al.*, 1994; Yuan and Giudice, 1997).

1.2.2 Endocrine regulation of ovarian function

Throughout reproductive life, follicle development and the cyclic changes in the reproductive tract (ovaries and uterus) is subject to hormonal regulation. The menstrual cycle can be divided into 2 periods, the follicular phase from onset of menses until ovulation, and the luteal phase from ovulation until the next menses. During the follicular phase a cohort of small antral follicles is stimulated to grow and eventually a single dominant follicle destined to ovulate is selected. After ovulation, a corpus luteum is formed by differentiated granulosa and theca cells from the ovulated follicle. Secretory products of the corpus luteum, predominantly steroid hormones, support the required environment of the fertilised ovum and early pregnancy.

A central role in the hormonal regulation of ovarian function is performed by the 2 gonadotrophins, FSH and LH. These hormones are produced by the gonadotrophes, which are localized in the lateral portions of the anterior pituitary gland. The pituitary secretion of gonadotrophins is controlled by gonadotrophin-releasing hormone (GnRH), a neurohormone secreted in a

pulsatile fashion by specific neurones in the hypothalamus (Speroff *et al.*, 1994). Gonadotrophins are glycoproteins, composed of two non-covalently linked protein subunits. All glycoproteins in the human (FSH, LH, thyroid-stimulating hormone [TSH] and human chorionic gonadotrophin [hCG]) share a common α subunit, while the β subunit is hormone specific. In FSH the β subunit consists of 111 amino acid residues, with a molecular mass of 33,000 Da. Two carbohydrate chains are linked to both the α and β subunits. Glycosylation of the subunits is involved in the biological activity and metabolic clearance of the hormones (Bishop *et al.*, 1995; Ulloa-Aguirre *et al.*, 1995).

Both gonadotrophins interact with specific receptors on the target cells. Theca cells express the specific LH receptor, whereas granulosa cells express the receptor for FSH and at a more advanced stage of development also the LH receptor (Hsueh *et al.*, 1984; Oh *et al.*, 1993b; Adashi, 1994a). Gonadotrophin receptors expressed at the surface of theca and granulosa cells belong to the family of G protein-coupled receptors. These glycoprotein receptors share general structural characteristics: an intracellular domain which interacts with G proteins, a transmembrane domain comprising 7 transmembrane segments and folded characteristically into 6 loops, and an extracellular part which determines the hormone specificity of the receptor (Strader *et al.*, 1995). Binding of the hormone to the extracellular domain is thought to result in conformational changes in the receptor, resulting in activation of the G protein complex coupled to the intracellular domain of the receptor. Stimulation of FSH receptors in granulosa cells results in activation of stimulatory G_s protein, which in turn activates the enzyme adenylate cyclase. The activated enzyme converts adenosine 5'-triphosphate in cyclic adenosine 3',5'-monophosphate (cAMP). Cyclic AMP serves as a second messenger within the cell, resulting in various biological responses through a cascade of events involving protein kinase A (PKA) activation and phosphorylation of enzymes and transcriptional activators (Leung and Steele, 1992; Burrin, 1994; Simoni *et al.*, 1997). The latter group comprise the cAMP response elements (CRE), binding proteins (CREBs) and modulators (CREMs). Activation or repression by these latter elements of CREs in the promoter region of specific genes results in an biological response (Simoni *et al.*, 1997). In addition, activation of FSH receptors may also result in intracellular increase in Ca^{++}, serving as a distinct second messenger. This increase is a result of a FSH-activated extracellular Ca^{++} influx, in which the PKA/cAMP pathway is involved also. Whether FSH receptor activation is coupled via G_q proteins and phospholipase C to the inositol-triphosphate/Ca^{++} pathway is unclear (Simoni *et al.*, 1997). Nevertheless, this dual signalling property of the FSH receptor may thus regulate distinct functions and biological responses by the same hormone (Gudermann *et al.*, 1992; Leung and Steele, 1992; Gorczynska *et al.*, 1994; Arey *et al.*, 1997; Sairam *et al.*, 1997).

Although the FSH receptor has been detected on preantral, secondary

follicles (Oktay *et al.*, 1997a), the role of FSH in the initiation of growth from primordial to antral stage is still under debate. Initial growth may be independent from FSH, since small follicles arrested in early antral stages can be encountered during pregnancy or after hypophysectomy, situations in which gonadotrophin levels are (very) low (Gougeon, 1996; Fauser and van Heusden, 1997). However, in patients with Kallmann's syndrome (hypogonadotrophic hypogonadism caused by lack of hypothalamic GnRH secretion) follicle growth arrest at the primordial stage has been shown, suggesting that total absence of gonadotrophins may block initiation of follicle development (Fauser and Hsueh, 1995). In addition, the androgen receptor and various growth factor receptors, including the receptor for IGF-I, have been shown to be present in human primary and secondary follicles (Gougeon, 1996; Hillier *et al.*, 1997b; Tetsuka and Hillier, 1997). After acquisition of FSH sensitivity, stimulation with FSH will result mainly in the induction of granulosa cell proliferation, whereas at later stages, after antrum formation (from a size of 2 - 5 mm onward), steroidogenesis is also induced; in particular oestradiol (E_2) production (Gougeon, 1996; van Dessel *et al.*, 1996b).

The observation that both theca and granulosa cells are necessary for the production of E_2 has led to the postulation of the 2 cell - 2 gonadotrophin concept (Falck, 1959; Ryan, 1979; Schoot *et al.*, 1992; Hillier *et al.*, 1994). According to this concept, theca-derived androgens, in particular androstenedione, serve as precursors for conversion into oestrogens by the granulosa cells. In both the granulosa and theca cells the enzyme complexes cytochrome P450 side chain cleavage (P450SCC) and 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) are expressed for the conversion of cholesterol into progestins. However, only the theca cells express the necessary 17α-hydroxylase/17-20 desmolase enzyme complex (P450C17) to convert progestins into androgens (Gore-Langton and Armstrong, 1988; Sasano *et al.*, 1989; Adashi, 1994a). In contrast, the expression of the enzyme necessary for aromatization of androgens into oestrogens, the cytochrome-P450aromatase, is confined to the granulosa cells in the ovary and is regulated by FSH (Steinkampf *et al.*, 1987).

1.3 Modulation of FSH action

1.3.1 General outline

Although a central role in the regulation of proliferation and function of granulosa cells is performed by FSH, the activity of FSH is subject to modification by a variety of factors (Fauser, 1996). These modifying factors may comprise systemic factors, which influence the endocrine regulation of the ovaries at a level distant from the target organ itself and factors which exhibit

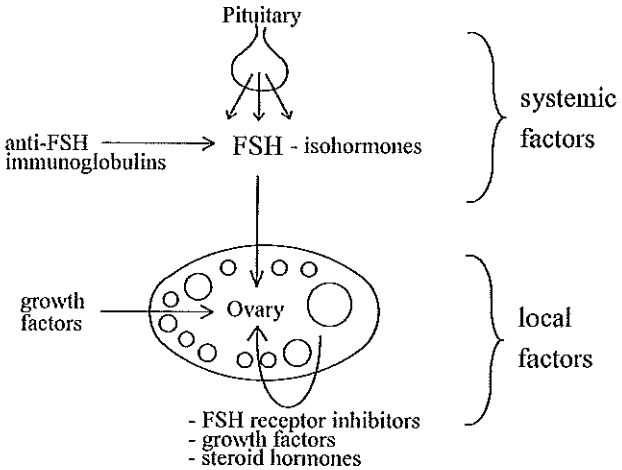

Figure 1.3.1
Schematic outline of modulation of FSH action.

local, intra-ovarian activity. The latter factors may be produced within or outside the ovary (Figure 1.3.1). The combined activity of these factors together determines the eventual biological response to stimulation.

1.3.2 Systemic factors

Multiple forms of FSH are secreted by the pituitary. Each isohormone varies in biopotency, as a result from differences in the glycosylation of the α and β subunits of the hormone (Ulloa-Aguirre *et al.*, 1995). Both subunits have 2 asparagine linked (N-linked) carbohydrate residues at amino acid positions 52 and 78 on the α subunit and at positions 7 and 24 of the β subunit. Using site-directed mutagenesis the specific roles have been assessed of each carbohydrate residue individually or in combination for hormone receptor interaction and activity, as well as for the metabolic clearance rate of the hormone (Cerpa-Poljak *et al.*, 1993; Bishop *et al.*, 1994; Flack *et al.*, 1994a; Flack *et al.*, 1994b; Bishop *et al.*, 1995). It has been demonstrated that circulating FSH isohormone profiles change during the menstrual cycle (Padmanabhan *et al.*, 1988; Wide and Bakos, 1993; Ulloa-Aguirre *et al.*, 1995). Furthermore, a decreased response to FSH stimulation due to specific anti-FSH antibodies has been described in patients treated with human gonadotrophins for induction of ovulation (Fauser, 1996). Thirdly, a variety of peptide and protein factors, such as immunoglobulins and growth factors have been described to inhibit or block FSH action (Chiauzzi *et al.*, 1982). Protein factors isolated from venous drainage of the human preovulatory follicle have been shown to suppress FSH-stimulated follicle development in rats (DiZerega *et al.*, 1982). However, since these factors display their effect either at the level of the FSH receptor or via other receptors on the granulosa cells they can be considered as local, intra-ovarian factors.

1.3.3 Intra-ovarian factors: FSH receptor inhibitors

A variety of intra-ovarian factors are operative in modifying FSH action (Figure 1.3.2). A variety of proteins and peptides of different molecular size, which inhibit specifically the binding of FSH to its receptor have been (partially) purified from serum and follicular fluid from various species, including human (Sanzo and Reichert Jr, 1982; Sluss and Reichert Jr, 1984; Lee *et al.*, 1990; Sluss and Schneyer, 1992; Lee *et al.*, 1993; Lee *et al.*, 1995). These FSH receptor inhibitors comprise a large group of receptor blocking proteins, either with or without receptor activating properties (Sluss *et al.*, 1987; Lee *et al.*, 1991). Some of these FSH receptor inhibitors are considered to be specific anti-receptor immunoglobulins (Chiauzzi *et al.*, 1982; Reznik *et al.*, 1998), and may play a role in the etiology of premature ovarian failure (van Weissenbruch *et al.*, 1990), although experimental and clinical data regarding this concept remain inconclusive (Anasti *et al.*, 1995; Lambert *et al.*, 1996).

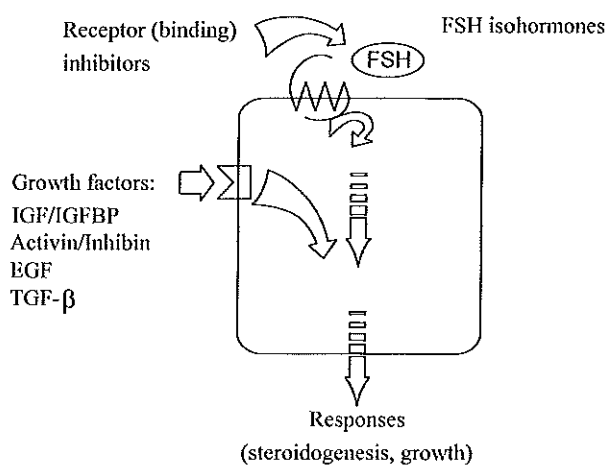

Figure 1.3.2
Schematic representation of intra-ovarian modulation of FSH activity at the level of the granulosa cells: at the level of FSH receptor interaction or at intracellular level.

1.3.4 Intra-ovarian factors: growth factors

In recent years, evidence has accumulated for the involvement of a variety of growth factors in the modification of FSH-induced responses in follicle growth development (Dorrington *et al.*, 1987; Fauser and Hsueh, 1988; Hammond *et al.*, 1991; Hillensjö *et al.*, 1992; Findlay, 1993; Greenwald and Roy, 1994; Giudice *et al.*, 1996). In contrast to FSH receptor inhibitors, growth factors act through their own receptors, and intracellularly their pathways merge with the FSH-activated pathways and thus modulate FSH-stimulated responses within the cell. Several of these factors fulfill the criteria required for auto- or paracrine

activity: 1) expression and production of the protein factor in ovarian tissue; 2) expression of its receptor on ovarian target cells; and 3) documented action *in vitro,* relevant to ovarian function (Giudice *et al.,* 1996). Numerous growth factors and cytokines have been demonstrated to contribute to the regulation of normal ovarian function. Most data result from animal studies *in vivo* or *in vitro,* in particular in rodents. A summary of several families of protein factors with auto- and paracrine activity in the ovary is shown in Table 1.2.1. Only a limited number of factors with intra-ovarian expression and activity is discussed here. Newly disclosed factors potentially involved in the regulation of ovarian function can continuously be added to this list, only indicating the complexity of this system. The role of the insulin-like growth factor (IGF) system and the inhibin/activin system in the ovary has been studied extensively in the human and will be discussed separately in more detail.

Epidermal growth factor (EGF) and its structural homologue transforming growth factor-α (TGFα) have been found in follicular fluid of antral follicles (Eden *et al.,* 1990; Westergaard *et al.,* 1990), and have been localized by immunohistochemistry and *in situ* hybridization in the theca cell layer of antral follicles and to a lesser extent in granulosa cells of small, antral follicles, although results are somewhat conflicting (Chegini and Williams, 1992a; Maruo *et al.,* 1993; Scurry *et al.,* 1994; Tamura *et al.,* 1995). EGF and TGFα share the same receptor, which is expressed on both granulosa and theca cells (Scurry *et al.,* 1994) and secreted by both granulosa cells and theca cells (Mason *et al.,* 1995). *In vitro,* EGF and TGFα stimulate mitosis of granulosa cells (Giudice *et al.,* 1996) and decreases FSH-stimulated aromatase activity and $P450_{aromatase}$ mRNA expression (Steinkampf *et al.,* 1988; Mason *et al.,* 1990).

Transforming growth factor-β (TGFβ), together with inhibin, activin, anti-Müllerian Hormone (AMH), belongs to the TGFβ superfamily of growth and differentiation factors. TGFβ mRNA is expressed in granulosa and theca cells of developing follicles (Chegini and Flanders, 1992a; Chegini and Williams, 1992b; Mulheron *et al.,* 1992). Although the precise role of TGFβ in the human ovary remains inconclusive, the expression increases with increasing follicle size, suggesting not only a proliferative role, but also a differentiating role (Yoshimura, 1997). *In vitro* studies indicate that TGFβ counteracts EGF/TGFα activity, as it enhances expression of the FSH receptor and FSH-stimulated aromatase activity (Adashi *et al.,* 1989; Giudice *et al.,* 1996).

Anti-Müllerian hormone, a glycoprotein which induces the regression of the Müllerian ducts in the male fetus (reviewed by Josso *et al.,* 1998), has also been found to be expressed in granulosa cells of preantral and small antral follicles in rat and human ovaries (Baarends *et al.,* 1994; Baarends *et al.,* 1995; Giudice *et al.,* 1996). Inhibitory effects on granulosa cell proliferation and aromatase activity *in vitro* have been described (Kim *et al.,* 1992; di Clemente *et al.,* 1994). Expression of AMH and AMH receptor mRNA at a high level in small antral

Table 1.2.1: Growth factors involved in the regulation of ovarian function.

Growth factor family	Factor	Main activities
Insulin-like Growth Factor family (IGF-I, IGF-II, IGF Binding Proteins, IGFBP proteases)[a,b,c,d,e]	IGF-I	Mitogenic and steroidogenic in granulosa and theca
	IGF-II	Granulosa proliferation and aromatase induction
	IGFBP-1	Modulation of IGF-I activity
	IGFBP-2	Inhibition of IGF-II activity
	IGFBP-3	Main carrier of IGF in serum
	IGFBP-4	Inhibition of IGF activity
	IGFBP-5	Potentiation of IGF-I activity
	IGFBP-6	
	Proteases	Increase local bioavailability of IGF's
Transforming Growth Factor-β family (TGF-β, Inhibin, Activin, Anti-Müllerian Hormone, growth differentiation factor-9)[f,g,h,i,j,H]	TGF-β	Stimulation of granulosa proliferation and steroidogenesis, inhibition of EGF activity
	Inhibin-A	Stimulation of androgen production in theca
	Inhibin-B	Inhibition of pituitary FSH secretion
	Activin	Stimulation of FSH receptor expression; stimulation of aromatase expression
	AMH	Gonadal differentiation, stimulation of granulosa cell proliferation
	GDF-9	Garnulosa cell proliferation, cumulus expansion and follicular rupture, stimualtion of steroidogenesis
Epidermal Growth Factor family (EGF, Transforming Growth Factor-α)[k,l,m,n,o,p,q,r]	EGF	Stimulation granulosa cell mitosis, inhibition of aromatase, increase IGFBP-1 expression
	TGF-α	Stimulation granulosa cell mitosis, inhibition of aromatase, decrease FSH receptor expression
Fibroblast Growth Factor family (acidic FGF, basic FGF)[s,t]	AFGF	Stimulation of granulosa cell mitosis, inhibition FSH stimulated steroidogenesis, suppression of apoptosis
	BFGF	
Cytokines (Tumour Necrosis Factor-α, Interleukins)[u,v,w,x,y,z]	TNF-α	Inhibition of gonadotrophin stimulated steroidogenesis
	Interleukin-I	Mediation of activity via macrophages, increased prostaglandin production in preovulatory follicle
Other:[A,B,C,D,E,F,G]		
- Renin/Angiotensin	RAS	Stimulation androgen secretion in theca and progesterone in lutein cells
- Plasminogen Activator	PA	Inhibition of IGF-I activity
- Vascular Endothelial Growth Factor	VEGF	Angiogenic in corpus luteum
- Leptin	Leptin	Decrease of IGF-I activity

[a] Hernandez *et al.*, 1992
[b] El-Roeiy *et al.*, 1993
[c] Giudice et al, 1996
[d] Lee et al, 1997
[e] Mason *et al.*, 1996
[f] Chegini and Flanders, 1992
[g] Jaatinen *et al.*,1991
[h] Martens *et al.*, 1997
[i] Mether and Mooore, 1997
[j] Baarends, 1995
[k] Steinkampf *et al.*, 1988
[l] Mason *et al.*, 1990
[m] Westergaard *et al.*, 1990
[n] Angervo *et al.*, 1992
[o] Chegini and Williams, 1992
[p] Maruo *et al.*, 1993
[q] Scurry *et al.*, 1994
[r] Mason *et al.*, 1995
[s] Hsueh *et al.*, 1994
[t] Yamamoto *et al.*, 1997
[u] Maudit *et al.*, 1993
[v] Jasper and Norman, 1995
[w] Montgomery Rice *et al.*, 1996
[x] Best and Hill, 1995
[y] Wang *et al.*, 1995
[z] Chang *et al.*, 1998
[A] Palumbo *et al.*, 1993
[B] Pepperell *et al.*, 1995
[C] Morris and Paulson, 1994
[D] Epifano *et al.*, 1994
[E] Zachow and Magoffin, 1997
[F] Yoshimura, 1997
[G] Karlsson *et al.*, 1997
[H] Elvin *et al.*, 1999

follicles represses aromatase activity at the onset of follicle development, whereas at a later stage of follicle development, increased FSH activity and stimulation of aromatase downregulates the expression of AMH and AMH receptor. On the basis of this model, a regulating role for AMH in dominant follicle selection has been proposed (Baarends, 1995). More recent studies also suggest a role for AMH in early follicle recruitment and depletion of the resting pool of primordial follicles (Durlinger *et al.*, 1999), although its role in postnatal human reproductive function is still inconclusive.

Another member of the TGFβ superfamily, growth differentiation factor-9 (GDF-9) is expressed at high levels in the mammalian oocyte. Animals deficient in GDF-9 appear to be infertile, due to a block in folliculogenesis at an early stage (Dong *et al.*, 1996). It has been demonstrated that GDF-9 stimulates growth and differentiation of early follicular development in a paracrine fashion by regulating the expression of gene products in granulosa cells, such as inhibin (Elvin *et al.*, 1999a; Elvin *et al.*, 1999b; Hayashi *et al.*, 1999). Other factors such as vascular endothelial growth factor (VEGF) (Yamamoto *et al.*, 1997a), the fibroblast growth factors (FGF) (Yamamoto *et al.*, 1997b), and cytokines (Terranova and Rice, 1997) may exhibit their specific roles in the regulation of corpus luteum formation and angiogenesis, prevention of apoptosis and luteolysis.

Dominant follicle development is closely associated with increased oestrogen secretion. *In vitro* studies of the rat model have demonstrated autocrine roles for E_2 in stimulation of granulosa cell proliferation, FSH and LH receptor expression and aromatase induction (Hsueh *et al.*, 1984; Kessel *et al.*, 1985; Fauser and van Heusden, 1997). In higher species the effects of oestrogens are less clear and may even be the opposite of effects in rodents (Koering *et al.*, 1994). The notion that follicle development in the human may be independent from local E_2 production is supported by clinical observations. In hypogonadotrophic patients follicle development occurs normally after stimulation with recombinant FSH despite very low intrafollicular E_2 levels (Schoot *et al.*, 1992). In addition, ovulation induction with FSH appeared successful in patients with 17α-hydroxylase/17-20 desmolase enzyme deficiency, characterised by low or absent androgen and oestrogen synthesis (Rabinovici *et al.*, 1989; Pellicer *et al.*, 1991). The lack of evidence for the presence of the oestrogen receptor in the ovary has further supported the minor or even absent role for E_2 in follicular development in the human, although some publications suggest otherwise (Iwai *et al.*, 1990; Hurst *et al.*, 1995). Recently a second type of the oestrogen receptor, the ERβ, has been identified. This receptor type is expressed specifically in the ovary and prostate, in contrast to the (classical) ERα (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996; Enmark *et al.*, 1997; Kuiper *et al.*, 1997). These findings may reopen the discussion regarding the autocrine role of E_2 in follicle development.

1.3.4.1 Insulin-like growth factor system

Much attention has been focussed on the insulin-like growth factors (IGF) and the IGF binding proteins (IGFBPs), which constitute an auto- and paracrine regulatory system, acting within the ovary in synergism with FSH (Giudice, 1992; Zhou and Bondy, 1993; Jones and Clemmons, 1995). IGF-I and IGF-II are mitogenic peptides which are structurally related to insulin (Giudice, 1992). Two receptor types are known to interact specifically with the IGFs. The type 1 receptor is present in a wide variety of cell types and mediates most of the biological effects of IGF-I and IGF-II *in vitro*; as well as that of insulin, if this is present in sufficiently high concentrations. The type 2 receptor is thought to play a role in IGF-II turnover and less in signal transduction (Jones and Clemmons, 1995). Both IGFs are potent stimulators of mitogenic activity, but also influence cell differentiation (in neural cells, osteoblasts and adipocytes) and hormone secretion of gonadal tissue. In addition, complementary to stimulation of cell proliferation, IGFs inhibit cell death or apoptosis (Jones and Clemmons, 1995). *In vitro*, IGF-I and -II stimulate synthesis of DNA and steroidogenesis in human granulosa cells and theca cells (Olsson *et al.*, 1990; Angervo *et al.*, 1991; Kamada *et al.*, 1992; Mason *et al.*, 1994b; Willis *et al.*, 1998a), and augment FSH-stimulated aromatase in granulosa cells (Steinkampf *et al.*, 1988; Erickson *et al.*, 1990; Bergh *et al.*, 1991; Erickson *et al.*, 1991). Insulin-like growth factor I synergizes with LH in the stimulation of androstenedione production in theca cells *in vitro* (Bergh *et al.*, 1993a).

Whereas IGF-I concentrations in follicular fluid do not differ between oestrogen dominant and androgen dominant follicles, IGF-II concentrations are significantly higher in oestrogen-dominant follicles and correlate positively with follicle size. Moreover, intra-ovarian IGF concentrations are not correlated to serum concentrations throughout the menstrual cycle, underscoring the auto- and paracrine role of the IGFs (van Dessel *et al.*, 1996a). Altogether, these data suggest that IGF-II is the major bioavailable IGF in the human ovary and is locally produced. Indeed, only expression of IGF-II mRNA has been demonstrated in the human ovary (Hernandez *et al.*, 1992; El-Roeiy *et al.*, 1993; Zhou and Bondy, 1993).

The IGFBPs are a group of structurally related proteins which specifically bind and modulate the actions of IGFs. To the present date, six IGFBPs have been identified and sequenced (Giudice, 1992; Jones and Clemmons, 1995). Apart from the function as transport-carrier proteins, the IGFBPs provide a means of tissue- and cell type-specific localisation and directly modulate interaction of the IGFs with the receptors, thereby indirectly controlling biological activity (Jones and Clemmons, 1995). At the cellular level, IGFBPs can augment and inhibit IGF activity. Inhibition of IGF action result from binding and thus prevention of IGF receptor interaction (Ui *et al.*, 1989; Bicsak *et al.*, 1990; Adashi *et al.*, 1992; Lee *et al.*, 1997). Potentiating actions may result from interaction of IGFBPs with cell surface proteins (Jones and Clemmons,

1995; Lee *et al.*, 1997). Furthermore, association of IGFBPs with extracellular matrix proteins may increase the local concentration of IGFs (Bicsak *et al.*, 1990; Oh *et al.*, 1993a; Jones and Clemmons, 1995; Lee *et al.*, 1997).

Five IGFBPs have been detected in follicular fluid from developing follicles (Giudice *et al.*, 1990; Holly *et al.*, 1990; Cataldo and Giudice, 1992a; Cataldo and Giudice, 1992b; Schuller *et al.*, 1993; Giudice *et al.*, 1996; van Dessel *et al.*, 1996a). IGFBPs are secreted by granulosa and theca cells *in vitro* (Jalkanen *et al.*, 1989; Suikkari *et al.*, 1989; Giudice *et al.*, 1991; Giudice, 1992; Mason *et al.*, 1996; Cwyfan-Hughes *et al.*, 1997). Both the expression of IGFBPs and IGF receptors in the human ovary has been demonstrated with *in situ* hybridisation and immunohistochemistry (El-Roeiy *et al.*, 1993; Zhou and Bondy, 1993). The IGFBPs are secreted by both granulosa and theca cells *in vitro*, regulated by IGFs and gonadotrophins (Jalkanen *et al.*, 1989; Adashi *et al.*, 1991; Dor *et al.*, 1992b; Cataldo *et al.*, 1993; Adashi *et al.*, 1994; Jones and Clemmons, 1995; Mason *et al.*, 1996). Whereas the IGFs have a stimulatory effect or synergism with gonadotrophins, IGFBPs counteract the action of IGF and gonadotrophins (Ui *et al.*, 1989; Bicsak *et al.*, 1990; Adashi *et al.*, 1992; Mason *et al.*, 1993; Jones and Clemmons, 1995; Mason *et al.*, 1998). In atretic or hyperandrogenic follicles, as opposed to healthy, oestrogenic follicles, follicular fluid levels of IGFBP-2 and IGFBP-4 have been found to be elevated and the expression of IGFBP-2 mRNA high (Cataldo and Giudice, 1992a), suggestive for inactivation of IGFs in these follicles. In addition, specific IGFBP proteases may also play a role in the regulation of IGF activity by means of regulating local IGFBP concentrations (Jones and Clemmons, 1995; Giudice *et al.*, 1996; Mason *et al.*, 1996). By local degradation of IGFBPs the bioavailability of IGF-II and IGF-I increases. Specific proteases have been related to follicular status, such as IGFBP-4 protease, which is mainly present in oestrogen-dominant follicles. Altogether, the components of the ovarian IGF system function as a local auto- and paracrine system, regulating FSH-induced follicle development.

1.3.4.2 Inhibin and activin

The inhibins and activins are dimeric proteins that belong to the TGFβ superfamily of growth factors. Originally these peptides were defined as gonadal hormones, regulating FSH secretion from the pituitary. With regard to inhibin, this concept has been already proposed 6 decades ago (see for review: de Jong, 1988). Today these factors are known to act also as auto- and paracrine regulators of growth and differentiation in a variety of tissues, including the reproductive system (Mather and Moore, 1997). The inhibins are heterodimers, consisting of an inhibin α and an inhibin βA or βB subunit, whereas activin is a hetero- or homodimer of 2 β subunits (βAβA, βBβB or βAβB) (Mather and Moore, 1997). The activin and inhibin subunits have been shown to be expressed in the human ovary in both the granulosa and theca cell compartment

(Jaatinen *et al.*, 1994). The expression appears to be dependent of the developmental stage of the follicle, small antral follicles expressing predominantly βB subunit, and healthy large follicles expresing βA and a subunits (Roberts *et al.*, 1993; Giudice *et al.*, 1996). *In vitro* activin augments FSH-stimulated aromatase activity (Hillier and Miró, 1993a), FSH receptor expression and inhibin production (Giudice *et al.*, 1996) in granulosa cells. Furthermore, *in vitro* granulosa cell proliferation is stimulated by activin (Mather and Moore, 1997). The major effect of inhibin upon steroidogenesis in the ovary is augmentation of androgen production by theca cells. Inhibin may act as an antagonist to some, but not all actions of activin. Recently, it has been proposed that inhibin may directly antagonize activin at the level of ligand binding to the receptor (Martens *et al.*, 1997). Both inhibin expression and secretion *in vitro* is highest by granulosa cells from large antral and preovulatory follicles and is regulated by FSH and not LH (Hillier *et al.*, 1991). On the basis of *in vitro* data, a model for the paracrine actions of activin and inhibin in the ovary has been proposed (Hillier and Miró, 1993b) (Figure 1.3.3). Activin produced by granulosa cells in immature follicles enhances the activity of FSH in stimulation of aromatase, at the same time suppressing androgen production in the theca cells. At later stages of follicle development, as inhibin expression increases parallel to the increase in aromatase activity under influence of FSH, androgen synthesis is stimulated by locally produced inhibin to sustain E_2 production in the preovulatory follicle. In essence, activin promotes granulosa proliferation and sensitization to FSH at early stages of follicle development, whereas inhibin is more likely to play a role in dominant follicle selection and maintenance of follicle dominance (Hillier and Miró, 1993b; Hillier *et al.*, 1997a).

A third member belonging to this group of autocrine regulatory proteins is follistatin, a monomeric, glycosylated protein. Follistatin has been isolated from follicular fluid and has been found to inhibit FSH secretion by pituitary cells *in vitro*. Expression of follistatin increases with preovulatory follicle development and is closely correlated to inhibin β subunit expression. Follistatin acts as a binding protein for activin and displays an inhibitory effect on activin stimulation of granulosa cells *in vitro*. Whether this is solely due to "entrapment" of activin by follistatin or to an intrinsic activity is unclear (Hillier and Miró, 1993b; Mather and Moore, 1997).

Whereas *in vivo* studies have demonstrated that administration of FSH results in increased serum inhibin levels (Hee *et al.*, 1993), measurement of total immunoreactive inhibin throughout the normal menstrual cycle showed that levels remained unchanged until the midcyclic LH surge and ovulation (Reddi *et al.*, 1990). Only after development of immunoassays capable of distinguishing between the subtypes of inhibin, inhibin-A and -B, inhibin-B was demonstrated to be the predominant form of inhibin in the developing follicle (Groome *et al.*,

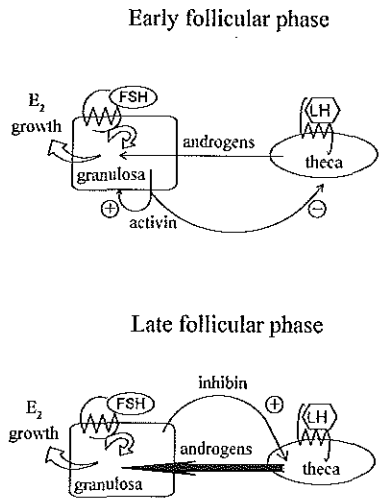

Figure 1.3.3
Schematic representation of the regulatory functions of activin and inhibin in the ovary (adapted from Hillier and Miro, 1993).

1994; Groome *et al.*, 1996). The serum inhibin-B level starts to increase shortly before the follicular phase, reaching a maximum in the mid-follicular phase coinciding with the decrease of the serum FSH level and drops after ovulation. The inhibin-A concentrations increases shortly before ovulation, to reach a maximum during the luteal phase (Groome *et al.*, 1996). These findings correspond with *in vitro* results regarding expression of inhibin subunits in the ovarian follicular cells during follicle development (Hillier *et al.*, 1997a), supporting the dual role of inhibin and activin as endocrine and paracrine regulators of follicle development.

In summary, a multitude of factors are involved in the regulation of FSH activity in the ovary. Some of these factors are true autocrine factors, which are induced in and excreted by the granulosa cells by FSH (IGF-II, IGFBPs or inhibin); others act in a more paracrine fashion and diffuse to the granulosa cells in order to exert their modulatory activity (activin, IGF-I, IGFBPs). These factors act as modulators of FSH action, through potentiation or inhibition of FSH-induced intracellular pathways.

1.4 Disturbed folliculogenesis

Anovulatory disorders are conditions in which normal follicle development and ovulation are disturbed or absent. An obvious cause is gonadal dysgenesis which is usually due to chromosomal abnormalities. Furthermore, several identified single gene defects affect the hypothalamic-pituitary-gonadal axis, resulting in

disturbed GnRH secretion or reception, in defective gonadotrophins or gonadotrophin receptors, or in disturbed gonadal and adrenal steroid biosynthesis (reviewed in: Fauser and Hsueh, 1995; Fauser *et al.*, 1999). A disregulation of the interplay between ovarian and hormonal factors involved in follicle development may thus cause anovulation, although only a small proportion of anovulatory disorders have yet been attributed to a particular genetic defect.

Patients with normogonadotrophic anovulatotion (type II anovulatory disorders, according to WHO classification) exhibit serum FSH and E_2 levels within normal limits in combination with cycle disturbances (oligomenorrhoea or amenorrhoea) (World Health Organization, 1993). This category represents the great majority of patients with cycle abnormalities. Characteristically in these patients follicle development is initiated from the primordial stage until the tertiary, antral stage at which follicle development ceases and selection of a dominant follicle with subsequent ovulation is absent (Fauser, 1994a). A proportion of these normogonadotrophic anovulatory women have polycystic ovaries syndrome (PCOS), i.e. polycystic ovaries, together with one ore more features such as obesity, hirsutism and elevated androgen or LH levels (van Santbrink *et al.*, 1997).

The pathogenesis and etiology of PCOS remain speculative. In its most typical form chronic anovulation is combined with hyperandrogenism and to a lesser degree with disregulated gonadotrophin secretion, i.e. elevated circulating LH levels. The importance of the latter factor in the etiology of PCOS has been emphasised recently in transgenic mice, in which elevated LH levels lead to polycystic ovaries and infertility (Risma *et al.*, 1995). The excess secretion of androgens may result from a disregulation of ovarian androgen biosynthesis, more specifically the expression of cytochrome P450C17 in the theca cells (Rosenfield *et al.*, 1990; Gilling-Smith *et al.*, 1994). Furthermore, diminished insulin sensitivity resulting in elevated insulin concentrations may also stimulate theca cell androgen synthesis (Nestler, 1997a; Nestler and Jakubowicz, 1997b). Effects of androgens on the polycystic transformation of ovaries has recently been demonstrated in *in vivo* monkey studies (Vendola *et al.*, 1998). However, since the most consistent feature of this pathological state is anovulation and cycle disturbances in combination with relatively normal circulating FSH concentrations, a disregulation of FSH activity at the intra-ovarian level may be at the basis of this disorder. Supporting evidence for this hypothesis can be found in both clinical and *in vitro* studies. It has been reported that in anovulatory patients elevation of serum FSH to maximum levels, as found in normal controls, stimulates normal follicle development (van Dessel *et al.*, 1996c). This indicates that the FSH threshold for ovarian stimulation in normogonadotrophic anovulatory women is not different from normal, and suggests a defect at intra-ovarian level. Since *in vitro* studies in which stimulation with FSH of granulosa cells from anovulatory or PCOS patients result in

aromatase induction not essentially different from normal cells (Erickson *et al.*, 1979; Mason *et al.*, 1994a), the primary defect may not be defective granulosa cells, but rather a disturbed local regulation of stimulation by FSH or enhancement of the FSH signal. Whether the disregulation of FSH activity is due to factors inhibitory to the interaction with FSH and its receptor or a disregulation of auto- and paracrine factors (the IGFs/IGFBPs among others), is a matter of debate and subject for further investigation.

1.5 Study objectives

FSH plays a decisive role in folliculogenesis. The biological activity of the hormone appears to be dependent on a variety of stimulatory and inhibitory factors, part of which reside in the ovary itself. The principal hypothesis of the present thesis is two-fold:

- First, disregulated follicle development in normogonadotrophic anovulation is caused by factors that inhibit FSH to interact with its receptor.
- Second, local enhancement of FSH action is indispensable for normal follicle development and ovulation and is a principal factor in determining the FSH sensitivity or FSH threshold level of the ovary.

In order to examine the first hypothesis, *in vitro* cultures of human granulosa cells are preferable. One of the objectives is to examine the applicability of these cells for an *in vitro* model. Alternatively, genetically modified Chinese hamster ovary cells, expressing the human FSH receptor, can be applied. With these models the effects of substances modifying FSH action, either at the level of the FSH receptor or intracellularly, can be studied. In addition to *in vitro* studies, the role of local enhancement of FSH action can be examined by studying follicle development and serum hormone levels during the normal menstrual cycle. Assessment of follicular development and serum hormone levels during the normal menstrual cycle may provide information on the relevance of the height of FSH increase in the early stages of the menstrual cycle and the role of local enhancement of FSH in follicle development and dominant follicle selection.

Chapter II: Inhibition of FSH receptor activation

2.1 Introduction

Disregulation of follicular growth can be caused by factors inhibiting FSH action through interfering with the interaction of FSH and its receptor. Over the past 15 years, specific inhibitors of FSH receptor binding have been detected in serum (Reichert Jr *et al.*, 1979; Sanzo and Reichert Jr, 1982) and follicular fluid (Lee *et al.*, 1990; Lee *et al.*, 1991) and a role for these factors in the pathophysiology of ovarian function has been proposed. In addition, a variety of growth factors, acting through their own receptors and intracellular pathways and thus bypassing the FSH receptor, modify FSH action (Carson *et al.*, 1989; Tonetta and DiZerega, 1989; Greenwald and Roy, 1994; Erickson and Danforth, 1995). The first aim of our studies was to develop an *in vitro* culture system by which the effect of various factors could be examined.

Within the follicle fluid, factors are present, which stimulate or inhibit granulosa cell function. The intention was to mimic this micro-environment in the laboratory by performing functional studies of cultured cells. On theoretical grounds, human granulosa cells would serve this purpose at best. However, primary cell cultures may demonstrate a significant variability in biological response, due to differences in subjects and follicles from which the cells are taken. With cells taken from inbred animal strains, part of these problems may be controlled and limited, whereas with genetically modified cell lines an even more stable *in vitro* model can be developed. However, both the animal cells and transfected cell lines may reflect the human physiology even less than cultured human cells. For this reason, the followed strategy in this thesis was: 1) to develop and validate an *in vitro* cell culture model using human granulosa cells, to study local regulation of granulosa cell function by growth factors; and 2) to validate an *in vitro* assay with a transgenic cell line, expressing the human FSH receptor and apply this assay to human serum and follicular fluid samples in order to measure specific FSH receptor activation inhibitors. For the first purpose, either granulosa cells isolated from follicular fluid after ovarian hyperstimulation in the course of IVF treatment, or granulosa cells from ovaries from normally menstruating women can be isolated. Whereas the primary option provides high amounts of cell material with relatively ease, these cells may already undergo differentiation, rendering them less suitable. Cells from normally cycling ovaries serve the purpose of the studies best; however the availability is limited and therefore a larger variation in *in vitro* behaviour can be expected.

In the following sections of this chapter two parallel lines of investigation are described. First, results from studies with cultured human granulosa cells, to be

used as an *in vitro* assay for the study of local regulation of FSH are reported. Second, results of the validation of an assay using a FSH sensitive transgenic animal cell line and the application of this system for measurement of inhibitory activity in serum and follicular fluid from anovulatory patients and controls.

2.2 Development of a human granulosa cell culture model with FSH responsiveness

2.2.1 Introduction

Follicle-stimulating hormone and luteinizing hormone play a central role in ovarian follicular growth and differentiation. Recent studies indicate that locally acting growth factors, like IGF and the IGFBPs may be involved in FSH-induced granulosa cell functions (Fauser and Hsueh, 1988; Erickson *et al.*, 1989; Adashi *et al.*, 1991; Mason *et al.*, 1992). In order to study factors involved in follicular growth and selection an *in vitro* culture system is indispensable. For practical reasons many studies have been performed using preovulatory human granulosa cells, obtained through follicle aspiration in patients undergoing controlled ovarian hyperstimulation for IVF (Fowler *et al.*, 1978; Veldhuis *et al.*, 1983; Sjögren *et al.*, 1987; McAllister *et al.*, 1990). For studies concerning differentiation of granulosa cells the usefulness of these type of cells might be limited, since these cells are obtained from a non-physiological *in vivo* environment due to hyperstimulation with gonadotrophins and are already luteinized due to the administration of human chorionic gonadotrophin. In an attempt to establish an *in vitro* model to examine regulation of human granulosa cells by FSH and modulation of FSH-induced cell functions, the validity of the use of preovulatory granulosa cells from women undergoing IVF procedures has been studied.

2.2.2 Materials and Methods

Reagents

Dulbecco's Modified Eagle's Medium was purchased from GIBCO (Grand Island, NY), and supplemented with 1% (vol/vol) Penicillin-Streptomycin-L-Glutamine (10,000 U/ml-10mg/ml-200mM, GIBCO). In certain cultures fetal calf serum (FCS, HyClone Laboratories Inc., Ogden, UT), Serumplus™ (Hazleton Biologics Inc., Lenexa, KS) or Ex-cell 320™ serum-free media (JRH Biosciences, Lenexa, KS) were used. Highly purified human FSH (NIDDK-hFSH-B-1, 1683 IU/mg biopotency; 5763 IU/mg RIA potency; 147 IU/mg LH contamination) and highly purified human LH (NIDDK-hLH-B-1, 4015 IU/mg biopotency, 11559 IU/mg RIA potency, 13 IU/mg FSH contamination)

were provided by the Hormone Distribution Program of the NIADDK. Percoll and androstenedione were obtained from Sigma Chemical Co. (St. Louis, MO). Fibronectin coated culture plates (2 µg human fibronectin/cm^2) were obtained from Collaborative Biomedical Products (Bedford, MA).

Granulosa cell isolation

Granulosa cells were obtained from preovulatory follicles of patients undergoing IVF at the University of Washington Medical Center. Patients received 1 mg Leuprolide Acetate (Lupron, TAP Pharmaceuticals, Chicago, IL) starting in the mid-luteal phase of the cycle prior to ovulation induction. After 10 days of treatment, daily Leuprolide dosage was decreased to 0.5 mg and daily injections of human menopausal gonadotrophin (150-225 IU, Pergonal; Serono Laboratories, Norwell, MA) combined with 150 IU follicle-stimulating hormone (Metrodin, Serono Laboratories) were administered. When ultrasound measurements revealed follicles with mean diameters - 16 mm and serum E_2 was approximately 250-300 pg/ml, 10,000 units human chorionic gonadotrophin (hCG-Profasi, Serono Laboratories) were administered i.m., and follicle retrieval took place 34 to 36 hours later. Follicles were aspirated, employing ultrasound directed needle aspiration. Follicular content was examined and oocytes were identified and removed. To obtain sufficient amounts of granulosa cells, follicular fluid of two or three patients were combined and centrifuged (150 x g for 5 min). The pellet, suspended in a small amount of media, was placed on a 40% Percoll gradient and centrifuged (300 x g for 25 min) to separate the granulosa cells from contaminating blood cells. The granulosa cells, obtained from the interface, were washed and resuspended in culture media. Dispersion of the cells was obtained by repeated gentle pipetting. Aliquots of the suspension were counted on a haemocytometer and viability was determined with trypan blue exclusion. Viability in these experiments varied from 70 to 90%, with approximately 1 - 2 x 10^6 cells per pool.

Culture procedure

Cells (10,000 viable cells/well) were cultured in plastic 96-well plates (Costar Corp., Cambridge, MA) with or without fibronectin coating in the presence or absence of fetal calf serum. Every 48 hours the media was removed, the wells were washed once to remove dead cells and debris, and fresh media and reagents were added. The media was stored frozen until assayed. Androstenedione was added to the culture media (10^{-7} M) as substrate for aromatase activity.

Radioimmunoassays

Progesterone and E_2 RIAs were performed using kits from Diagnostic Systems Laboratories Inc. (Webster, TX). The P assay had a sensitivity of 10 pg/ml, and using media standards the intra- and interassay coefficients of variation were 6.0

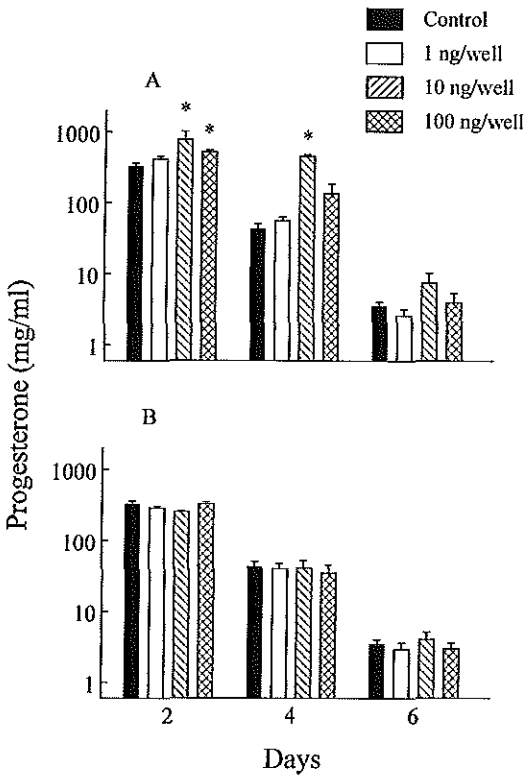

Figure 2.2.1
Progesterone production from freshly retrieved granulosa cells. Human granulosa-lutein cells were cultured in serum-free media and stimulated with LH (1, 10 and 100 ng/well) (*upper panel*) or FSH (1, 10 and 100 ng/well) (*lower panel*). Media was assayed for progesterone after 2, 4 and 6 days. Data are the mean ± SEM from a representative experiment in triplicate. * Significantly different from control ($P < 0.05$).

and 7.5%, respectively. The sensitivity of the E_2 assay was 15 pg/ml, and using media standards the intra- and interassay coefficients of variation were 6.2 and 7.1%, respectively.

Statistical analysis
RIA data were analysed with a program that used a weighted logit-log regression analysis (Burger et al., 1972). Values for P and E_2 by RIA were calculated using analysis of variance, and are presented as the mean ± SEM of three or four cultures from one representative experiment. Experiments were repeated at least 2 times. Values were considered significantly different when $P < 0.05$.

2.2.3 Results

Response of freshly retrieved human granulosa cells to FSH and LH stimulation
On the day of retrieval, human granulosa cells were cultured in serum-free

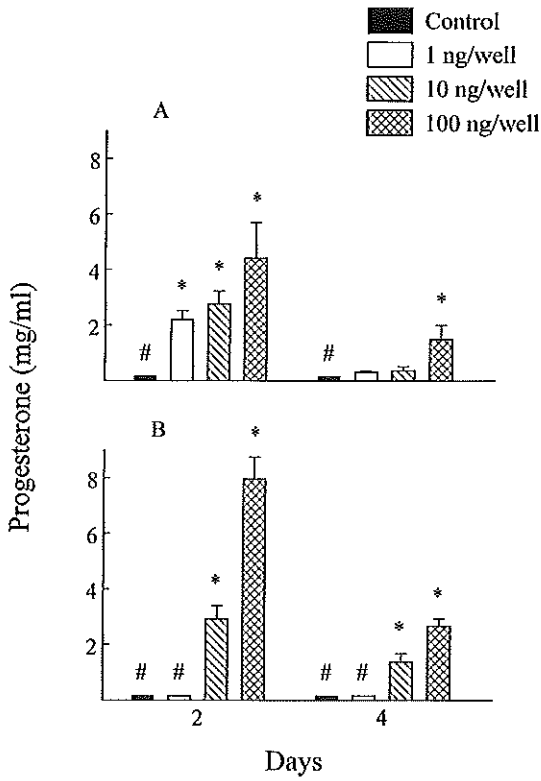

Figure 2.2.2
Effect of preincubation on progesterone production. Human granulosa-lutein cells were preincubated for 7 days with 10% serum, and then stimulated in serum-free media with LH (1, 10 and 100 ng/well) (*upper panel*) or FSH (1, 10 and 100 ng/well) (*lower panel*) for 2 and 4 days. Control values were below detectable limit (#: 0.2 ng/ml). Data are the mean ± SEM from a representative experiment in triplicate. * Significantly different from control ($P < 0.05$).

medium on plastic multi well plates (10,000 cells/well). The cells were stimulated with increasing concentrations of gonadotrophins for 6 days. The cultures initially displayed high basal P production, which decreased sharply in the course of 6 days (Figure 2.2.1, solid bars). With addition of 10 and 100 ng/well of LH, P production increased significantly after 2 and 4 days, but not after 6 days (Figure 2.2.1 *upper panel*). As seen in Figure 2.2.1 *lower panel*, addition of increasing concentrations of FSH (1, 10 and 100 ng/well) did not stimulate P production above basal levels.

Effects of preincubation on basal and gonadotrophin-stimulated progesterone production
Freshly retrieved granulosa cells were preincubated in media with 10% FCS for a period of 7 days, and media was changed every 2 days. After preincubation, the granulosa cells were cultured in serum-free media with increasing doses of LH or FSH (1, 10 and 100 ng/well). The basal P levels in these cultures were low or below

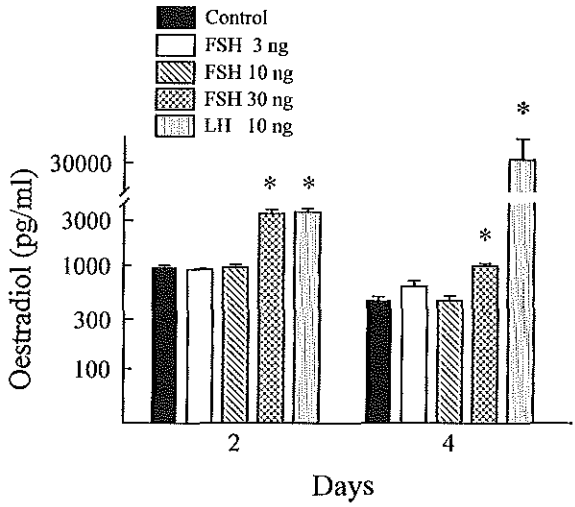

Figure 2.2.3
Oestradiol production from preincubated granulosa cells. Human granulosa-lutein cells were preincubated for 7 days with 10% serum. After preincubation, the cultures were stimulated in serum-free media with FSH (3, 10 and 30 ng/well) or LH (10 ng/well) for 2 and 4 days. Data are the mean ± SEM from a representative experiment in triplicate. * Significantly different from control ($P < 0.05$).

detectable limits (Figure 2.2.2; 0.2 ng/ml) addition of LH resulted in a significant increase of P production in a dose-dependent manner. After 4 days, a significant increase over basal could only be obtained with 100 ng LH. As shown in Figure 2.2.2 *lower panel*, addition of FSH resulted in a dose-dependent stimulation of P during 4 days. Moreover, in contrast to freshly retrieved cells, stimulation with FSH resulted in relatively higher P levels than stimulation with LH.

Oestradiol production of granulosa cells stimulated with FSH and LH after preincubation

Granulosa cells were preincubated in the presence of FCS (10%) for 7 days and media was changed every 2 days. During preincubation, basal E_2 levels remained high (> 150 ng/ml, data not shown). After preincubation, cells were cultured in serum-free media and stimulated with FSH (3, 10 and 30 ng/well) and LH (10 ng/well). After 2 days in serum-free media, basal E_2 levels decreased to 950 pg/ml, with a further decrease after 4 days (Figure 2.2.3). Significant stimulation with FSH was observed with 30 ng/well, after 2 - 4 days. Addition of LH (10 ng/well) raised E_2 production over basal after 2 days, with a further increase after 4 days. In contrast to FSH, LH stimulated E_2 levels were 10-fold higher after 4 days, as compared to the levels after 2 days.

Effects of extracellular matrix on progesterone production

It has been reported that the use of extracellular matrix *in vitro* promotes

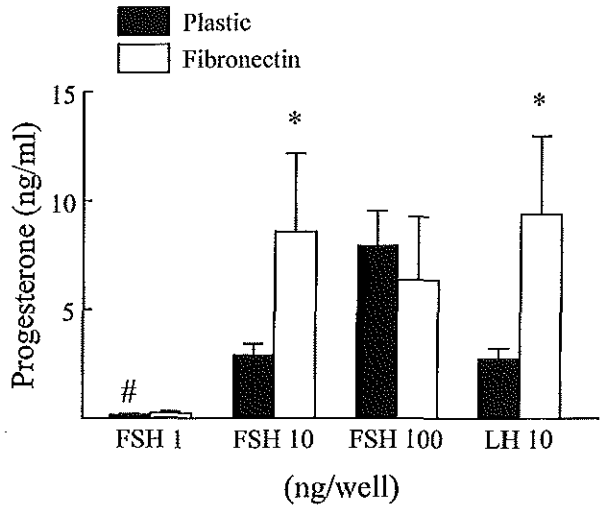

Figure 2.2.4
Effect of extracellular matrix on progesterone production. Human granulosa-lutein cells were plated on plastic or human fibronectin coated wells with 10% serum. After 7 days, cells were stimulated with FSH (1, 10 and 100 ng/well) or LH (10 ng/well) in serum-free media for 2 days. Data are the mean ± SEM from a representative experiment in triplicate. * Significantly different from cultures on plastic ($P < 0.05$). Basal P levels on both plastic and fibronectin were below detectable limit (0.2 ng/ml, data not shown)

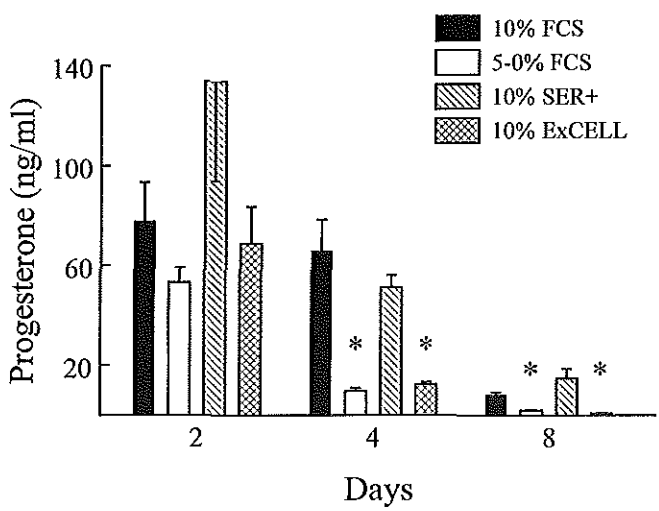

Figure 2.2.5
Effect of serum additives on progesterone production. Human granulosa-lutein cells were cultured for 8 days in media containing 10% fetal calf serum (10% FCS), 5% fetal calf serum decreasing to 0% (5-0% FCS), 10% Serum-Plus (10% SER+) or ExCell 320 (10% ExCell). Data are the mean ± SEM from a representative experiment in triplicate. * Significantly different from cultures in 10% FCS ($P < 0.05$).

biochemical and morphological differentiation of cells in culture. Therefore, preincubated granulosa cells cultured on plastic were compared to cells cultured on human fibronectin. Freshly retrieved granulosa cells were preincubated for 7 days in media with 10% FCS. After preincubation, FSH (1, 10 and 100 ng/well) or LH (10 ng/well) were added to the cultures in serum-free media. Basal P production was low or below detectability (0.2 ng/ml) on both types of plates (data not shown).
Addition of FSH resulted in an increase of P production on both plastic and fibronectin. The maximal stimulatory dose on fibronectin appeared to be lower than on plastic (Figure 2.2.4). Addition of LH (10 ng/well) resulted in a significant increase on both types of plates, with P levels on fibronectin significantly higher than on plastic.

Effects of varying concentrations of serum in culture media on progesterone production in cultured human granulosa cells
To investigate the influence of factors present in serum on the proliferation and

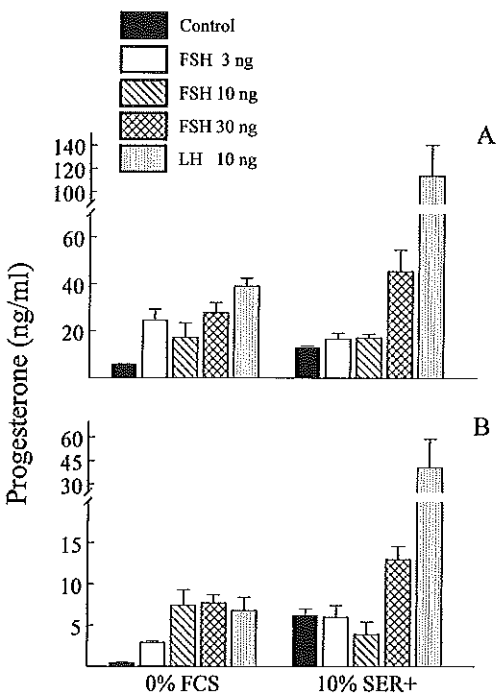

Figure 2.2.6
Effect of media additives on gonadotrophin stimulated progesterone production. Human granulosa-lutein cells were cultured in either serum-free media, after 7 days preincubation in 10% FCS (0% FCS); or in Serum-Plus, after 7 days preincubation in Serum-Plus (10% SER+) and stimulated with FSH or LH for 2 (*upper panel*) and 4 (*lower panel*) days. Data are the mean ± SEM from a representative experiment in triplicate. * Significantly different from serum-free value ($P < 0.05$).

differentiation of granulosa cells *in vitro*, cells cultured in various types of media were examined. Freshly retrieved granulosa cells were plated on fibronectin coated plates and preincubated in media containing constant (10%) or decreasing (5-0%) concentrations of FCS and two commercially available media additives: Serum-Plus (low concentration fetal bovine serum) and Ex-cell 320 (serum-free medium, with added factors). Basal P levels decreased gradually during the preincubation period in all media types (Figure 2.2.5). After 8 days preincubation, the cultures with Serum-Plus displayed significantly higher P levels than cultures in other media types.

After preincubation in media as described above, granulosa cells were cultured for 4 days in serum-free media (preincubation with FCS), Serum-Plus (preincubation in Serum-Plus) or Ex-cell 320 (preincubation in Ex-cell 320) and stimulated with LH (10 ng) or FSH (3, 10 and 30 ng). Both basal and stimulated P levels from cultures in Ex-cell 320 were below detectable limits (data not shown). Cultures preincubated in decreasing concentrations of FCS displayed low basal P levels, which could be increased with FSH and LH after two days. However, after four days basal and stimulated P levels were undetectable (data not shown). After preincubation in 10% FCS, P levels increased significantly over basal levels in response to FSH (3,10 and 30 ng) and LH (10 ng) after 2 and 4 days (Figure 2.2.6). Cultures in Serum-Plus displayed significant P increases over basal after stimulation with 30 ng FSH and 10 ng LH. LH stimulated P levels were significantly higher in Serum-Plus, while FSH-stimulated P levels did not differ significantly.

2.2.4. Discussion

These studies were undertaken to establish an FSH responsive *in vitro* granulosa cell culture model. Freshly retrieved cells from women undergoing IVF procedures were stimulated *in vitro* with FSH and LH, either directly or after 7 days of preincubation. Effects of extracellular matrix and presence of FCS in culture media on P production were investigated to establish culture conditions for maximal stimulation by FSH with minimal potential interference by other factors.

The results obtained using freshly retrieved granulosa cells and the effects of gonadotrophic stimulation on P production are comparable with previous reported studies (Veldhuis *et al.*, 1983; Hillensjö *et al.*, 1985; Erickson *et al.*, 1991). The low response to LH and FSH in the first days of culture suggests an already highly differentiated granulosa cell population with a maximally stimulated steroidogenic capacity, due to *in vivo* stimulation with high doses gonadotrophins prior to retrieval. With prolonged culturing, the cells seem to regain some sensitivity to LH. However, the maximal effective dose of 10 ng/well in combination with decreasing P production at higher doses LH,

indicates a cell population which can be easily overstimulated. With respect to the lack of response to FSH, Erickson *et al.* (1991) demonstrated a marked difference in response to FSH of granulosa cells from stimulated or unstimulated follicles, suggesting that FSH is not a physiological regulator of P synthesis in differentiated cells. After a preincubation period of 7 days, granulosa cells displayed very low basal steroid concentrations which remained constant during the subsequent 4 days. The cells appeared to retain their steroidogenic capacity, as suggested by the dose-dependent effects of LH and FSH.

It is known that aromatase activity of granulosa cells *in vitro* can be stimulated by FSH and LH (Hsueh *et al.*, 1984). In this study, we found significant stimulatory effects of both LH and FSH with respect to E_2 production in preincubated cultures. The significant increase in E_2 after LH stimulation on day 4, compared to day 2 suggests induction of aromatase by LH. In a previous study, a significant increase in E_2 production with LH stimulation and to a lesser extent FSH has been noted also (Wickings *et al.*, 1986). The authors concluded that the effects of FSH were caused by contamination with LH. The LH and FSH preparations used in this study had a cross-contamination of 0.3 and 8.7%, respectively. This could explain the increase in production of both P and E_2 in the preincubated cultures with the highest dose of FSH. Moreover, the bioactivity of the used LH preparation was higher than that of the FSH, expressed as IU per mg, which could explain the differences E_2 in response. Since the basal E_2 levels decreased dramatically during preincubation, the increase following stimulation could also suggest changes in the cell population *in vitro*, as has been suggested by several studies (Schmidt *et al.*, 1984; Tapanainen *et al.*, 1987). It can be hypothesized that an already highly differentiated, highly steroidogenic population overshadows less differentiated cells at the onset of preincubation. During preincubation these cells could die, lose activity or fall back to a less differentiated state. This concept could explain the observed increase in both P and E_2 following stimulation with FSH. However, the high E_2 levels after LH stimulation suggest that LH receptors already have been induced, meaning that the cells have already reached a certain level of differentiation. This could be caused by low concentrations of FSH and growth factors present in serum, which maintain LH receptors or give rise to LH-receptor induction on less differentiated cells during the period of preincubation. It has been reported that granulosa cells from preovulatory follicles tend to luteinize spontaneously in culture media containing serum, while cells from smaller follicles do not (Channing, 1970). In an attempt to minimize influences of serum factors during preincubation, cells were cultured in different culture media. When cultured in Ex-cell 320 (serum-free media with added factors), P production decreased to undetectable levels without measurable increase after stimulation. The basal and LH stimulated P production were highest in Serum-Plus. With prolonged culturing, the levels

stayed relatively high as compared to cultures preincubated in FCS and stimulated in serum-free media (data not shown). Since Serum-Plus contains bovine serum and other protein factors, the continuous exposure could itself have stimulatory effects on the cells, which could explain the high basal P levels in this media. The influence of factors present in Serum-Plus could account for the high response to LH, when cultured in this media. This might suggest an ongoing differentiation of the cultured cells, rather than a selection of less differentiated cells as indicated by the first experiments. Although serum-free media was used during stimulation the inherent effect of unknown factors would make the use of serum or serum containing media additives during preincubation less desirable.

Several studies have shown that the use of extracellular matrix has permissive effects on proliferation and differentiation of granulosa cells (Furman *et al.*, 1986; Ben-Rafael *et al.*, 1988; Amsterdam *et al.*, 1989; Molskness *et al.*, 1991). In these experiments, cells appeared to be more sensitive to gonadotrophic stimulation when cultured on human fibronectin extracellular matrix than on plastic. Although the basal P production did not differ, stimulation with FSH and LH resulted in higher P production when cultured on extracellular matrix. Moreover, maximal stimulation took place at a lower FSH dose, suggesting a higher sensitivity to stimulation due to better culture conditions.

One aspect that needs further evaluation, is the decreasing P production in the time course. This could be due to a decrease in cell number, lack of steroid precursor or changes in gonadotrophin receptor number. In one separate experiment, cell numbers from non-stimulated cultures preincubated 7 days in presence of serum and subsequently cultured 2 days in serum-free media, were counted with a haemocytometer after detaching the cells with 0.25% trypsin. The cell numbers were 30 to 50% lower at the end of the culture period, compared to the number initially plated (data not shown). In contrast to several studies we could not show proliferation of the cells (Tapanainen *et al.*, 1987; McAllister *et al.*, 1990) although in a more recent study (Yong *et al.*, 1992), it has been shown that granulosa-luteal cells do not proliferate *in vitro*. However, the applied method using trypsin appeared not to be efficient and accurate enough for the cell numbers (10,000/well) used in this study. In addition, since the decrease in P and E_2 production was also found during preincubation in the presence of FCS and androstenedione, lack of substrate seems unlikely to be the sole cause of the decrease in steroid production.

In conclusion, this study has shown that preovulatory human granulosa cells obtained after ovarian hyperstimulation, can be successfully cultured in serum-free conditions. Preincubation of the cells without stimulation leads to a change in responsiveness to gonadotrophins. Cells appear to become more sensitive to FSH with respect to P but not to E_2 production, probably due to the advanced stage of differentiation. The use of extra cellular matrix can enhance sensitivity

to gonadotrophins. Although serum in culture media appears to have effects on steroidogenic function, low concentrations of serum during a limited time, might be necessary to maintain cultures. One has to be cautious with the interpretation of data obtained through this widely used culture system, employing highly differentiated cells for the study of local regulation of follicular maturation and development.

2.3 Preliminary experience with cultured human granulosa cells from ovaries without exogenous stimulation

2.3.1 Introduction

In order to study local factors involved in modification of FSH action *in vitro*, cultured granulosa cells isolated from preovulatory follicles after administration of hCG in IVF patients are less suitable. These cells display only little responsiveness to FSH stimulation due to luteinization (Schipper *et al.*, 1993). A limited number of *in vitro* studies with human granulosa cells from women without exogenous stimulation have been reported (Ryan and Petro, 1966; Erickson *et al.*, 1979; Erickson *et al.*, 1990; Olsson *et al.*, 1990; Mason *et al.*, 1990; Bergh *et al.*, 1991; Woodruff *et al.*, 1993; Mason *et al.*, 1994a; Montgomery Rice *et al.*, 1996; Willis *et al.*, 1996, Willis *et al.*, 1998a); and only a few have investigated the modulation of FSH action by growth factors. The principal aim of this study is to examine the role of locally acting growth factors in the proliferation and differentiation of granulosa cells, in particular in connection with arrested follicle development in polycystic ovary syndrome. In order to study local modification of FSH action we aimed at isolation and culture of human granulosa cells from unstimulated ovaries.

2.3.2 Materials and methods

Subjects

Granulosa cells were isolated from patients (age 20 - 50 years) with regular menstrual cycles, undergoing abdominal surgery for benign gynaecological disorders, unrelated to endocrine disturbances or ovarian pathology. Only patients undergoing surgery during the follicular phase of the menstrual cycle (1 - 15 days after the onset of the preceding menses) were included. The subjects had not received hormonal medication or treatment for at least 3 months prior to surgery. Informed written consent was obtained prior to the procedure from all patients. The study protocol was approved by the Ethics Review Committee of the Dijkzigt Academic Hospital.

Isolation of granulosa cells

Several methods for the isolation of granulosa cells have been applied. Initially, follicles were punctured and follicular fluid and cells aspirated with a subcutaneous needle and syringe. The samples were kept at 0° C for maximally 30 minutes after puncture and cells were isolated after centrifugation at 300 G for 5 minutes. Cell suspensions of several follicles from one patient were combined. Secondly, cells were isolated by flushing the follicular cavity with phosphate buffered saline (PBS) or scraping the intra-follicular wall with either a needle or a small, blunt, cornea curette. The third method used, involved the excision of one or more antral follicles in toto. The ovarian tissue fragment was kept in sterile ice-cold PBS and transferred within 30 - 60 minutes after the excision to the laboratory. In the laboratory the surrounding interstitial tissue was removed under sterile conditions, while immersed in PBS. Follicles with a yellowish-brownish coloured wall and evident vascularisation of the wall were considered healthy and non-atretic (H.D. Mason, personal communication) and were used for isolation of cells. The follicular size was measured and the follicle was opened with micro-scissors. The inner follicular wall was gently scraped with a small platinum loop and cells were transferred to culture medium (serum-free Medium-199, with the addition of 200 mM L-glutamine, 0.1% (w/v) bovine serum albumin and antibiotics [penicillin and streptomycin]; Gibco, Breda, The Netherlands). Cell clumps were dispersed either mechanically by gentle pipetting (Mason *et al.*, 1990), or enzymatically with collagenase/dispase (Themmen *et al.*, 1991; Woodruff *et al.*, 1993) or trypsin (Bergh *et al.*, 1993b). Viability of cells was estimated by the trypan blue exclusion test (0.4% trypan blue in PBS), as described previously (Erickson *et al.*, 1990; Mason *et al.*, 1990).

Granulosa cell culture

Cells were plated in 96 multi-well culture disks in a volume of 200 ml serum-free culture media (M-199 with 0.1% bovine serum albumin). A standard cell density of 10,000 cells/well (= 0.3 cm^2) was used and cells were incubated at 37° C and 5% CO_2 . Culture plates were coated with 1 µg/well human fibronectin (Boehringer Manheim BV, Almere, The Netherlands) prior to cell plating to enhance cell attachment. Cells were allowed to adhere to the culture dish for 24 h, followed by renewal of media. Subsequently the cells were cultured for periods of 48 h, up to 96 h. Culture media were stored at minus 20° C, until assayed for E_2 or cAMP. Aromatase activity was induced with human recombinant FSH (recFSH), with or without IGF-I (Boehringer Manheim BV, Almere, The Netherlands).

Radioimmunoassays

E_2 was measured using a radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). This immunoassay, which was originally developed for measurement of E_2 in serum samples, has been validated for use

Table 2.3.1: Aromatase activity in cultured human granulosa cells

No.	Follicle size (mm)	Total cell number (x 1000)	Viability (%)	Basal E_2 (pmol/well)	Maximum FSH conc. (IU/l)	Maximum stimulation factor
1	12	720	60	3.0	30	4.5
2	8	600	50	2.4	30	5.3
3	8	500	40	1.4	300	2.6
4	11	800	80	54.1	300	1.2
5	6	900	40	1.3	100	2.9
6	9	400	40	0.1	100	5.6
7	8	600	50	3.9	100	2.5
8	7	800	70	0.1	100	9.6
9	5	800	40	0.4	100	2.4

with culture media samples. For details of the cAMP assay, see previous publications (Schipper *et al.*, 1996).

2.3.3 Results

Cell yield and cell viability

Follicle puncture and isolation of granulosa cells from the follicular fluid resulted in very small amounts of granulosa cells, which were heavily damaged (viability 0 - 10%). Flushing or scraping of the follicular cavity *in situ* did not improve cell yield, but rather resulted in blood contamination and spill of follicular content. Eventually the excision of single antral follicles or small parts

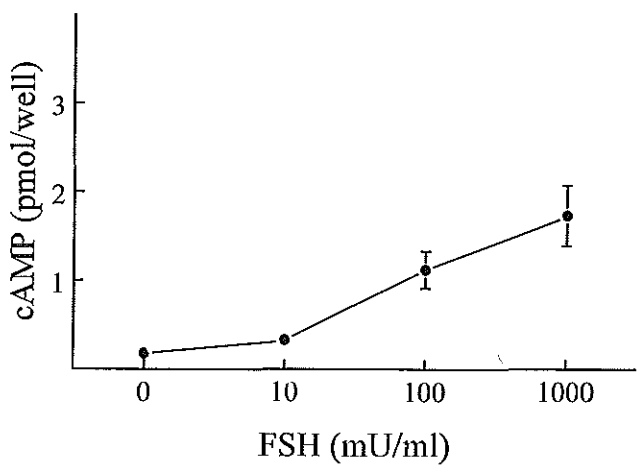

Figure 2.3.1
Cyclic AMP production in human granulosa cells. Freshly isolated cells were cultured 24 h prior to stimulation with recFSH for 2 h in the presence of 0.1 nM IBMX. Results are mean ± SD of 2 estimations, each performed in triplicate.

of ovarian tissue proved to be the best method. Over a period of 27 months material from 26 patients was obtained. Recovery of granulosa cells from morphologically healthy follicles (6 - 12 mm in diameter) ranged between 0.3 to 1.1 x 10^6 cells. However, the viability of these cells was low and varied from 10 - 80%. The viability was not related to the total cell number isolated (Table 2.3.1). If the cell viability was below 40% the cells were discarded and not used for culture experiments. Since this low viability may be caused by mechanical dispersion of cell aggregates, dispersion with enzymes was applied also. However, this did not result in an improved viability of the isolated cells. Twenty four hours after plating, only part of the cells attached to the culture dish and displayed a stellate-like shape, and even in these cells viability was only 80%. The unattached, non viable cells, were removed prior to investigations of hormonal effects on cell function.

FSH-stimulated cAMP production

After 24 h in culture, granulosa cells were stimulated with increasing concentrations of recFSH for 2 h in the presence of 0.1 mM isobutyl-methyl-xanthine and cAMP production was measured (Figure 2.3.1). After incubation with 10 mU/ml recFSH cAMP production was increased up to 1.8 times basal production (0.29 ± 0.06 pmol/l). Cyclic AMP production increased further at higher concentrations of recFSH and at 100 mU/ml recFSH, cAMP production was stimulated 6-fold (1.74 ± 0.14 pmol/well, $P < 0.05$, Student's *t*-test).

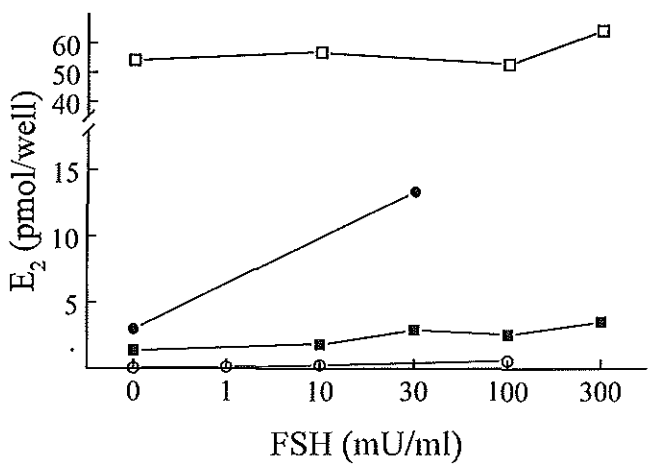

Figure 2.3.2
Oestradiol production in human granulosa cells stimulated with recFSH for 48 h in the presence of 100 nM Androstenedione. Results (mean of triplicate wells) of 4 similar stimulation experiments are given. Symbols refer to the follicle number as given in Table 3.3.1: ● follicle 1; ■ follicle 3; □ follicle 4 and ○ follicle 8.

FSH-stimulated E_2 production

Cells were incubated in the presence of 100 nM androstenedione, serving as a substrate for aromatase. This concentration proved to result in maximal aromatase activity, as assessed in initial experiments (data not shown). For stimulation of aromatase in the cultured granulosa cells, cells were incubated for 48 h in the presence of increasing concentrations recFSH. The aromatase activity and degree of stimulation in the granulosa cell preparations showed large variation, with more than 100-fold differences in basal E_2 production between cell cultures from different follicles and patients. Moreover, stimulation with high doses of recFSH increased the aromatase activity only 2 - 3 fold, and a dose-dependent increase could not always be measured. In Figure 2.3.2 the results from 4 granulosa cell cultures are given, with cross reference to Table 2.3.1. The differences in aromatase activity of the granulosa cells were not related to the initial cell viability after isolation of the granulosa cells. Aromatase activity in cultured granulosa cells was also stimulated with 100 ng/ml IGF-I (1.8-fold increase in E_2, as compared to basal levels [$P = 0.8$]), but synergism with FSH could not be shown.

2.3.4 Discussion

The principal aim of our study was to examine the effects of (growth) factors on FSH-stimulated granulosa cells *in vitro*. As the data show, our attempts to isolate and culture granulosa cells from human ovarian tissue without exogenous stimulation to investigate this was unsatisfactory. Due to practical and logistical problems only a limited amount of healthy ovarian tissue could be obtained. Excision of follicles form ovaries *in situ* during surgical procedures appeared to be the best method to obtain reasonable amounts of follicular material. However, the quality of the isolated cells was mostly poor. Even if the follicles appeared morphologically healthy (Mason *et al.*, 1990), and the granulosa cells could be easily flushed off or scraped off with a platinum loop from the inner follicular wall, the quality of the granulosa cells was poor. In general the cell viability, as tested with trypan blue, was low (on average 40%). Since the trypan blue test only measures changes in the permeability of the cell membrane, and not intracellular damage, it may very likely underestimate the cellular damage inflicted by the isolation procedure. The notion that after 24 h in culture only 80% of the attached cells remained viable may underscore this.

Although stimulation of cAMP production provides more information on cell metabolism and expression of functional FSH receptors as compared to trypan bleu exclusion, this response can even be shown in granulosa cell homogenates or cell membrane preparations, provided that sufficient ATP is present (Fletcher *et al.*, 1982). It is therefore a primitive response which does not provide sufficient information on cellular integrity as a whole. The induction of

aromatase is a more complex response, requiring more intact cellular functions, such as gene expression and protein synthesis. However, the poor aromatase response after stimulation with FSH, with a maximum stimulation factor of only 2 - 5 times basal aromatase activity, is an indication for a disturbed function of the cultured granulosa cells. As reflected by follicular phase E_2 levels in serum and follicular fluid, a similar or stronger response is elicited *in vivo* by a less elevation in FSH (Sanyal *et al.*, 1974; McNatty *et al.*, 1976; van Dessel *et al.*, 1996b).

We have not been able to improve the viability of the isolated granulosa cells, whereas we could isolate responsive granulosa cells from preovulatory follicles (Schipper *et al.*, 1993) and FSH responsive Sertoli cells from rat testes (Schipper *et al.*, 1996). It appears therefore that the low viability of human granulosa cells is more a reflection of fragile cells, rather than of an improper isolation procedure. Other investigators also report a low viability of isolated human granulosa cells, varying between 50 and 80% (Bieszczad *et al.*, 1982; Sjögren *et al.*, 1988; Mason *et al.*, 1990; Bergh *et al.*, 1991; Woodruff *et al.*, 1993). Furthermore, stimulation of aromatase as reported by others (Erickson *et al.*, 1979; Mason *et al.*, 1990; Mason *et al.*, 1993), and the large variations within and between cultures (Montgomery Rice *et al.*, 1998) is in the same order of magnitude compared to our results.

It seems that the relatively low aromatase response *in vitro* is accepted as an intrinsic property of the cells. However, it is more likely that isolated and cultured cells are damaged and only poorly reflect their *in vivo* cellular properties. The low *in vitro* responsiveness does not allow to measure subtle modulation of FSH action and therefore it may impossible to study finetuning of FSH action. This shortcoming of the isolation procedure should receive more attention when conclusions from *in vitro* studies are applied to physiology. The isolation of granulosa cells from their microenvironment disrupts the intercellular structure and the matrix in which the cells are embedded, thus distorting the auto- and paracrine regulation by (growth) factors. Newly developed techniques in the fields of molecular biology and cell biology, may provide alternatives to elucidate the local regulatory mechanisms. An option may be fluorescence microscopy and molecular probing, by which small quantities of tissue and cells within their microenvironment can be studied *in vitro* (Sanders, 1995).

2.4 Application of a Chinese hamster ovary cell line, transfected with the human FSH receptor for the measurement of specific FSH receptor activation inhibitors in human serum

2.4.1 Introduction

Follicle-stimulating hormone (FSH) induces aromatase activity in granulosa cells (Gore-Langton and Dorrington, 1981; Garzo and Dorrington, 1984), luteinizing hormone (LH) receptor expression and cell proliferation (Hsueh *et al.*, 1984). The effects of FSH appear to be modulated by para- and autocrine factors, such as epidermal growth factor, transforming growth factor-b and the insulin-like growth factors (IGFs) and IGF-binding proteins (Fauser and Hsueh, 1988; Tonetta and DiZerega, 1989; Woodruff *et al.*, 1993). A disregulation of FSH actions by local factors might play a role in the pathogenesis of 'normo'gonadotrophic anovulation, in particular polycystic ovary syndrome, as suggested by several authors (Erickson *et al.*, 1990; Mason *et al.*, 1990; Fauser, 1994a; Mason *et al.*, 1994b).

Inhibition of FSH-induced aromatase in cultures of rat granulosa or Sertoli cells by non-specific factors present in serum has been shown by different authors, and even minor (1-5%) amounts of serum decrease the FSH-induced aromatase substantially (Jia and Hsueh, 1986a; Padmanabhan *et al.*, 1987). It is not clear whether this inhibition of FSH bioactivity is being caused by specific inhibitors acting directly on the FSH receptor, by growth factors acting on the aromatase enzyme through growth factor receptors or by cytotoxic effects of the serum. Since induction of aromatase may be regulated by FSH in combination with other factors, we have evaluated whether cAMP production may be a more specific and suitable endpoint for measuring activation or inhibition of the FSH receptor.

Various FSH receptor binding inhibitors have been detected in serum (Reichert Jr *et al.*, 1979; Sanzo and Reichert Jr, 1982) and follicular fluid (Lee *et al.*, 1991; Lee *et al.*, 1993). Although these partly purified factors can bind to the FSH receptor in radioreceptor assays under hypotonic conditions, it is not known how they affect the functional properties of the receptor, in particular under normal (isotonic salt) conditions and when present in diluted form in serum. The scope of this study is to determine the biological activity of putative inhibitors in serum by measuring the inhibition of adenylate cyclase activity in a Chinese hamster ovary cell line, stably transfected with the human FSH receptor (CHO-F3B4). Inhibition of FSH receptor activation by serum factors is examined in buffers with normal ionic strength as well as in buffers of low ionic strength, such as are usually used in radioreceptor binding assays.

2.4.2 *Materials and Methods*

Hormones and reagents

Recombinant human FSH (recFSH) (Org. 32489, bioactivity 8413 IU/mg as assessed by *in vivo* bioassay, relative to reference preparation IS 70/45; immunoactivity 12000 IU/mg) was a generous gift from NV Organon Oss, The Netherlands. Isobutyl-methyl-xanthine (IBMX), forskolin, choleratoxin and media additives were purchased from Sigma Chemie (Bornhem, Belgium).

A pool of hypogonadotrophic serum (HS) was obtained from high-dosed (50 µg ethinyl-oestradiol daily) combined oral contraceptive pill users. The FSH level of the serum pool was <0.5 IU/l, as assessed by immunoradiometric assay (Medgenix, Fleurus, Belgium).

Cell cultures

A Chinese hamster ovary cell line, stably transfected with the human FSH receptor was used. This cell line has been developed and provided generously by NV Organon, Oss, The Netherlands. The human FSH receptor (hFSH-R) cDNA was cloned from a human testis cDNA library and inserted in the eukaryotic pKCR expression vector. After stable transfection in CHO cells, a single cell clone (CHO-F3B4) was obtained after successive selection by neomycin and 10 µM $CdCl_2$, respectively. The transfected cells expressed 3000 FSH receptor molecules per cell with a binding affinity (Kd) of 25 pmol/l. Specificity of receptor activation was assessed by ligand activated adenylate cyclase. Compared to FSH-stimulated cAMP production, addition of a 1000-fold excess in concentration of human chorionic gonadotrophin or luteinizing hormone did not reveal stimulation of adenylate cyclase, suggesting a high ligand specificity of the transfected receptor.

CHO-F3B4 cells were cultured for 48 hours on multi-well plates (Ø 1.2 cm) in 0.5 ml Dulbecco's Modified Eagle's Medium/HAM-F12 (1:1) (DMEM/F-12, GIBCO Europe BV, Breda, The Netherlands) with the addition of 100 U/ml penicillin and 100 µg/ml streptomycin and 10% (v/v) fetal calf serum (FCS, Sebak GmBH, Adenbach, Germany). The culture medium was replaced by serum-free media prior to stimulation with FSH.

Rat Sertoli cells were isolated as described previously (Themmen *et al.*, 1991). In brief: testes were isolated from 21-day old rats. The tissue was dispersed by incubation in collagenase (1 mg/ml), trypsin (1 mg/ml), hyaluronidase (1 mg/ml) and DNAse (5 µg/ml). The tubule fragments obtained, were washed with PBS and incubated once more for 20 minutes in the presence of the indicated enzymes. The resulting tubule fragments were dispersed with a dounce homogenizer. The aggregates of Sertoli- and germinal cells were plated on 24-well culture dishes in Eagle's Minimal Essential Medium (MEM, GIBCO Europe BV, Breda, The Netherlands) supplemented with non-essential amino a-

cids, penicillin (100 U/ml), streptomycin (100 µg/ml) and 1% FCS. After 48 hours incubation, germinal cells were removed by osmotic shock in 10-fold diluted MEM in water for 2 minutes. After this procedure, fresh culture medium was added to the wells and Sertoli cells were cultured for another 24 hours prior to stimulation experiments.

FSH receptor activation

After removal of culture media, CHO-F3B4 cells were incubated at 37° C in a volume of 100 µl DMEM/F12, containing 0.1% BSA and 0.1 mM IBMX, recFSH. For measurement of inhibition by serum factors of FSH-induced cAMP production, various amounts of serum were added to the cells. At the end of the incubation period, medium was removed and stored at -20° C until cAMP analysis.

Effects of plating density and incubation time on recFSH-stimulated adenylate cyclase activity were assessed initially. Sensitivity for FSH-stimulated cAMP production was not different when between 5 and 20 x 10^3 cells per well (area: 1.1 cm^2) were initially plated, while a decrease in sensitivity was observed when 60 x 10^3 cells were plated. For further experiments 20 x 10^3 cells were plated in each well.

FSH-stimulated cAMP production rate reached a steady state in less than 15 minutes and remained constant for at least 6 hours. An incubation period of 4 hours was taken for further experiments. After 4 hours incubation 90-95% of the total amount of cAMP was present in the medium. Therefore, extracellular cAMP was taken as a reflection of adenylate cyclase activity.

Adenylate cyclase activation in rat Sertoli cells was measured after 1 hour stimulation with recFSH in MEM containing 0.1% BSA and 0.1 mM IBMX. For measurement of aromatase activity, Sertoli cells were incubated for 48 hours in the presence of 10^{-7} M 19OH-Androstenedione as substrate, with or without addition of HS.

Cell viability tests

In all experiments DNA content in the wells was measured by a fluorometric method as described previously (Downs and Wilfinger, 1983). The intra-assay coefficient of variation for DNA content/well was less than 12%. To test effects of HS on cellular integrity of CHO-F3B4 cells, cells were incubated 4 or 24 hours in the absence or presence of 10 or 90% HS. After removal of the incubation medium the percentage of propidium-iodide (0.5 µg/ml final concentration) stained cells was taken as an index of membrane damage. To assess possible cytotoxic effects of serum factors, CHO-F3B4 cells were incubated in absence or presence of 10 or 90% HS. After 4 hours the incubation medium was removed and choleratoxin (CT, 20 µg/ml) or forskolin (10 µM) activated cAMP production was measured in fresh, serum-free medium.

FSH receptor activation in CHO-F3B4 cells in low salt buffer

Since several FSH receptor binding inhibitors have been described to be present in serum, it was attempted to test the effect of inhibition by HS under similar, hypotonic, conditions as applied in many receptor binding studies. For this purpose, CHO-F3B4 cells were stimulated for 4 hours with FSH in a low salt buffer, containing 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$ and 25 mM HEPES. To compensate for the low osmolarity 200 mM sucrose was added. This buffer with an osmolarity of 290-310 mosmol/kg was designated KRB-Na+S.

In order to remove salts from the serum samples, HS was dialysed in distilled water (volume ratio HS/water: 1/200) for 36 hours. The retentate (fraction >10kDa) was subsequently dialysed against a large volume of KRB-Na+S for 24 hours until the osmotic value was similar to that of untreated serum. The <10kDa molecular weight serum fraction was obtained after ultrafiltration of serum using Centriprep concentrators, with a cutoff value of 10kDa (Amicon Inc., Beverly, MA).

Radioimmunoassays

Cyclic AMP was assayed as described previously (Harper and Brooker, 1975). In brief, after acetylation, the samples were incubated overnight with cAMP antibody (purchased from Prof Dr J. Stoof, Free University, Amsterdam, The Netherlands). The assay was validated for the use of culture media and corrected for addition of serum in the samples. All samples were assayed in duplicate. Sensitivity of the assay was 0.125 pmol/ml. Inter- and intra-assay coefficients of variation were 20% and 8%, respectively.

E_2 was measured using a radio-immunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). To compensate for the different amounts of serum in the incubation media, samples were extracted in hexane-ether and redissolved in steroid free serum to be assayed against serum standards. Recovery of E_2 after extraction was 67%. The inter- and intra coefficients of variation were less than 5 and 7% respectively.

Data analysis

Dose-response curves are expressed as a percentage of the maximal stimulation, which was taken as 100%. In experiments in which serum was added to the cells, the resulting responses are expressed as a percentage of the response in serum-free conditions, which was taken as 100%. For reference, basal responses in terms of amount of cAMP produced are given in the results section or figure legends. In all experiments, cAMP production is expressed as pmol/µg DNA. Mean values ± standard error (SEM) of at least 3 independent bioassays are given. For each individual point within one bioassay, triplicate incubations were used.

2.4.3 Results

FSH activation of adenylate cyclase and aromatase activity

Adenylate cyclase in CHO-F3B4 cells could be stimulated reproducibly by different FSH doses (Figure 2.4.1a). Basal levels of cAMP were 0.89 - 3.45 pmol/µg DNA and maximal stimulated levels were 100 - 230 times higher. The sensitivity, defined as the dose of recFSH eliciting a response of more than twice the standard deviation (SD) of the basal cAMP production, was 0.4 mU/ml.

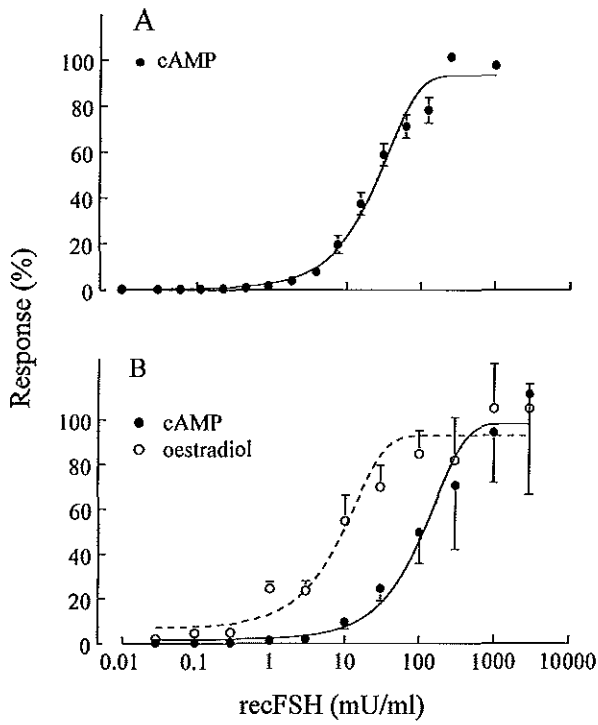

Figure 2.4.1

A: FSH-induced adenylate cyclase activity in CHO-F3B4 cells. CHO-F3B4 cells were incubated with recFSH for 4 hours in the presence of 0.1 mM IBMX in DMEM/F-12 medium. Results are expressed as a percentage of the maximal stimulation. Mean values (± SEM) of 3 independent bioassays, each point performed in triplicate, are shown. Basal cAMP production varied from 0.89 - 3.45 pmol/µg DNA. Maximally simulated cAMP production was 100 - 230 times higher.

B: FSH-stimulated adenylate cyclase production and aromatase activity in rat Sertoli cells. Isolated rat Sertoli cells were stimulated with recFSH in the presence of 0.1 mM IBMX. After 1 hour incubation a medium sample was taken for measurement of cAMP (●). After incubation for 48 additional hours, E_2 (○) was assayed in the medium. Mean values (± SEM) of 3 similar experiments are expressed as percentage of maximal stimulation. Basal cAMP production after 1 hour varied from 0.1 to 0.16 pmol/µg DNA, and maximally stimulated levels were 200-230 times higher. Basal E_2 production varied from 0.038 to 0.041 pmol/µg DNA. Maximal stimulated aromatase activity was 33 to 39 times higher.

The half maximal stimulation of cAMP production (ED_{50} value) was obtained at 24.9 mU/ml. The intra-assay coefficient of variation at the ED_{50} level was less than 16%.

In rat Sertoli cells cAMP production was also stimulated significantly by recFSH. Basal production was 0.1-0.16 pmol/µg DNA, and maximally stimulated levels were 200 to 230 times higher. The ED_{50} value for recFSH-stimulated cAMP in rat Sertoli cells (60-80 mU/ml) was 2-3 times higher than in CHO-F3B4 cells (Figure 2.4.1b). Basal E_2 production was 0.04 pmol/µg DNA and the maximal stimulated production was 33-39 times higher. The ED_{50} value for FSH-stimulated E_2 production in rat Sertoli cells (10 mU/l) is 5-

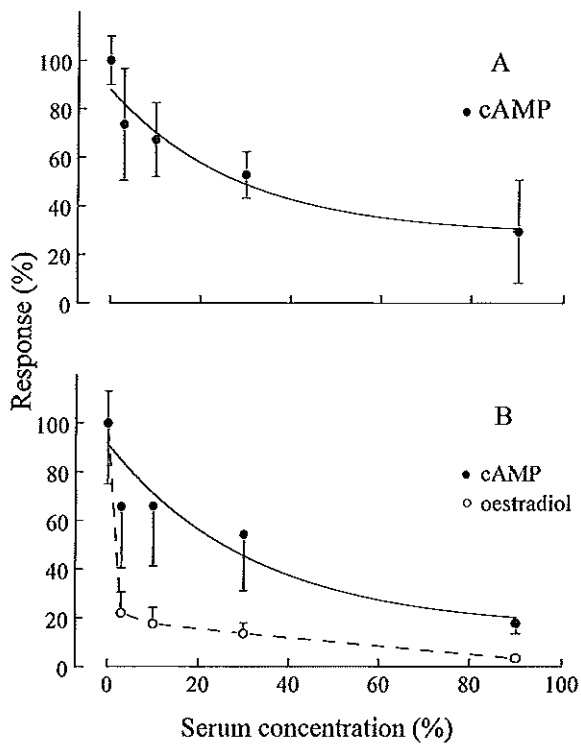

Figure 2.4.2
A: Inhibition cAMP production in CHO-F3B4 cells. CHO-F3B4 cells were stimulated with 30 mU/ml recFSH in the presence of 0.1 mM IBMX and various amounts of hypogonadotrophic human serum were added (3 - 90% v/v). Results are expressed as percentage of cAMP production ± SEM in the absence of serum, which was taken as 100%.
B: Inhibition of cAMP and oestradiol production in rat Sertoli cells. Isolated Sertoli cells were stimulated with 30 mU/l FSH in the presence of 0.1 mM IBMX and 100 nM 19OH-androstenedione. After 1 hour incubation a medium sample was taken for measurement of cAMP (●). After incubation for 48 additional hours, E_2 (○) was assayed in the medium. Results are expressed as percentage of the response in the absence of serum, which was taken as 100%.

8 fold lower than the ED_{50} for cAMP production. Therefore stimulation of E_2 production can be measured with a higher sensitivity. The intra-assay coefficient of variation at the ED_{50} level for cAMP and E_2 production was less than 23 and 19% respectively.

Inhibition of stimulated cAMP production and aromatase activity by human serum
To assess inhibitory effects of HS on FSH-stimulated cAMP production, CHO-F3B4 cells were incubated with a fixed stimulatory dose of recFSH of 30 mU/ml (ED_{50} value) and increasing amounts of HS were added. FSH-stimulated cAMP production decreased with 30-40% after the addition of 3-10% HS. At higher concentrations of HS (up to 90%) a further decrease in cAMP accumulation was observed, up to a maximal inhibition of approximately 70% (Figure 2.4.2a). Horse serum or FCS (3 - 90%), as well as mixtures of various concentrations of sera, inhibited FSH-stimulated cAMP production to a similar extent as various concentrations of HS alone (data not shown). Addition of increasing concentrations of pure bovine or human serum albumin up to 7% (w/v) did not inhibit FSH-stimulated cAMP production in CHO-F3B4 cells (data not shown).

The FSH-induced cAMP production in rat Sertoli cells as in CHO-F3B4 cells was inhibited by HS to a same extent (Figure 2.4.2b). However, in rat Sertoli cells, FSH-stimulated aromatase activity was inhibited to a much greater extent, when compared to cAMP production. Addition of minimal amounts of serum (1-3%) already inhibited the E_2 response for approximately 80% and at a serum concentration of 90%, the stimulated E_2 production (0.07 pmol/µg DNA) could hardly be discriminated from basal E_2 production (0.04 pmol/µg DNA).

Specificity of inhibition by human serum
In order to assess whether the presence of HS affects cell integrity, both DNA content and cell viability were measured. Concentrations up to 90% HS for 24 hours, had no effect on DNA content of the wells and the cellular integrity, as measured by propidium-iodide exclusion. Moreover, preincubation of CHO-F3B4 cells in the presence of 10 or 90% HS for 4 hours, did not affect the stimulatory effect of choleratoxin or forskolin in serum-free medium and only the stimulatory effect of FSH (30 mU/ml) was diminished after preincubation with 90% HS (Figure 2.4.3). Due to serum protein binding of choleratoxin and forskolin these experiments could not be carried out when choleratoxin and forskolin were present together with serum. In addition, incubation of CHO-F3B4 cells in presence of increasing concentrations of HS (3 - 90%) without stimulating doses of recFSH did not increase basal cAMP production. Only at 90% HS a slight increase in cAMP (up to 2-fold basal production) was observed, which was not significantly higher than in absence of serum (data not shown).

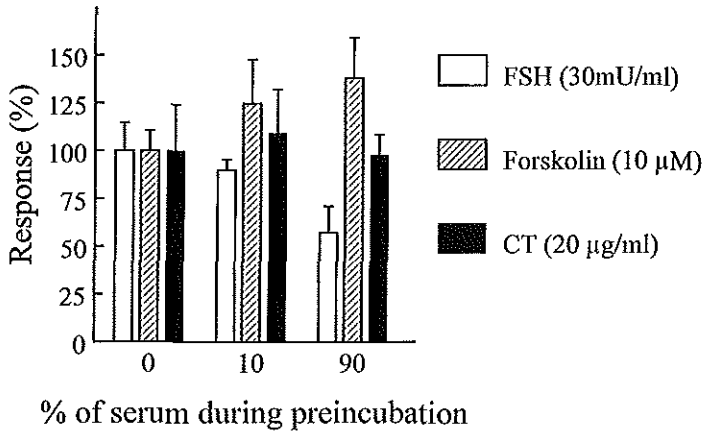

Figure 2.4.3
Specificity of FSH receptor inhibition by human hypogonadotrophic serum. CHO-F3B4 cells were incubated for 4 hours in the presence of 0.1 mM IBMX and absence or presence of human hypogonadotrophic serum (10 or 90%), prior to stimulation with recFSH (30 mU/ml), choleratoxin (20 μg/ml) or forskolin (10 μM) for 2 additional hours. Responses are expressed as percentage (± SEM) of cAMP production after 4 hours incubation in absence of serum, which was taken 100%.

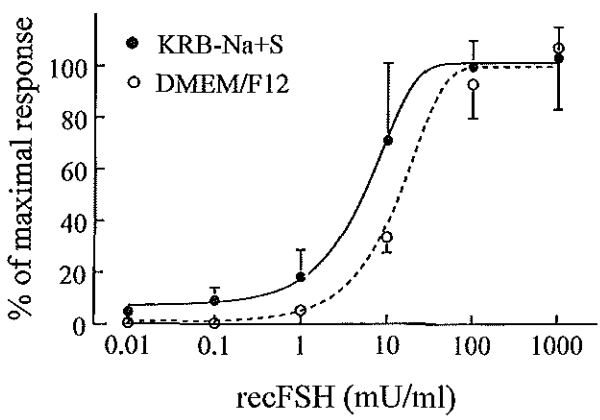

Figure 2.4.4
Stimulation of CHO-F3B4 cells in low salt buffer. CHO-F3B4 cells were incubated with recFSH in low salt buffer (●, KRB-Na+S, see Materials and Methods) or normal incubation medium (○) for 4 hours in the presence of 0.1 mM IBMX. Results are expressed as percentage of maximal stimulation. Data points are mean ± SEM of 3 experiments, each performed in triplicate. Basal cAMP production in low salt buffer varied from 4.0 to 7.6 pmol/μg DNA, maximally stimulated levels were 45 to 60 times higher.

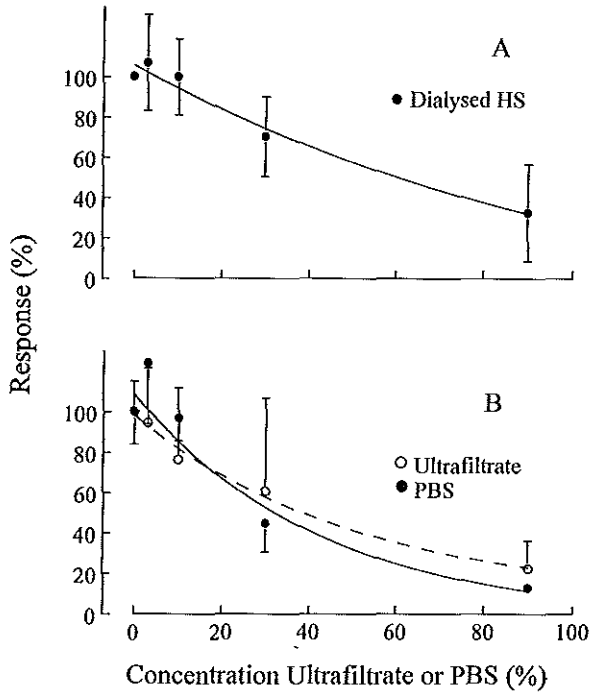

Figure 2.4.5
A: Inhibition of FSH-stimulated cAMP production in CHO-F3B4 cells by dialysed hypogonadotrophic human serum. CHO-F3B4 cells were incubated with 3 mU/ml recFSH in the presence of 0.1 mM IBMX in KRB-Na+S, in absence or presence of various amounts (3 - 90%) of dialysed hypogonadotrophic serum.
B: Inhibition of FSH-stimulated cAMP production in CHO-F3B4 cells by ultrafiltrated hypogonadotrophic human serum or phosphate-buffered saline. CHO-F3B4 cells were incubated with 3 mU/ml recFSH in the presence of 0.1 mM IBMX in KRB-Na+S, in absence or presence of various amounts (3 - 90%) of ultrafiltrated hypogonadotrophic human serum (<10kD,○) or phosphate-buffered saline (PBS,●).

Effects of serum on FSH-stimulated cAMP production in low salt buffer
Stimulation of CHO-F3B4 cells with recFSH was also possible in a low-salt buffer (KRB-Na+S, see Materials and Methods). Basal levels of cAMP in KRB-Na+S were higher (4.0-7.6 pmol/μg DNA) compared to standard medium, while maximal stimulation was lower (45-60 times basal). Although the stimulation factor was less, a 3 to 4 fold increase in sensitivity (ED_{50}: 5-8 mU/ml recFSH) could be observed under these conditions (Figure 2.4.4). The cells remained functionally active for at least 4 hours and incubation for 6 hours in KRB-Na+S did not affect gross morphology of the cells.

After addition of dialysed HS to CHO-F3B4 cells in KRB-Na+S, the FSH-induced cAMP response was inhibited in a similar fashion as in normal incubation buffer, although inhibition at lower serum concentrations seemed to be less pronounced (Figure 2.4.5a). Addition of an ultrafiltrate of HS,

containing serum components of a molecular weight <10kD, including ions, inhibited the FSH-induced cAMP response with 80-90%. This inhibitory effect could be reproduced by addition of various concentrations of phosphate buffered saline (PBS) and additional inhibitory effects of low molecular serum components could not be demonstrated (Figure 2.4.5b). When 90% of PBS was added to the cells (corresponding with a NaCl concentration of 135 mM), cAMP response was similar to the response in normal culture medium.

2.4.4 Discussion

There are many indications that intra-ovarian regulation of FSH action is important for normal follicular development (Ujita *et al.*, 1987; Lee *et al.*, 1990), and intra-ovarian factors may also play a role in the pathogenesis of disturbed follicular growth (Fauser, 1994a; Mason *et al.*, 1994b). Abnormal follicular development may be the consequence of insufficient stimulation of granulosa cells by FSH as a result of interference of these local factors. Whether these factors disturb the interaction between FSH and its receptor or act through other (growth factor) receptors remains unclear. In order to distinguish between effects of inhibitors acting on the FSH receptor and those on other receptors, we have measured FSH-stimulated cAMP production as a reflection of FSH receptor activation in CHO-F3B4 cells stably transfected with the human FSH receptor. While other transfected cell lines have been used for measuring bioactivity of FSH (Kelton *et al.*, 1992; Tilly *et al.*, 1992; Albanese *et al.*, 1994), effects of serum are unknown in most cases. Recently, strong inhibitory effects of serum were reported (Gudermann *et al.*, 1994), using a mouse cell line, transfected with the rat FSH receptor (Ltk^-).

In confirmation of other studies (Jia and Hsueh, 1986a; Padmanabhan *et al.*, 1987) we observed a strong inhibition by serum of the FSH-induced aromatase activity in cultured rat Sertoli cells. However, the effects of serum on FSH-stimulated cAMP levels in these cells are less pronounced. These observations support the hypothesis that aromatase activity is being controlled by other receptors and intracellular pathways in addition to the FSH receptor and cAMP alone (Hsueh *et al.*, 1984). Although activation of aromatase can be measured more sensitively than cAMP production, it is a less specific endpoint for FSH action in the presence of serum components. Adenylate cyclase activation, being a response more proximal to FSH receptor activation, seems to be more specific.

Data from this study indicate that serum proteins exert an inhibitory effect at the level of FSH receptor-mediated activation of adenylate cyclase. It was attempted to test such serum factors in a wide range of concentrations, up to a concentration which can be expected in physiological conditions, i.e. pure serum. We could neither demonstrate a loss of cell viability nor diminished choleratoxin- and forskolin-stimulated adenylate cyclase activity after

preincubations of cells in the presence of serum prior to stimulation with various stimulators. The FSH-induced cAMP production was inhibited slightly less, when compared to the experiments in which FSH and serum were continuously present. This suggests that the inhibitors do not bind tightly to the receptor.

It must be realized, that other components, present in serum may also activate adenylate cyclase after interaction with their respective (G protein-coupled) receptors. However, the strong response of the CHO-F3B4 cell line to FSH stimulation (50 to 100-fold stimulation at 30 mU/ml), in combination with the undetectable stimulation by hypogonadotrophic serum alone, indicates that the contribution of other hormones or ligands to the observed response, is relatively small. Furthermore, when HS was added at different doses of FSH, the cAMP response was always inhibited up to 40 - 60% of the serum-free response at the highest HS concentrations (30 or 90%), irrespective of the FSH dose used. Only at low HS concentrations (<10%) differences in response were observed (data not shown). At these concentrations high doses of FSH (up to 100 mU/ml) were inhibited to a lesser extent than low FSH doses, suggesting a partially competitive mechanism at the level of the FSH receptor.

In a study by Fletcher a partial purified low molecular mass fraction of bovine follicular fluid was capable of inhibiting the NaF-stimulated adenylate cyclase activity in membrane preparations, presumably acting on the G proteins (Fletcher *et al.*, 1982). Although these observations indicate that inhibitors of adenylate cyclase are present in follicular fluid, the physiological relevance is less clear, since G proteins are not accessible in intact cells. In contrast with our findings, others have reported that, for Ltk^- cells, transfected with the rat FSH receptor, cytotoxic effects of serum contributed largely to the inhibition of stimulated cAMP production (Gudermann *et al.*, 1994). These results may reflect a specific characteristic of the cell type used. Therefore it may be possible that the choice of cell type for transfection is of paramount importance for the development of bioassay systems.

Although the mechanism of the observed inhibition in our studies remains unclear, the results indicate the inhibitory effect to be specific with respect to the site of action, since it acts mainly via the FSH receptor. On the other hand, sera from different species (human, equine or bovine) all showed similar inhibitory effects. This points to a non-specific origin of the inhibitory components in serum. The inhibition appears not to be the result of a high concentration of extracellular proteins *per se*, since human albumin in high concentrations (up to 7% w/v) did not show an inhibitory effect. While serum concentrations greater than 10% inhibit FSH receptor activation, similar serum concentrations are required for optimal cell culturing *in vitro* and obviously do not inhibit cell growth. The question remains therefore, what is the optimal protein concentrations for the measurement and detection of elevated levels of inhibitory factors in serum.

The presence of FSH receptor binding inhibitors has been demonstrated in several fractions of serum and follicular fluid using FSH radioreceptor assays (Reichert Jr *et al.*, 1979; Sluss *et al.*, 1987; Sluss *et al.*, 1989; Lee *et al.*, 1990). Since these fractions were obtained after several purification steps, and have been investigated in a concentrated form, it is difficult to predict the biological activity of combinations of these inhibitors and activators when present in serum in diluted form.

Binding of FSH to its receptors can only be measured in low-salt buffers (0.05 M Tris) and not in normal buffers, when the affinity for the receptor appears to be much lower (van Loenen *et al.*, 1994a). Moreover, high affinity receptor binding appears not to be required for correct receptor activation (van Loenen *et al.*, 1994b). In this respect it is not known whether inhibitors of high affinity binding will also influence receptor activation. Since FSH binding studies have been carried out exclusively at low-salt concentrations, we have also studied the inhibition of receptor activation in low-salt buffers with addition of sucrose to compensate for low osmolarity. When stimulated in a low-salt buffer, CHO-F3B4 cells display a slightly higher sensitivity, which decreased after addition of increasing concentrations of salts to the medium. We have concluded from the results, that FSH receptor inhibition by dialysed serum at low ionic strength is comparable with receptor inhibition in normal buffers. A low-molecular-mass fraction of HS (<10 kDa), obtained after ultrafiltration, showed a dose-dependent inhibition that was similar to the dose-dependent inhibition by PBS. The observed inhibition of receptor activation by the low-molecular-mass fraction can therefore be attributed to the presence of the salts in this fraction. The absence of an additional inhibitory effect of the low-molecular-mass fraction of HS suggests that this fraction does not contribute significantly to inhibition of FSH receptor activation.

In conclusion, the CHO-F3B4 cells transfected with the human FSH receptor show good dose-response characteristics for serum-dependent inhibition of FSH receptor activation. Data from this study show that high concentrations of serum, both under normal culture conditions and in the presence of low concentrations of salts, exhibits only modest inhibitory effects on FSH-dependent cAMP production. The CHO-F3B4 cells can be used to assess whether FSH inhibitory activity is increased in serum or follicular fluid from patients with disregulation of FSH action, such as in polycystic ovary syndrome.

2.5 Low levels of FSH receptor activation inhibitors in serum and follicular fluid from normal controls and anovulatory patients with or without polycystic ovary syndrome

2.5.1 Introduction

A large proportion of women presenting with anovulation and infertility exhibit serum FSH concentrations within the normal range. The majority of these patients can be diagnosed as suffering from polycystic ovary syndrome (PCOS), on the basis of the sonographic appearance of the ovaries and endocrine serum parameters (Fauser *et al.*, 1991; Franks, 1995). A common finding in PCOS is normal early follicle development, whereas selection of the dominant follicle is absent (Pache *et al.*, 1992a; Fauser, 1994a). The underlying mechanism of follicle maturation arrest is unclear. The possibility of abnormal circulating FSH could be ruled out by demonstrating normal bioactivity, as assessed by an *in vitro* rat granulosa cell aromatase bioassay (Fauser *et al.*, 1991; van Dessel *et al.*, 1996c). Because normal follicular growth, selection and ovulation can be induced in some PCOS patients by administration of exogenous gonadotrophins (Schoot *et al.*, 1993), and granulosa cells of these patients show normal (Erickson *et al.*, 1979) or even elevated (Mason *et al.*, 1994a) FSH-induced E_2 production *in vitro*, it may be postulated that locally active factors, rather than defective granulosa cells are involved in arrested follicle growth in PCOS patients.

There are many indications that local regulation of FSH action may play a role in normal and disturbed follicular development (Hsueh *et al.*, 1984; Tonetta and DiZerega, 1989; Greenwald and Roy, 1994; Fauser, 1996). Growth factors, such as insulin-like growth factors (IGF) (Giudice, 1992) or activin (Miró and Hillier, 1992) act as potentiators of FSH action *in vitro*. Ongoing growth of the dominant follicle, despite a decrease in FSH serum levels in the late follicular phase (van Santbrink *et al.*, 1995b), may be attributed to the enhancement of FSH action by these growth factors, acting in a para- or autocrine fashion. On the other hand, growth factors may also exert inhibitory actions, as has been described for epidermal growth factor (Steinkampf *et al.*, 1988; Mason *et al.*, 1990). Although stimulatory or inhibitory effects of growth factors are mediated by their specific receptors and pathways, inhibition of FSH action may also be caused by specific FSH receptor inhibitors (Fauser, 1996). Several studies report the presence of specific FSH receptor binding inhibitors of unknown origin in human serum (Reichert Jr *et al.*, 1979; Sanzo and Reichert Jr, 1982) and follicular fluid (Fletcher *et al.*, 1982; Sluss *et al.*, 1983; Lee *et al.*, 1991; Lee *et al.*, 1993). Part of these inhibitors may be of immunological nature (van Weissenbruch *et al.*, 1991) and could lead to premature ovarian failure. It may be postulated that partial inhibition of FSH receptor activation causes arrested

follicular growth and absent dominant follicle selection in PCOS patients.

The aim of this study was to assess the level of inhibitors of FSH receptor activation in serum and follicular fluid of normogonadotrophic anovulatory patients with or without PCOS, as compared with normal controls. For this purpose a Chinese hamster ovary-cell line, transfected with the human FSH receptor (Schipper *et al.*, 1996) has been used. Inhibition of FSH-stimulated cAMP production has been studied at physiological concentrations of FSH and at a range of concentrations of serum or follicular fluid.

2.5.2 Materials and Methods

Patient serum samples

Serum samples were collected from 29 anovulatory patients, attending the Dijkzigt Hospital outpatient clinic for infertility evaluation. Inclusion criteria were; infertility, oligo- or amenorrhea and serum FSH levels within normal limits (1 - 10 IU/l) The involvement of human subjects in these investigations was approved by the Ethics Review Committee of the Dijkzigt Academic Hospital and Erasmus University Medical School, and informed consent was obtained from all subjects participating. Patient samples were divided into 2 subgroups, representing normogonadotrophic anovulation either without (n = 13) or with PCOS (n = 16). Patients were diagnosed as having PCOS on the basis of rigid criteria including elevated serum LH levels (> 8 IU/l) and elevated serum androgens [androstenedione (AD) > 15.0 nmol/l; and/or testosterone (T) > 3.0 nmol/l; and/or a Free Androgen Index (FAI: [T x 100]/Steroid Hormone Binding Globulin ratio) > 5] (Fauser *et al.*, 1991) and polycystic ovaries, as assessed by transvaginal sonography (Pache *et al.*, 1992b). Patients diagnosed as anovulatory (without PCOS) had normal LH and androgen concentrations and normal ovaries on ultrasound. In addition, FSH levels in all serum samples were

Table 2.5.1: Population and endocrine characteristics (mean ± SD) of study subjects from which serum was obtained

	Controls (n=8)	Normogonadotrophic anovulation (n=13)	PCOS (n=16)
Age (years)	25.5 ± 4.2	30.4 ± 6.4	26.9 ± 5.3
BMI (kg/m²)	23.2 ± 2.4	25.4 ± 5.1	26.0 ± 3.4
IRMA-FSH (IU/l)	5.4 ± 0.2	4.3 ± 1.2	4.8 ± 0.8
IRMA-LH (IU/l)	3.5 ± 1.3	4.0 ± 1.9	10.5 ± 1.2
Testosterone (nmol/l)	1.8 ± 0.5	1.3 ± 0.5	3.1 ± 1.2
Androstenedione (nmol/l)	10.5 ± 3.2	7.9 ± 2.8	23.9 ± 9.8
FAI	3.1 ± 1.0	2.6 ± 1.3	10.3 ± 5.7

BMI = body mass index, weight/ square length; IRMA-FSH = immunoreactive FSH; IRMA-LH = immunoreactive LH; FAI = Free Androgen Index, calculated as (Testosterone x 100)/Steroid Hormone Binding Globulin.

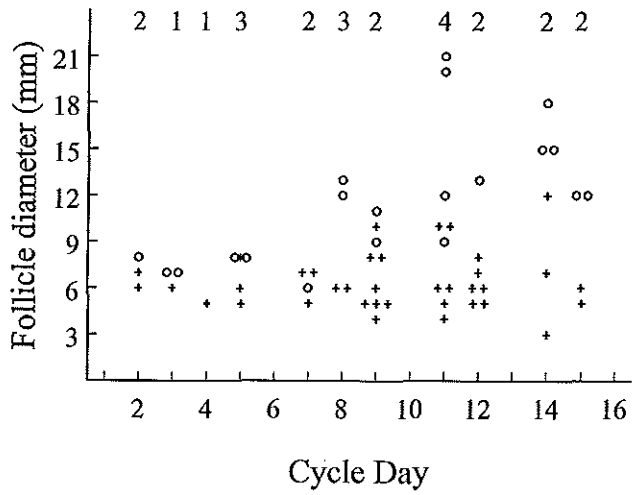

Figure 2.5.1
Follicle diameter and menstrual cycle day in 57 follicles, obtained from 25 normally cycling women. Healthy follicles (AD/E2 ratio < 4): À%; atretic follicles (AD/E2 ratio (4): +. The numbers on top indicate number of subjects from whom samples were obtained on a given cycle day.

measured by immunoradiometric assay (IRMA-FSH; Medgenix, Fleurus, Belgium). Serum samples selected for this study had IRMA-FSH levels between 3 and 6 IU/l in order to fulfill the FSH receptor activation assay requirements (see below).

Normal ovulatory women in the early follicular phase of the menstrual cycle (8 - 10 days before the LH surge) served as controls (n = 8). These volunteers were recruited by advertisement and paid for their participation (van Santbrink *et al.*, 1995b). Mean cycle length was 28 ± 2 [SD] days. Again, control samples exhibiting IRMA-FSH levels between 3 and 6 IU/l were chosen. See Table 2.5.1 for population and endocrine characteristics of all subjects studied.

Follicular fluid samples

Follicle fluid was obtained from 22 women, undergoing laparotomy for reversal of tubal sterilization or adhesiolysis and from 3 women undergoing laparoscopic tubal ligation. Informed consent was obtained from all subjects participating. All subjects were regularly cycling women with a mean cycle length of 27 ± 2 [SD] days and of normal weight (mean body mass index: 24 ± 2 [SD] kg/m^2). Mean age was 33 ± 4 [SD] years, and all patients had a history of proven fertility. The day of the menstrual cycleat the time of the follicle puncture was assessed from the day of onset of the last menstrual period. Follicle fluid was obtained from individual follicles (between 1 and 8 per patient; Figure 2.5.1) and assayed for E_2 and AD as described previously (van Dessel *et al.*, 1996b). Follicular fluid samples were classified into 5 different categories. The classification was on the

Table 2.5.2: Follicles (n = 83) obtained from 25 normally cycling subjects and 5 PCOS patients, divided into categories on the basis of cycle day, size and AD/E_2 ratio

	Category	Number	Cycle Day	Size (mm)	A/E_2 ratio
Normal cycle	**I** (EFP;<10 mm; atretic)	10	5 (2-7)	6 (5-8)	42 (4-238)
	II (EFP;<10 mm; healthy)	6	4 (2-7)	7 (5-8)	0.7 (0.3-2)
	III (LFP;<10 mm; atretic)	23	11 (8-15)	6 (3-8)	30 (4-256)
	IV (LFP;≥10 mm; atretic)	4	11 (9-14)	10 (9-12)	127 (62-173)
	V (LFP;≥10 mm; healthy)	14	11 (8-15)	13 (9-25)	0.2 (0.04-3.3)
PCOS	**A** (<10 mm; atretic)	7	-	5 (5-8)	9.5 (5.0-46.9)
	B (<10 mm; healthy)	4	-	7 (3-8)	2.4 (0.1-3.2)
	C (≥10 mm; atretic)	4	-	17 (16-18)	7.4 (4.3-12.8)
	D (≥10 mm; healthy)	10	-	16 (9-20)	0.1 (0.1-0.7)

EFP = early follicular phase; LFP = late follicular phase; AD/E_2 ratio < 4; atretic = AD/E_2 ratio ≥ 4. Values given are median and range.

basis of 3 criteria: 1) the menstrual cycle phase in which patients underwent surgery [early follicular phase (EFP): cycle day 1 - 8 or late follicular phase (LFP): cycle day 9 - 15]; 2) follicle size [non-dominant: < 10 mm or dominant: ≥ 10 mm] (van Santbrink *et al.*, 1995b; van Dessel *et al.*, 1996b); and 3) AD/E_2 ratio [healthy: < 4, or atretic: ≥ 4] (van Dessel *et al.*, 1996b; McNatty *et al.*, 1979b).

In addition, follicular fluid was obtained from 5 anovulatory, infertile patients diagnosed as PCOS (diagnostic criteria the same as for PCOS serum study). In 2 patients 2 - 3 follicles were punctured during laparoscopy. In 1 patient 6 follicles were punctured during elective surgery for fallopian tube correction. The remaining 2 patients were stimulated with human menopausal gonadotrophins, for infertility treatment. Due to multiple follicle development (more than 3 follicles larger than 16 mm), stimulation was cancelled. In these

patients follicular fluid samples, 5 and 9 respectively, were obtained through sonographically guided transvaginal punction. See Table 2.5.2 for the classification and endocrine characteristics of the follicular fluid samples used.

Cell cultures, hormones and reagents

Recombinant human FSH (recFSH; Org 32489) and the Chinese hamster ovary cell line, stably transfected with recombinant human FSH receptor cDNA (CHO) was generously provided by NV Organon (Oss, The Netherlands). Cell cultures and FSH receptor activation assays were performed as described in paragraph 2.2.2.

For the measurement of the inhibitory effect in the serum samples a fixed concentration of 6 mIU/ml FSH was used. To obtain this concentration of FSH in each dilution of the serum samples, endogenous FSH serum levels (range: 3 to 6 IU/l) were corrected by the addition of recFSH. Each serum sample was tested in duplicate in 2 independent FSH receptor activation assays. Results are expressed as the percentage of the cAMP production, relative to the cAMP response in serum-free conditions, which was set at 100% at the given stimulatory dose of recFSH (6 mIU/ml).

Because of small volumes available, the CHO assay was adapted, to allow analysis of follicular fluid samples. For this purpose, CHO cells were cultured in 96-multiwell dishes, at a cell density of 10^4 cells/cm^2 and incubated in a volume of 50 µl/well. The sensitivity for recFSH and the degree of stimulation of the cAMP production in this assay, applying small volumes, was similar to the assay performed in 48-wells (data not shown). The small volumes of individual follicular fluid samples allowed for testing at only 2 concentrations (10 and 30% vol/vol) in triplicate. The inhibitory effect of follicular fluid was assessed by stimulating CHO cells with 3 mIU/ml recFSH, instead of 6 mIU/ml. This FSH concentration was taken in order to approximate the endogenous FSH levels in the follicular fluid samples (McNatty, 1979a; Erickson *et al.*, 1992; Fauser, 1994a; Mason *et al.*, 1994a). Because volumes of the tested follicular fluid samples were small (in some cases only 30 µl), it was not possible to measure the FSH content.

In all experiments DNA content of the wells was measured by a fluorometric method as described previously (Downs and Wilfinger, 1983). The intra-assay coefficient of variation for DNA content/well was less than 12%. In all FSH receptor activation assays the cAMP levels were normalized on the basis of the DNA content of the wells.

Data analysis

Experimental data are presented as the mean ± SEM if they are normally distributed and as median and range if distributed otherwise. The inhibition of FSH-stimulated cAMP production is expressed as the percentage of the cAMP response in absence of serum or follicular fluid, which is set as 100%. Results

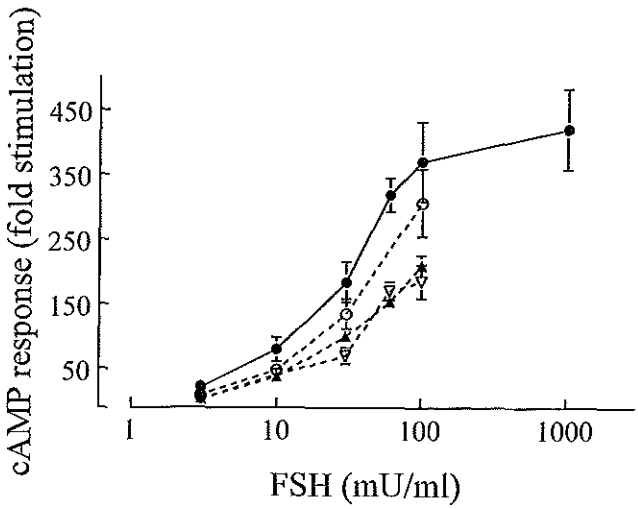

Figure 2.5.2
Effect of human serum on FSH-stimulated cAMP response in CHO cells. Cyclic AMP response, expressed as the stimulation factor times basal production (mean ± SEM of 3 assays in triplicate) in CHO cells, stimulated with increasing concentrations of recFSH in the presence of 0.1 mM IBMX and increasing concentrations of human hypogonadotrophic serum (● serum-free; ○ 10%; ▲ 30%; ▽ 90% vol/vol).

were evaluated using one-way ANOVA comparing responses as percentages of the serum-free response. Correlation of follicle characteristics with experimental results were analysed using Spearman's rank order test. *P* values given are two-sided, with 0.05 taken as the limit for statistical significance.

2.5.3 Results

Effect of hypogonadotrophic human serum

The dose-dependent stimulation of adenylate cyclase in CHO cells is shown in Figure 2.5.2. Basal levels of cAMP were 0.9 - 2.1 pmol/µg DNA and maximally stimulated levels were 350 to 420 times higher. The sensitivity, defined as the dose of recFSH eliciting a response of more than twice the standard deviation of the basal cAMP production, was 0.4 mIU/ml. The half maximal stimulation of cAMP production (ED_{50} value) was obtained at a dose of 24.9 mIU/ml FSH. The intra-assay coefficient of variation at different stimulatory concentrations of FSH was less than 16%. To study the effects of serum at different doses of recFSH, CHO cells were incubated in the presence of different concentrations of human hypogonadotrophic serum (10, 30 and 90% vol/vol), and stimulated with increasing doses of recFSH (3 - 100 mIU/ml). Basal cAMP production was not affected by the addition of hypogonadotrophic serum, while in the presence of 90% vol/vol hypogonadotrophic serum stimulated cAMP production decreased to 49 - 68% of responses under serum-free conditions (Figure 2.5.2).

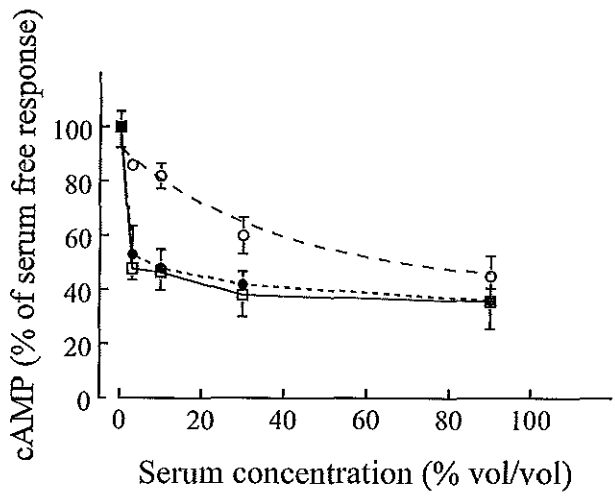

Figure 2.5.3
Inhibitory effect of human serum on FSH-stimulated cAMP response in CHO cells. Decreased cAMP production by CHO cells, stimulated with 30 mIU/ml (○); 6 mIU/ml (●) or 3 mIU/ml (□) of recFSH, incubated in the presence of increasing concentrations (3 - 90% vol/vol) of hypogonadotrophic human serum. Results (mean ± SEM of 3 experiments in triplicate) are expressed as the percentage of the response under serum-free conditions (100%).

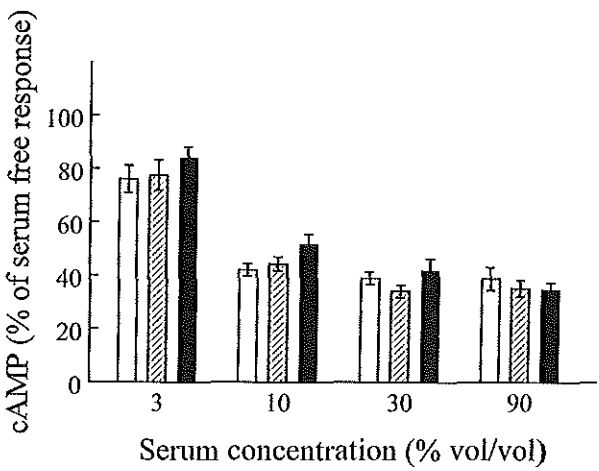

Figure 2.5.4
Inhibitory effect of serum from anovulatory patients on FSH-stimulated cAMP production. Cyclic AMP production expressed as percentage of the production under serum-free conditions (mean ± SEM of 2 experiments in duplicate) by CHO cells, stimulated with a fixed dose of FSH (6 mIU/ml) in the presence of increasing concentrations of serum (3 - 90% vol/vol) from regularly cycling women (n = 8; *open bars*); normogonadotrophic anovulatory patients without PCOS (n = 13; *hatched bars*) and PCOS patients (n = 16; *solid bars*).

When cAMP response at any given concentration of recFSH is expressed relative to cAMP production under serum-free conditions, addition of hypogonadotrophic serum at concentrations > 10% vol/vol inhibits cAMP production independent from the concentration of serum (Figure 2.5.3). At a high FSH concentration (30 mIU/ml) a competition between FSH and serum components is apparent over the whole serum concentration range, while at low FSH concentrations (3 or 6 mIU/ml) this competition occurs only at serum concentrations < 3% vol/vol. At serum concentrations > 10% vol/vol a saturation of the inhibitory effect is observed. Despite the slight inhibition by serum factors, CHO cells remain sensitive to stimulation by FSH. Even in the presence of 90% vol/vol of hypogonadotrophic serum, addition of 3 mIU/ml recFSH results in a 5- to 8-fold stimulation of the cAMP production (data not shown).

Effects of serum from normal controls, normogonadotrophic anovulatory patients and PCOS patients

Serum samples obtained from normally cycling individuals or anovulatory patients, with or without PCOS, inhibited cAMP production up to 39 ± 4%, 35 ± 2% and 34 ± 3% of the response under serum-free conditions, respectively when tested at 90% vol/vol. Within each group a significant further increase in inhibition was absent ($P > 0.38$) when serum concentrations were increased from 10 to 90% vol/vol. Furthermore, no statistically significant differences were found between both patient groups and normal controls ($P > 0.08$), with regard to the inhibition of FSH-stimulated cAMP production at each concentration of serum (Figure 2.5.4).

Effect of follicular fluid from normal controls and PCOS patients

Adapted bioassay conditions were used to measure the effects of small amounts of follicular fluid (see Materials and Methods section). The intra-assay coefficient of variation of the adapted assay was greater (< 2%), compared to the "48-well" assay (<16%). FSH-stimulated cAMP production was clearly inhibited in the presence of 10 or 30% vol/vol of follicular fluid, although in several samples a concentration dependent decrease in cAMP was not observed. The mean relative cAMP production was lower at a concentration of 30% vol/vol, as compared with 10% vol/vol (55 ± 2% and 68 ± 2%, respectively; $P < 0.001$). No differences in inhibition of the cAMP response were found when comparing various classes of follicular fluid samples from normally developing follicles (Figure 2.5.5, *upper panel*). The inhibitory effect of follicular fluid did not correlate with follicle size, with the AD/E_2 ratio of the follicular fluid, nor with the menstrual cycle phase in which the samples were obtained (data not shown). Similar results were obtained with follicular fluid from PCOS patients, as compared with follicular fluid from regularly cycling women (Figure 2.5.5, *lower panel*).

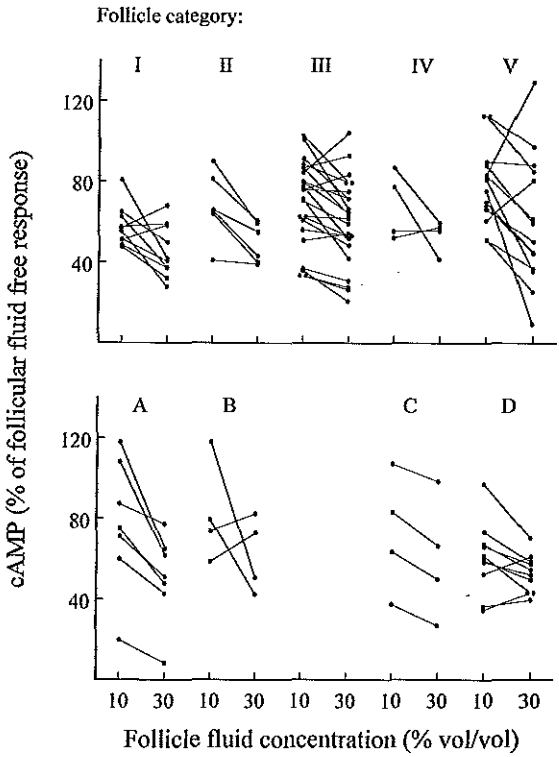

Figure 2.5.5
Inhibitory effect of follicular fluid on FSH-stimulated cAMP production. Cyclic AMP response in CHO cells stimulated with 3 mU/ml recFSH in the presence of follicular fluid from regularly cycling women (*upper panel*) and PCOS patients (*lower panel*). The response is expressed as a percentage of the response in the absence of follicular fluid. *Dots* indicate responses of individual samples (tested in duplicate), with a *solid line* connecting the responses of individual samples at 10 and 30% vol/vol follicular fluid concentration. Follicle category in *upper panel:* I = early follicular phase (EFP), < 10 mm, atretic; II = EFP, < 10 mm, healthy; III = late follicular phase (LFP), < 10 mm; IV = LFP, ≥ 10 mm, atretic and V = LFP, healthy. Follicle category in *lower panel:* A < 10 mm, atretic; B: < 10 mm, healthy ; C: ≥ 10 mm, atretic and D ≥ 10 mm healthy. See also Table 2.5.2.

2.5.4 Discussion

Inhibition of FSH action at the level of granulosa cells in the ovaries may underlie anovulation in women presenting with normal circulating FSH concentrations. The aim of this study was to compare levels of FSH receptor inhibitory activity in serum and follicular fluid from regularly cycling women and patients with normogonadotrophic anovulation. The inhibitory activity was assessed by measuring the FSH-induced adenylate cyclase activity in cultured CHO cells, stably transfected with the human FSH receptor (Schipper *et al.*, 1996). A similar system has been applied recently to assess bioactive FSH

concentrations in serum throughout the menstrual cycle (Christin-Maitre *et al.*, 1996). In the present study, CHO cells were stimulated with physiological concentrations of FSH in the presence of untreated serum or follicular fluid. A wide range of concentrations of serum or follicular fluid were tested, from 3 up to 90% vol/vol. High concentrations were applied to approach protein concentrations that occur under physiological conditions.

Primary cultures of rat granulosa (Jia and Hsueh, 1986a) or Sertoli cells (Padmanabhan *et al.*, 1987) have been used as *in vitro* bioassays for FSH. These bioassays are hampered by a strong inhibition of the FSH response, caused by unspecified factors in serum. An inhibition of more than 80% of the induced E_2 response can be observed in the presence of only 4% serum (Jia and Hsueh, 1986a; Schipper *et al.*, 1996). In contrast, currently used CHO cells show only a limited inhibition of FSH-induced cAMP production by serum. Even in the presence of 90% vol/vol human serum the cAMP response is inhibited up to 60% only. Although this inhibition might be caused by metabolic changes in the cells, this seems less likely because prolonged incubation of the cells in serum affected neither cell viability nor basal cAMP production (Schipper *et al.*, 1996). In addition, the cells can be stimulated to produce large amounts of cAMP despite preincubation in 90% serum (Schipper *et al.*, 1996). It is of interest that in the presence of serum concentrations > 10% vol/vol, the percentage inhibition of FSH-dependent receptor activation (at a fixed FSH concentration) is independent of the concentration of serum. This fixed magnitude of inhibition over a wide range of serum concentrations (10 to 90% vol/vol) suggests a saturating effect of the inhibitory components in serum. On the other hand, the CHO cells still respond to elevation of the FSH levels, irrespective of serum concentrations. This suggests that a competition exists between inhibiting factors and FSH for the FSH receptor. Although the mechanism of inhibition is unclear, this may be attributed to a non-specific protein matrix effect since bovine and equine serum show similar inhibition curves (Schipper *et al.*, 1996). Comparing sera from regularly cycling women with normogonadotrophic anovulatory patients (with or without PCOS) no differences in inhibitory activity were observed. This argues against the presence of increased levels of inhibitors of FSH receptor activation in serum from these patients.

Because factors inhibiting FSH receptor activation may be present exclusively in the follicular compartment, follicular fluid obtained from individual follicles throughout the follicular phase of the menstrual cycle and from PCOS patients were also tested. Follicular fluid samples were classified according to the stage of the menstrual cycle in which they were obtained, as well as by size and steroid content (expressed as the AD/E_2 ratio). Different classes of normally developing follicles did not reveal differences with regard to inhibition of FSH receptor activation. Neither did follicular fluid from PCOS patients, compared with matched control follicles, show any difference in inhibition of FSH receptor

activation. Results obtained with follicular fluid displayed a larger degree of variation, compared with results obtained from the addition of serum samples. Part of this variation may be caused by the higher intra-assay coefficient of variation of the assay applying small volumes. Another explanation may be found in differences in the endogenous FSH levels of the individual follicular fluid samples. In the present study, FSH content in follicular fluid could not be assessed due to the small volumes available. It was therefore not possible to normalize for potential differences in FSH levels. In an attempt to limit the variation in FSH levels in the follicular fluid assays, a small amount of FSH (3 mIU/ml) was added to each follicular fluid dilution. The choice for this FSH concentration was on the basis of data from the literature (Erickson *et al.*, 1992; Fauser, 1994a; Mason *et al.*, 1994a) that report levels of intrafollicular immunoreactive FSH ranging between 0.3 and 6 IU/l. This uncertainty in the final FSH concentration could explain the reversal of inhibition observed in some samples at increased concentration. However, despite these uncertainties the observed responses did not correlate with any follicle characteristic, such as size or steroid content, or with menstrual cycle phase or PCOS diagnosis. Although inhibitory effects of subfractions in serum or follicular fluid have not been tested separately, the total net effect of potentially different combinations is similar in both controls and anovulatory patients. Therefore it seems unlikely that FSH receptor inhibition plays a significant role in the (patho)physiology of normal or disturbed follicle development.

Specific FSH receptor binding inhibitors have been described in partly purified fractions of human serum and follicular fluid (Sanzo and Reichert Jr, 1982; Lee *et al.*, 1990; Lee *et al.*, 1993). However, not all FSH binding-inhibitory activity results in inhibition of FSH, since part of these binding-inhibitors have FSH agonist activity (Lee *et al.*, 1991). Results of the present study show that the biological activity of FSH in the context of serum proteins is approximately 50% of the activity in the absence of serum. The mechanism underlying this reduction in bioactivity is not known, but it may be postulated that FSH is partly bound to plasma proteins with low affinity, thereby reducing the amount available for receptor activation. On the other hand serum proteins may also bind reversibly to the receptor, thereby reducing the number of receptors available for interaction with FSH. One other aspect to be considered is that, in the present study, only adenylate cyclase activity has been taken as the endpoint of FSH receptor activation. It is known that FSH receptor activation results in formation of intracellular messengers other than cAMP, like calcium ions (Leung and Steele, 1992; Sharma *et al.*, 1994). Although it is not yet clear whether this Ca^{2+} pathway functions independently from cAMP (Gorczynska *et al.*, 1994; Sharma *et al.*, 1994), it could be postulated that specific FSH receptor inhibitors have a preference for one pathway leaving the other less inhibited, which could thus result in expression of different cellular functions. Possible

inhibition of different pathways is illustrated by findings that immunoglobulins in serum from women with premature ovarian failure block FSH-induced DNA synthesis in granulosa cells *in vitro* (van Weissenbruch *et al.*, 1991), while they do not affect FSH-induced steroidogenesis (Anasti *et al.*, 1995).

Our conclusion from these *in vitro* studies that FSH receptor inhibition is a constant and therefore may be of only limited significance for regulation of follicle development is being supported by recent findings from clinical studies. Growth and selection of the dominant follicle is induced by increasing levels of FSH during the luteo-follicular transition in the menstrual cycle. Although normogonadotrophic anovulatory patients display bioactive and immunoreactive FSH levels comparable to follicular phase levels during the normal menstrual cycle (Fauser *et al.*, 1991; van Dessel *et al.*, 1996c), these patients lack the intercycle rise. Normal mono-follicular development and ovulation can be induced in normogonadotrophic anovulatory patients by the administration of exogenous FSH, applying a decremental-dose regimen (Schoot *et al.*, 1993; van Santbrink *et al.*, 1995a). Although the administration of gonadotrophins in these patients suggests an increased "FSH treshold" level that needs to be surpassed to ensure follicle development, it appears that normal follicle development can be achieved in normogonadotrophic anovulatory patients without increasing FSH to supra physiological concentrations (van Dessel *et al.*, 1996c). These findings dispute the presence of an increased "FSH treshold" level in normogonadotrophic anovulatory patients, which could be caused by locally acting factors blocking FSH action. It could well be that the absence of the transient increase in FSH levels in anovulatory patients is an important factor in the etiology of arrest of follicle development. It may be of interest that in particular bioactive FSH levels increase during the luteo-follicular transition, as has been demonstrated recently (Christin-Maitre *et al.*, 1996). Although the bioactive-to-immunoactive FSH ratio remains constant during the early follicular phase in regular cycling women (van Dessel *et al.*, 1996c; Christin-Maitre *et al.*, 1996) and is not different in anovulatory patients (van Dessel *et al.*, 1996c), the change in FSH levels and the bioactive-to-immunoreactive ratio in the late luteal phase may be of significance for follicle recruitment and selection. Furthermore, under normal conditions ongoing growth of the dominant follicle occurs despite decreasing levels of FSH in the late follicular phase (van Santbrink *et al.*, 1995b). This suggests an enhancement of FSH action, allowing the dominant follicle to continue its development, whereas FSH levels drop below the threshold for the remaining less mature follicles. This enhancement of FSH action may be the result of locally active growth factors such as IGF-I and -II and the IGF-binding proteins (Giudice, 1992). These factors act through their own specific receptors and therefore exert their effect through intracellular pathways and not directly through the FSH receptor. The potential significance of IGF-II as an intra-ovarian factor involved

in the development of the dominant follicle in the human has been stressed recently (van Dessel *et al.*, 1996a).

In conclusion, analysis of inhibition of FSH receptor activation by serum or follicular fluid samples from both regularly cycling women and PCOS patients indicate that increased levels of FSH receptor inhibitors do not play a significant role in the regulation of normal and abnormal follicle development. This is in concert with findings that normogonadotrophic anovulatory patients do not exhibit an increased FSH treshold level *per sé*. Both the dynamics in FSH levels in the early follicular phase and locally active growth factors, acting through their own receptors may be of greater significance for follicle growth and selection.

2.6 The action of inhibitors of FSH receptor activation is buffered in serum.

2.6.1 Introduction

Follicle-stimulating hormone (FSH) stimulates granulosa cell proliferation and differentiation in the ovary, and its activity is subject to modulation by various locally acting factors, acting through their own receptors and pathways (Greenwald and Roy, 1994; Erickson, 1996; Giudice *et al.*, 1996; Fauser and Heusden, 1997). Specific inhibitors of FSH receptor binding have been demonstrated in serum and follicular fluid of several species (Sluss *et al.*, 1989; Lee *et al.*, 1991; Lee *et al.*, 1993). It has also been shown that deglycosylated gonadotrophins (Bishop *et al.*, 1994), specific immunoglobulins (Dattatreyamurty *et al.*, 1990), synthetic peptides (Lee *et al.*, 1995) and several organic compounds (Wilks *et al.*, 1986; Heindel and Chapin, 1989; Grasso *et al.*, 1993; Treinen *et al.*, 1993) can inhibit FSH receptor activation under *in vitro* conditions (for review see: Fauser, 1996). Moreover, it is well known that serum at concentrations of no more than a few percent causes non-specific inhibition of FSH bioactivity in most bioassays (Jia and Hsueh, 1986b; Padmanabhan *et al.*, 1987; Gudermann *et al.*, 1994). As a result, it seems cumbersome to measure the FSH bioactivity in the presence of high concentrations of serum. These observations have substantiated the notion that inhibitors of FSH action may be important for the modulation of FSH action at the level of the target cell in the ovary. However, although there is abundant information on effects of specific FSH inhibitors *in vitro*, it is not clear whether such inhibitors play a role in ovarian physiology or pathophysiology.

In a recent study regarding inhibition of FSH receptor activation using Chinese hamster ovary (CHO) cells transfected with the human FSH receptor, we demonstrated that high serum protein concentrations (up to 90 % v/v)

inhibited receptor activation maximally for not more than 50 % (Schipper *et al.*, 1996). This inhibition was concentration independent when the amount of serum in the incubation media was above 30 % (v/v). Moreover, we were unable to demonstrate any increased inhibitory activity in serum samples from patients with anovulation and normal immunoreactive serum FSH concentrations (Schipper *et al.*, 1997). These results indicate that serum constituents may act as a buffer against inhibitors of FSH receptor activation. Since ovarian granulosa cells are probably exposed to high levels of serum constituents *in vivo*, inhibition of FSH receptor activation *in vivo* might also be subjected to the buffering activity of serum proteins. This would argue against an important role for inhibitors of FSH action in serum or follicular fluid.

We intended to assess the effects of high concentrations of serum proteins on specific inhibitors of FSH receptor activation *in vitro*. In spite of various efforts we have not been successful in obtaining well characterised serum fractions or modified hormones such as deglycosylated FSH, which have been demonstrated to exert inhibition of FSH activity *in vitro*. Unpublished observations indicated that protamine, a cationic-rich polypeptide with proven inhibition of the activity of G protein-coupled receptors (Bang Olsen *et al.*, 1988), would inhibit FSH receptor activation (R. Meloen, personal communication). We have therefore decided to examine the effects of serum proteins on the activity of a FSH receptor inhibitor, using protamine as a receptor inhibitor.

2.6.2 Materials and Methods

Hormones and reagents

Recombinant human FSH (Org. 32489, bioactivity 8413 IU/mg as assessed by *in vivo* bioassay, relative to reference preparation IS 70/45; immunoactivity 12000 IU/mg) was a generous gift from NV Organon (Oss, The Netherlands). Protamine, isobutyl-methyl-xanthine (IBMX), forskolin, human serum albumin (HSA) and media additives were purchased from Sigma Chemicals (Bornhem, Belgium). A pool of hypogonadotrophic serum was obtained from high-dosed (50 µg ethinyl-oestradiol daily) combined oral contraceptive pill users. The FSH level of the serum pool was < 0.5 IU/l, as assessed by immunoradiometric assay (Medgenix, Fleurus, Belgium).

Cell cultures

A CHO cell line, stably transfected with the human FSH receptor, was provided generously by NV Organon, Oss, The Netherlands. CHO cells were cultured as described previously (Schipper *et al.* 1996). In brief, cells were plated on 48-well culture dishes (10^4 cells/cm^2) in Dulbecco's Modified Eagle Medium/Ham-F12 (DMEM/F12, 1:1) (GIBCO Europe BV, Breda, The Netherlands) with the addition of 100 U/ml penicillin and 100 µg/ml streptomycin and 10% v/v fetal

calf serum (FCS) (Sebak GmbH, Adenbach, Germany). After 2 days in culture the CHO cells reached semiconfluence and were used for the FSH receptor activation assay.

FSH receptor activation assay

Validation of this assay has been described previously (Schipper *et al.* 1996). In brief, cultured CHO cells were incubated in 48-well culture dishes. The culture medium was replaced by FCS-free DMEM/F12 containing 0.1 mM IBMX and 0.1 % (w/v) bovine serum albumin. For the measurement of inhibition of adenylate cyclase activity the cells were incubated at a fixed concentration of 30 mU/ml FSH (corresponding to the ED_{50} for FSH-stimulated cAMP production) (Schipper *et al.*, 1996) in a final incubation volume of 100 µl. Substances to be tested were added individually or in combinations to the culture media. Human serum was added in concentrations of 3 - 90% (v/v) and HSA in concentrations of 7, 40 or 70 mg/ml. Protamine concentrations ranged from 2 to 100 µg/ml. After 4 hours of incubation in air with 5% CO_2, the medium was removed and stored at -20° C until analysis for cAMP. Specificity of the inhibition was tested by stimulation of adenylate cyclase with forskolin (10 µM) or FSH (30 mU/ml), either directly or after 4 h preincubation with 10

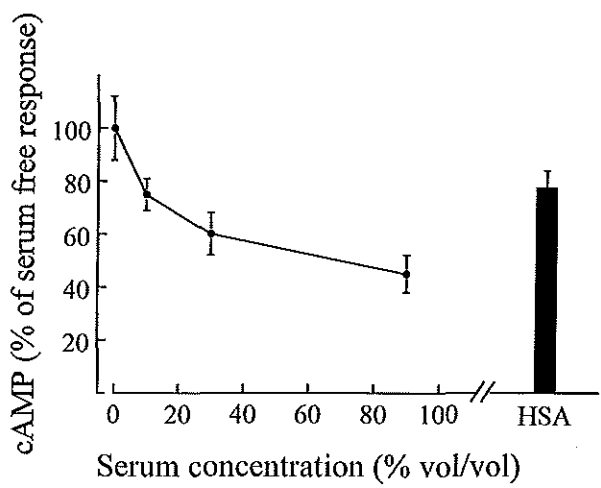

Figure 2.6.1
Inhibition of FSH receptor activation by serum proteins. CHO cells were incubated for 4 h with 30 mU/ml FSH in the presence of 0.1 mM IBMX and various concentrations of hypogonadotrophic human serum (*line*) or human serum albumin (*bar*). Results (mean ± SEM of 3 independent assays, each in triplicate) are expressed as a percentage of the cAMP production in the absence of serum, which was taken as 100%. Addition of serum (10 - 90% v/v), as well as HSA (70 mg/ml) resulted in a significantly lower cAMP production compared to serum-free conditions (ANOVA, $P < 0.05$). Cyclic AMP production in presence of 10% v/v serum was not different from HSA ($P = 0.88$), with significantly lower cAMP production at higher serum concentrations.

µg/ml protamine and media change. In all experiments DNA content of the wells was measured by a fluorometric method as described previously and the degree of FSH receptor activation were normalized on the basis of the amount of DNA (Schipper *et al.* 1996). In a distinct set of experiments the viability of the cells was assessed by measuring the index of cellular membrane damage as represented by the percentage of propidium-iodide (0.5 µg/ml final concentration) stained cells.

Radioimmunoassays
Cyclic AMP in the media was assayed as described previously (Schipper *et al.* 1996). In brief, after acetylation, the samples were incubated overnight with cAMP antibody (purchased from Dr J. Stoof, Free University, Amsterdam, The Netherlands). The assay was validated for the use of culture media and corrected for addition of serum in the samples. All samples were assayed in duplicate. Sensitivity of the assay was 0.125 pmol/ml. Inter-and intra-assay coefficients of variation were 20% and 8%, respectively.

Data analysis
In all experiments, cAMP production is expressed as pmol/µg DNA. Dose-response curves are expressed as a percentage of the maximal stimulation, which was taken as 100 %. The inhibitory effect of added compounds is expressed as a percentage of the cAMP response under basal conditions, which was taken as 100%. Mean values ± standard error (SEM) of 3 independent assays are given. For each individual point within one bioassay, triplicate incubations were used. Results were analysed using two-way ANOVA and Student's t-test. Differences with P values < 0.05 were considered statistically significant.

2.6.3 Results

Inhibition of stimulated cAMP production by human serum proteins
As described previously (Schipper *et al.*, 1996), the cells remained viable and sensitive to stimulation with FSH in the presence of high concentrations of serum. Non-specific toxic effects of serum components on adenylate cyclase activity could be excluded by incubation with forskolin and choleratoxin. Increasing concentrations of hypogonadotrophic serum inhibited the FSH-stimulated cAMP production in CHO cells, incubated with a fixed stimulating dose of FSH of 30 mU/ml (ED_{50} value). Basal cAMP production in the absence of serum varied in the different experiments between 0.26 and 1.1 pmol/well, with a 85 to 232-fold stimulation factor at 30 mU/ml FSH. At concentrations between 30 and 90 % v/v hypogonadotrophic serum (20 - 60 mg protein/ml) a maximal inhibitory effect was obtained, decreasing the cAMP production to 41 ± 5% of the production in the absence of serum (Figure 2.6.1). As demonstrated

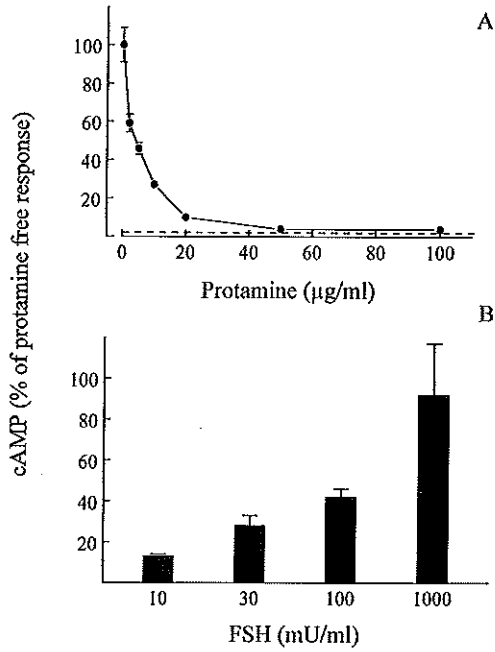

Figure 2.6.2
A: CHO cells were stimulated with FSH (30 mU/ml) in the presence of 0.1 mM IBMX and increasing concentrations of protamine. Results (mean ± SEM of 3 independent assays, each in triplicate) are expressed as a percentage of the cAMP production in the absence of protamine, which was taken as 100% (stimulation factor: 117 ± 17 times basal cAMP production). The *dotted line* indicates basal cAMP production in the absence of FSH.
B: Inhibition of cAMP production in CHO cells stimulated with 10, 30, 100 or 1000 mU/ml FSH in the presence of 0.1 mM IBMX and 10 µg/ml protamine.

previously (Schipper *et al.*, 1996), this inhibition appears to be specific at the receptor level and is not caused by a decrease in cell viability. In order to examine the possibility of non-specific effects of high concentrations of proteins, the addition of human serum albumin (HSA) to the incubation media was tested. Only at a concentration of 70 mg/ml HSA (equal to the total amount of proteins in serum) there was a slight inhibition of cAMP production to 79 ± 7% of the original activity (P = 0.03), whereas at lower concentrations of HSA (7 and 40 mg/ml) inhibitory effects were absent (data not shown).

Inhibition of FSH-activated adenylate cyclase by protamine
Increasing concentrations of protamine (0 - 100 µg/ml) inhibited the FSH (30 mU/ml) stimulated cAMP production to almost basal activity (Figure 2.6.2a). Although the viability of CHO cells decreased substantially (> 50 % non-vital cells) when high levels of protamine (50 - 100 µg/ml) were used, incubation of the cells for 24 h at a protamine concentration of 10 µg/ml decreased cell

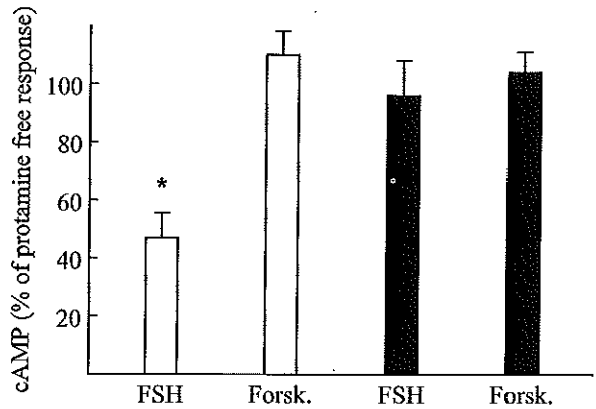

Figure 2.6.3
Specificity of FSH receptor inhibition. CHO cells were stimulated with FSH (30 mU/ml) or Forskolin (10 μM) for 4 h in the presence 10 μg/ml protamine (*open bars*). Responses are expressed as the percentage (mean ± SEM of 3 independent assays) of cAMP production after 4 hours incubation in the absence of protamine, which was taken 100%. In addition, CHO cells were preincubated for 4 h with protamine and subsequently stimulated for 4 h after media change (*solid bars*). *Asterisk* indicates significantly lower cAMP production ($P < 0.05$) compared to stimulation in the absence of protamine.

viability with only 15 % (mean of 3 estimations), compared to protamine free conditions. During an incubation period of 4 h there were no indications for decreased cell viability. Therefore, a concentration of 10 μg/ml protamine was used for further experiments. At a concentration of 10 μg/ml protamine the FSH-stimulated cAMP production diminished to 28 ± 5 % of the activity in the absence of protamine. At higher concentrations of FSH (100 and 1000 mU/ml) the inhibition of cAMP production at the same concentration of protamine (10 μg/ml) was decreased (42 ± 4 % and 93 ± 25 % of the cAMP production in the absence of protamine respectively; Figure 2.6.2b).

The specificity of the inhibition of cAMP production by protamine was tested by comparing the effect of protamine on cAMP production stimulated with FSH (30 mU/ml) and forskolin (10 μM). The FSH-activated adenylate cyclase was inhibited to 47 ± 9 % by protamine, whereas cells stimulated with forskolin were not inhibited (activity 110 ± 9 % of controls; Figure 2.6.3). The inhibitory effects of protamine were reversible since after preincubation of the cells for 4 h with 10 μg/ml protamine and subsequent removal of the protamine prior to stimulation with the FSH and forskolin, the stimulated cAMP production was completely restored (96 ± 9 % and 103 ± 8 % of control values respectively). Preincubation of cells with 10 μg/ml protamine for 24 h revealed similar results for subsequent stimulation of cAMP production with FSH or forskolin (data not shown).

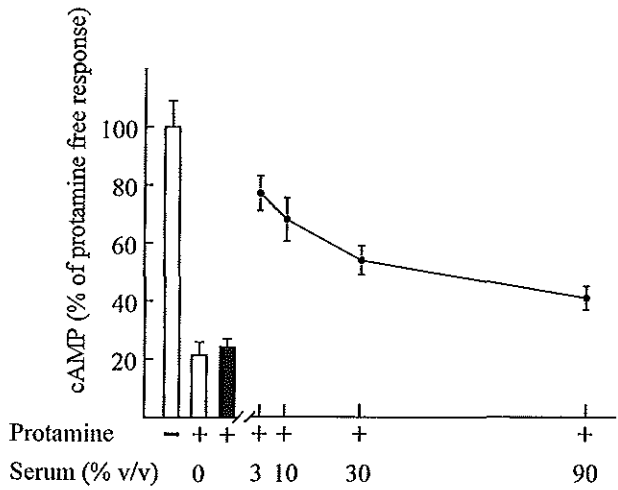

Figure 2.6.4
Effect of serum proteins on the inhibitory effects of protamine. CHO cells were incubated for 4 h with 30 mU/ml FSH without (-) or with (+) protamine (10 µg/ml) in the absence of hypogonadotropic human serum (*open bars*), with human serum albumin (70 mg/ml, *solid bar*) or in the presence of hypogonadotropic human serum (3 - 90% v/v; *line*). Results (mean ± SEM of 3 independent assays, each in triplicate) are expressed as a percentage of the cAMP production in the absence of serum and protamine, which was taken as 100%.

Effect of serum proteins on the inhibitory effect of protamine

The strong inhibitory effect of 10 µg/ml protamine was completely negated by the addition of hypogonadotrophic serum (in concentrations ranging from 3 to 90 % v/v; Figure 2.6.4). A comparison of the results as presented in Figures 2.6.1 and 2.6.4 revealed that there were no differences between inhibition by hypogonadotrophic serum alone or hypogonadotrophic serum in combination with protamine (P = 0.78). Although the diminished inhibitory effect of protamine in the presence of serum could be due to non-specific binding of protamine to proteins, addition of HSA to the incubation media in a concentration of 70 mg/ml (equal to the total protein concentration in 100 % serum) did not show a diminished inhibitory effect of protamine (Figure 2.6.4).

2.6.4 Discussion

Results from the present study demonstrate that protamine has an inhibitory activity of FSH receptor activation. The addition of 10 µg/ml of protamine to cultured CHO cells inhibited the FSH-stimulated cAMP production by approximately 70 %. This inhibition by protamine is presumably through a disturbance of FSH receptor activation since forskolin-stimulated adenylate

cyclase activity was not affected. Protamine inhibition could be reversed by addition of increasing concentrations of FSH, suggesting a competition between protamine and FSH for the receptor. Following the addition of human serum, at concentrations ranging between 3 and 90 % v/v of the incubation media, the inhibitory effect of protamine was indistinguishable from the inhibition by serum alone.

Results from our previous studies (Schipper *et al.*, 1996; Schipper *et al.*, 1997) on inhibitory activity of serum on FSH receptor activation have shown that at serum concentrations above 20 % (v/v) the degree of FSH receptor activation becomes independent from the serum concentration and stabilizes at a level of level of approximately 50 % of the cAMP production under serum-free conditions. Despite an increase in serum concentration up to 90 % v/v, no further inhibitory effect is seen. The nature of this stabilisation at 50 % inhibition is unknown; neither do we know how the different serum constituents contribute to this effect. Whereas concentrations of serum ranging from 5 - 20 % v/v, are required for normal *in vitro* cell growth, high serum protein concentrations approximate the physiological environment of (granulosa) cells more closely. We have been able to demonstrate that the inhibitory effects of serum are not caused by non-specific toxic effects , since cells remained highly responsive to FSH in the presence of high concentrations of serum and only a 50 % reduction of the 100 to 230-fold stimulation factor was observed. Moreover, the forskolin and choleratoxin dependent activation of adenylate cyclase was not affected by serum (Schipper *et al.*, 1996). Since cAMP production was constant over a period of 6 h, there were no indications of alterations in the FSH receptor content. Similar effects were observed with horse- and FCS samples (Schipper *et al.*, 1996). Application of this assay to examine follicular fluid samples from controls and patients with polycystic ovary syndrome gave similar results as in serum samples and did not reveal differences in inhibitory activity related to follicular development or disease state (Schipper *et al.*, 1997). Altogether, our previous data indicate that yet unknown constituents in serum and follicular fluid can buffer the action of also unknown compounds which inhibit FSH receptor activation. With protamine as a defined inhibitor (although not physiological) of FSH receptor activation, we could prove this buffering action of serum. It was not possible to carry out the experiments with hypogonadotrophic follicular fluid.

The mode of action of protamine as inhibitor of FSH receptor activation is not known, nor can we provide satisfactory explanation for the annihilation of the inhibitory effect of protamine by serum constituents. It seems that protamine is not bound non-specifically to negatively charged plasma proteins, since high concentrations of human serum albumin, which is acidic and negatively charged at physiologic pH, do not diminish the inhibitory effect of protamine. The quench of the inhibitory effect by protamine must therefore be

mediated by interaction with serum constituents other than albumin. This neutralising capacity of serum seems to be high, since already at a low dose of serum (3 % v/v) the inhibitory effects by protamine appear to be absent.

The inhibitory effect of protamine itself can be the result of binding to FSH, thereby preventing the hormone to interact with the FSH receptor. On the other hand, protamine might interact with the FSH receptor itself and thus interfere with receptor activation. Both the receptor and FSH itself contain negatively charged regions (Reichert Jr, 1994), which may interact with protamine. Although binding of protamine to FSH may in theory explain the present observations, direct interactions between protamine and G protein-coupled receptors have been demonstrated also (Bang Olsen *et al.*, 1988; Siciliano *et al.*, 1994). Furthermore, another positively charged, arginine-rich protein, histone H2A, has been described as a potent inhibitor of FSH-stimulated cAMP and P production in rat granulosa cells (Aten and Behrman, 1989).

Whatever the explanation for the mechanism of protamine inhibition of FSH receptor activation is, the results of the present study show that serum constituents can counteract the inhibiting effects of protamine. If a similar buffering activity is operative against naturally occurring (positively charged) inhibitors, it almost precludes that variations in the concentration of such inhibitors play an important role in the physiology. Since this conclusion about the relative role of naturally occurring inhibitors of FSH receptor activation contrasts with the current notion on FSH binding inhibitors and inhibitors of FSH bioactivity, these latter two issues will be discussed separately.

Firstly, in most reports regarding inhibitors of FSH receptor binding, purified fractions from serum or follicular fluid have been used (Sanzo and Reichert Jr, 1982; Lee *et al.*, 1990; Lee *et al.*, 1993). FSH receptor binding inhibitors (0.5 - 5 kDa molecular mass) have been isolated and purified from human follicular fluid. When examined in radio-receptor assays 30 µg/ml of such partly purified fraction was required for a 50% inhibition of FSH receptor binding (Lee *et al.*, 1993). Since this fraction was purified more than 100-fold, these inhibitors could theoretically inhibit FSH binding for only 0.5 %, if present at a normal, physiological concentration. Moreover, binding properties of the receptor for FSH or binding inhibitors may not always be related to the functional properties of the receptor under physiological conditions because studies regarding FSH receptor binding employ low ionic conditions while receptor activation is always measured at normal ionic strength. It has been reported that the affinity of FSH for the receptor when measured at low ionic strength decreases more than 20-fold when the salt concentration is restored to normal physiological levels (van Loenen *et al.*, 1994a). Also for other glycoprotein hormones conditions for measuring ligand binding and receptor stimulation are not similar and many discrepancies have been observed (Combarnous *et al.*, 1986; van Loenen *et al.*, 1994b).

Secondly, for the measurement of FSH bioactivity, primary cultures of rat granulosa or Sertoli cells have been used frequently (Jia and Hsueh, 1986b; Padmanabhan *et al.*, 1987); and it has been found that the FSH-stimulated E_2 production is almost completely inhibited when these cells are incubated in the presence of only 5 - 10 % (v/v) serum (Jia *et al.*, 1986a; Schipper *et al.*, 1996). This could indicate non-specific toxic effects of serum. However, as has been shown in our previous study, if cAMP production was used as a direct index of FSH receptor activation in rat Sertoli cells, the inhibitory effects are much less (Schipper *et al.*, 1996). Part of the inhibition of E_2 production is probably due to (auto- and paracrine) effects of growth factors or steroids influencing the induction of aromatase via their own specific receptors and not via the FSH receptor (Harlow *et al.*, 1988; Ui *et al.*, 1989; Bicsak *et al.*, 1990; Agarwal *et al.*, 1996). Application of cells transfected with the human FSH receptor and the use of specific direct indices of FSH receptor activation (cAMP or cAMP responsive gene products such as luciferase) (Albanese *et al.*, 1994; Gudermann *et al.*, 1994; Christin-Maitre *et al.*, 1996) show that results from previously used, less specific, bioassays may have overemphasised the importance of inhibitors of FSH receptor activation. It may be the components in serum such as growth factors, that play a decisive role in the expression of FSH-induced activity, such as aromatase induction. These effects would be at an intracellular level more distant from receptor activation. A detailed discussion regarding growth factor modulation of the various expressions of FSH action is, however, beyond the scope of this paper.

The major conclusion from the present study is that the postulated buffering capacity of serum against naturally occurring, but ill defined, inhibitors in serum is supported by our observations with protamine as a chemically defined inhibitory compound for FSH receptor activation. The results indicate that the bioactivity of FSH, as measured in this CHO bioassay, depends not only on the concentration of FSH in serum but also on the inhibitory and buffering actions of serum compounds. A competition between activating and inhibitory principles may also occur in follicular fluid at the level of the granulosa cells. Information on the inhibitory actions of follicular fluid can therefore be essential for understanding the regulation of the FSH receptor activation.

Chapter III: The FSH threshold/window concept and follicle growth

3.1 Introduction

As FSH levels increase during the luteo-follicular transition of the menstrual cycle, a cohort of small antral follicles is rescued from atresia and stimulated to grow. From this recruited cohort of growing follicles one will be selected to gain dominance. It has been hypothesized that differences in sensitivity for FSH or the "FSH threshold" level of the ovary, at the time of recruitment of a cohort of follicles, determines the number of follicles selected to gain dominance (Brown, 1978; Schoemaker *et al.*, 1993). However, intra-ovarian growth factors may increase the sensitivity of follicles for FSH (Hodgen, 1982; Erickson, 1996). Through enhancement of FSH action by these growth factors, follicle development and dominant follicle selection may occur despite decreasing FSH concentrations during the follicular phase of the menstrual cycle (Figure 3.1.1). Whether the increase in FSH concentration at the onset of the follicular phase is solely the determining factor for stimulation of follicle development and selection of a dominant follicle, or that induction of locally active factors play a decisive role in these events, are the subject of the studies described in the following sections.

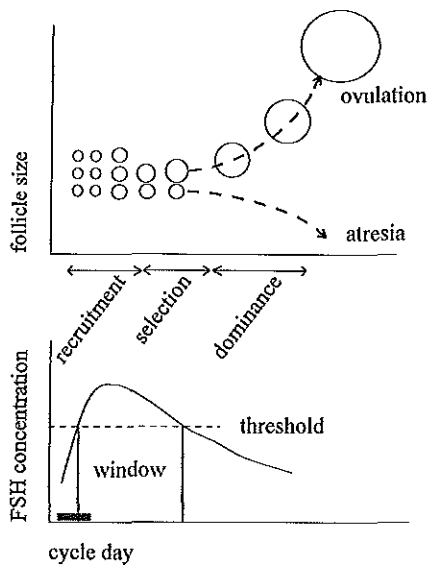

Figure 3.1.1
Schematic representation of the intercycle rise in serum FSH and follicle development, according to the FSH threshold/window concept.

3.2 The FSH threshold/window concept examined by different interventions with exogenous FSH during the follicular phase of the normal menstrual cycle: duration rather than magnitude of FSH increase affects follicle development

3.2.1 Introduction

As FSH levels increase during the luteo-follicular transition of the menstrual cycle, a cohort of small antral follicles is prevented from undergoing atresia and stimulated for further development (Hodgen, 1982; Baird, 1987; Gougeon, 1996; Fauser and van Heusden, 1997). The FSH threshold concept has been proposed, emphasizing the need for serum FSH to surpass a distinct concentration to induce 'ovarian activity' (Brown, 1978; Schoemaker *et al.*, 1993; Ben-Rafael *et al.*, 1995). Based on observations during gonadotrophin induction of ovulation, Brown suggested that an elevation of FSH concentrations only 10 - 30% above the threshold level is sufficient to stimulate normal follicle development, whereas a further increase causes excessive stimulation (Brown, 1978). Indeed, multiple follicle development and ovulation have been attributed to higher elevations of FSH above the threshold level (Messinis and Templeton, 1990; van der Meer *et al.*, 1994).

Circulating FSH levels decrease in the late follicular phase of the normal menstrual cycle, supposedly due to increased secretion of ovarian factors such as estradiol (E_2) and Inhibin-B, which exert negative feedback at the hypothalamic-pituitary level (Hotchkiss and Knobil, 1994; Groome *et al.*, 1996). The strict relationship between decreasing FSH concentrations and dominant follicle development in the normal menstrual cycle has been established recently by our group (van Santbrink *et al.*, 1995b), emphasizing the importance of decreasing FSH levels in securing single dominant follicle selection. Apparently, the maturing dominant follicle requires less FSH to continue its growth (Zeleznik and Kubik, 1986; Hall *et al.*, 1991). This may be due to intra-ovarian changes in growth factors, which up-regulate FSH sensitivity of follicles in more advanced developmental stages (Erickson, 1996). As a consequence, other recruited follicles lack sufficient stimulation by FSH and go into atresia. In this regard, the "FSH gate" (Baird, 1987) or "FSH window" (Fauser, 1994a) concept has been proposed, which adds the element of time to the FSH threshold theory and emphasizes the significance of a transient increase of FSH above the threshold level for single dominant follicle development. Indeed - as has been demonstrated in primates - interference with the decrease in FSH levels in the mid follicular phase overrides selection of a single dominant follicle (Zeleznik *et al.*, 1985). Moreover, administration of gonadotrophins for induction of ovulation in the human applying a decremental dose regimen, has proven to be successful in reducing the incidence of multiple follicle development (van

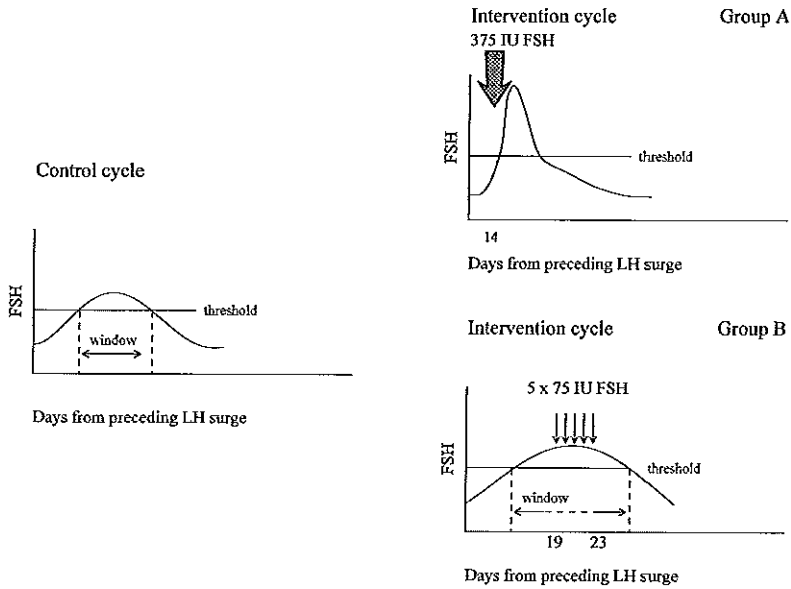

Figure 3.2.1
Outline of FSH dosage schedules administered to regularly cycling women for the study of the FSH threshold/window concept (see text).

Santbrink *et al.*, 1995a; van Santbrink and Fauser, 1997).

Further insight in the significance of the pattern of FSH stimulation for recruitment and dominant follicle selection may provide a basis for understanding mechanisms underlying follicle maturation arrest in polycystic ovary syndrome patients, for improved treatment regimens for gonadotrophin induction of ovulation, and for effective suppression of residual ovarian activity during the pill-free interval of steroid contraception (Fauser and van Heusden, 1997). The principal aim of the present study was to examine to what extent elevation of FSH levels - differing in duration and magnitude - would affect follicle development. For this purpose FSH was administered at different times and dosages to women exhibiting normal ovarian function, to elicit either an increase in FSH concentrations far above the threshold level in the early follicular phase or a moderate, but prolonged FSH increase in the mid-to late follicular phase (Figure 3.2.1).

3.2.2 Materials and Methods

Subjects and Study Design

This study was approved by the Ethics Review Committee of the Academic Hospital and Erasmus University Medical School, Rotterdam. A total of 26 study subjects was selected from respondents to an advertisement in a local newspaper. Inclusion criteria were: age between 20 and 33 years; regular

menstrual cycles for at least 3 months prior to the study (cycle lengths between 26 and 31 days), and no oral contraceptive use or other medical or hormonal treatment for at least 3 months prior to study initiation. In addition, subjects were required to be of normal weight (BMI [body mass index] between 19 and 25 kg/m^2) and to have never been under treatment for infertility. During the study, subjects were required to use sufficient contraceptive measures such as intra-uterine devices, tubal ligation or condoms. Informed written consent was obtained from each participant, and all 26 subjects were paid for their participation. Fourteen subjects had been pregnant previously.

Subjects were studied during 3 subsequent cycles. From 10 days after the onset of menses subjects assessed the luteinizing hormone (LH) surge, using a urinary LH test (Clear-plan One Step®, Unipath Ltd., Bedford, UK) according to manufacturers instructions. Six or seven days after a positive urinary LH test a blood sample was taken for assessment of mid-luteal progesterone (P). From 12 days after the LH surge a series of daily transvaginal ultrasound scans (TVS) and blood sampling was performed (control cycle). Daily investigations for each subject took place between 12.00 - 17.00 h and were concluded on the day of sonographically assessed ovulation (decrease in size of > 50% of the largest [≥ 18 mm] follicle). Normal ovulation was confirmed by assessment of elevated P levels (> 18 nmol/l) 6 or 7 days later (van Santbrink *et al.*, 1995b).

A second series of daily TVS and blood sampling started 10 days after the onset of the LH surge (intervention cycle). The day of the LH surge (Day_{LH}) was assessed by measurement of serum LH in the control cycle, and was defined as the day on which the LH level was highest or as the first LH level being at least 3 times higher than the level of the previous day. At the 12th day after the LH surge, subjects were randomly assigned to one of 2 intervention schedules (designated as Group A and Group B, respectively). Randomisation was performed by number in blocks of 3 pairs by drawing of a sealed envelope indicating the assigned group. The administration of urinary FSH (Metrodin HP of the same badge, kindly provided by Serono Benelux BV, The Netherlands) was performed by a third party, with the assigned protocol unknown to the observer (I.S.). Group A received 375 IU (= 5 ampules) FSH as a single SC injection on the 14th day after the LH surge, effectively increasing FSH concentrations far above the presumed threshold level for a brief period of time in the early follicular phase. Group B received 75 IU (= 1 ampule) of FSH SC daily during 5 consecutive days from 19 to 23 days after the LH surge, thereby preventing decremental serum FSH concentrations in the mid- to late follicular phase, thus widening the FSH window. FSH was administered by qualified nurses shortly after the daily blood withdrawal, with the next blood withdrawal 24 h later. Daily TVS and blood sampling continued until the day of ovulation in the intervention cycle. Again, normal ovulation was confirmed by assessment of elevated P levels 7 days later.

Sonographic examinations were performed by the same observer (IS) using a 6.5 MHz transvaginal transducer (EUB-415, Hitachi Medical Corporation, Tokyo, Japan) as described previously (Pache *et al.*, 1990; van Santbrink *et al.*, 1995b). In brief, follicle diameter was calculated as the mean diameter measured in 2 dimensions if both diameters were ≤ 9.0 mm and in 3 dimensions if at least 1 diameter was > 9.0 mm.

Hormone estimations

Blood samples were centrifuged within 2 h after withdrawal and stored at -20° C until assayed. FSH and LH levels were measured by immunoradiometric assay (Medgenix, Fleurus, Belgium) as described previously (Fauser *et al.*, 1991). P levels were assessed by radioimmunoassay (RIA) as described before (de Jong *et al.*, 1974), and E_2 levels were estimated using RIA kits provided by Diagnostic Products Corporation (Los Angeles, CA). Intra- and interassay coefficients of variation were < 3% and < 8% for FSH, < 5% and < 15% for LH, < 16% and < 17% for P, and < 15% and < 18% for E_2, respectively. All samples from one subject were run in the same assay.

Data analysis

Data are presented as the mean ± SD if distributed normally or as the median and range if distributed otherwise. The time scale used is based on the day of the preceding LH surge (Day_{LH}). Day of selection of the dominant follicle is defined as the day on which a given follicle was ≥ 10 mm together with an enlargement of this follicle during subsequent days until ovulation, as described previously (Pache *et al.*, 1990; van Santbrink *et al.*, 1995b;). The day of E_2 rise is defined as the day during the follicular phase on which E_2 concentrations were significantly higher as compared to the preceding days and continued to increase thereafter until ovulation. This was estimated using piece-wise linear regression relating $\log(E_2)$ to cycle day as described previously (van Santbrink *et al.*, 1995b).

The effects of both interventions were estimated by comparison of the area under the curve (AUC) of FSH and E_2 for a given number of days. AUC's were calculated for each subject individually during 2 periods of the follicular phase of the menstrual cycle using the trapezoidal rule method. Period 1 was arbitrarily defined to comprise Day_{LH+14} until Day_{LH+19}, and Period 2 Day_{LH+19} until Day_{LH+24} based on FSH administration regimens. As a reflection of follicle growth the total number of follicles observed (divided into size classes of 8 - 10 mm and ≥ 10 mm) was calculated for the same periods.

Statistical analysis was performed using Wilcoxon's test for matched pairs for comparison between the control cycle and the intervention cycle for each group. Comparisons of outcome measures between both randomized groups were performed using Mann-Whitney U test. Power analysis (β= 0.20) showed that

the sizes of both intervention groups allowed for detection (α= 0.05) of differences in outcome parameters of 1.1 SD. Correlation coefficients given are Spearman's. *P* values are two-sided with 0.05 taken as the limit for statistical significance.

3.2.3 Results

FSH and E_2 levels during control and intervention cycles

Two of the 26 subjects were excluded from further analysis due to an irregular control cycle and/or anovulation during the control cycle. A third subject was excluded due to multiple persisting cysts at the onset of the intervention cycle, in combination with an insufficient luteal phase in the preceding control cycle. All remaining 23 subjects were ovulatory in both the control and the intervention cycle, as assessed by TVS and elevated serum P levels (38 ± 10 nmol/l) 7 days after sonographically assessed ovulation. After the control cycle, 11 subjects were randomized for the intervention cycle into Group A, and 12 into Group B. With regard to the distribution of age and BMI no significant differences were found between the 2 groups (data not shown).

Due to the design of the study, FSH serum levels are presented with the LH surge from the preceding cycle as the point of reference. This is for reasons of comparison between the control and intervention cycles and does not represent accurately individual patterns of serum FSH concentrations. In the intervention cycle in Group A (Figure 3.2.2a, *top right panel*), in all subjects FSH levels reached a maximum level 1 day after SC administration of 375 IU FSH on Day_{LH+14} with a median concentration of 10.1 IU/l (range 7.9 - 13.9). FSH levels remained significantly elevated ($P < 0.01$) for 2 days and returned to levels similar to the control cycle on Day_{LH+17} (median levels 5.0 IU/l [range 2.4 - 7.3] and 5.5 [range 4.1 - 9.6] in control and intervention cycles, respectively). In all subjects a second, endogenous increase in FSH reached its maximum level on Day_{LH+21} (range Day_{LH+18} and Day_{LH+30}). The median concentration of these maximum FSH levels in the intervention cycle was not different from the corresponding levels in the control cycle (5.7 IU/l [3.7 - 7.4] and 5.7 IU/l [4.2 - 6.6], respectively). In Group B, daily SC administration of 75 IU FSH on Day_{LH+19} until Day_{LH+23} resulted in FSH levels 15% [6 - 37%] higher compared to matching days during the control cycle ($P < 0.05$) (Figure 3.2.2a, *bottom right panel*).

In response to administration of 375 IU FSH on Day_{LH+14} in Group A, E_2 levels were significantly elevated on Day_{LH+15} and Day_{LH+16}, compared to levels prior to injection ($P < 0.01$) and to levels on matching days in Group B ($P < 0.01$). After this increase, E_2 levels returned to basal levels on Day_{LH+17} (Figure 3.2.2b, *top right panel*). One day after administration of the first injection of 75 IU FSH in Group B, E_2 levels increased significantly as compared to both the control cycle and the intervention cycle of Group A on matching days ($P <$

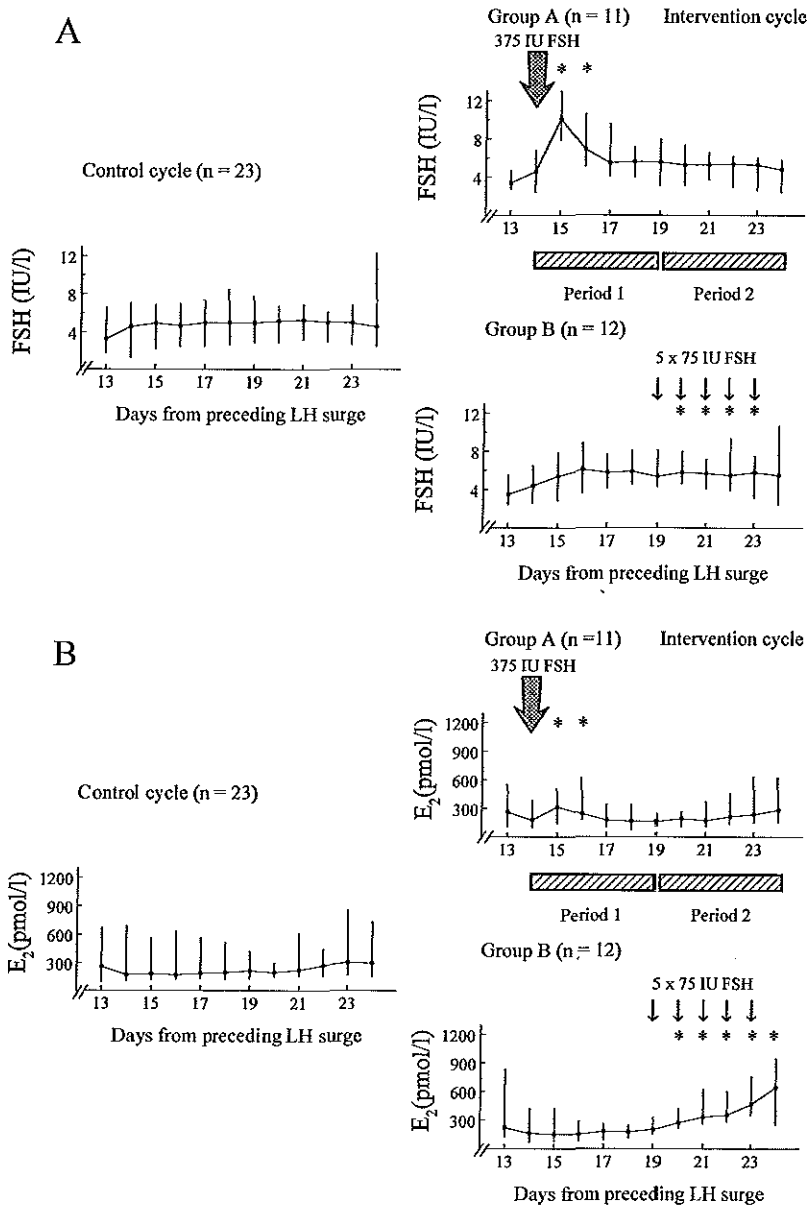

Figure 3.2.2
Daily follicular phase FSH (A) and E_2 (B) serum concentrations during control (*left panel*) and intervention cycles in 23 regularly cycling women. Data are shown as median and range and the time scale is expressed as days from the preceding LH surge (Day_{LH}). Intervention cycle Group A (*right upper panel*) represents administration of a single SC injection of 375 IU FSH on Day_{LH+14}, whereas Group B (*right lower panel*) represents 5 SC injections of 75 IU FSH daily from Day_{LH+19} until Day_{LH+23}. The intervention cycle has been divided arbitrarily into 2 periods; Period 1 (Day_{LH+14} until Day_{LH+19}) and Period 2 (Day_{LH+19} until Day_{LH+24}). Asterisks (*) indicate statistically significant higher levels compared to matching days in the control cycle ($P < 0.05$)

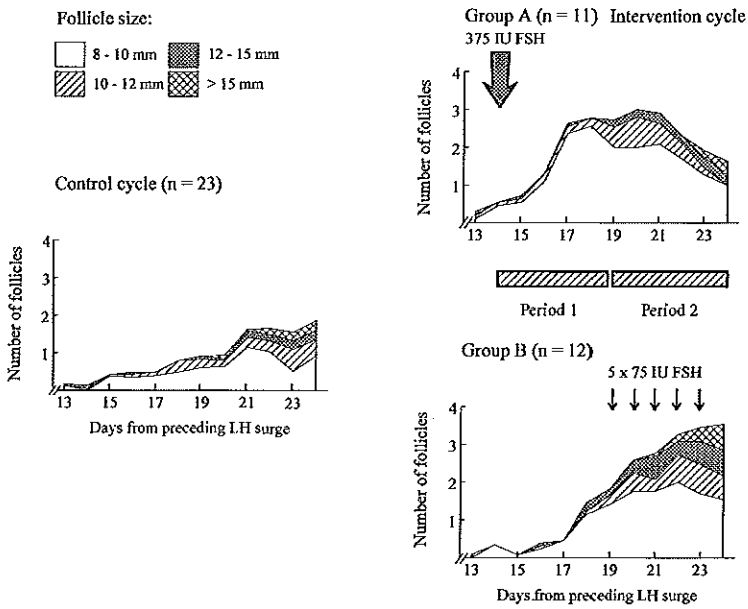

Figure 3.2.3
Number of follicles (> 8 mm) during the follicular phase of control (*left panel*) and intervention cycles in 23 regularly cycling women. Areas represent the mean number of follicles in both ovaries on a given day in all subjects, with shaded areas representing different size classes (see legend). The time scale is expressed as days from the preceding LH surge (Day_{LH}). Intervention cycle Group A (*right upper panel*) represents administration of a single SC injection of 375 IU FSH on Day_{LH+14}, whereas Group B (*right lower panel*) represents 5 SC injections of 75 IU FSH daily from Day_{LH+19} until Day_{LH+23}. The intervention cycle has been divided arbitrarily into 2 periods; Period 1 (Day_{LH+14} until Day_{LH+19}) and Period 2 (Day_{LH+19} until Day_{LH+24}).

0.01). Although the Day of E_2 rise occurred earlier in the intervention cycle of Group B compared to the control cycle ($P = 0.03$), the median preovulatory E_2 levels were not statistically different (1040 [380 - 1800] pmol/l and 900 [390 - 1300] pmol/l, respectively). Furthermore, the median preovulatory E_2 levels in Group B were not statistically different from Group A (810 [550 - 1100] pmol/l).

Follicle development during control and intervention cycles
Figure 3.2.3 shows the mean number of follicles ≥ 8 mm in both ovaries during the follicular phases of the control cycle and intervention cycle of Group A and B. Follicles < 8 mm have been omitted from the graphs since analysis of follicle numbers in smaller size classes did not display significant differences (data not shown).

After administration of 375 IU FSH on Day_{LH+14} in Group A an increase in the number of follicles of 8 - 10 mm was observed ($P < 0.05$), however without

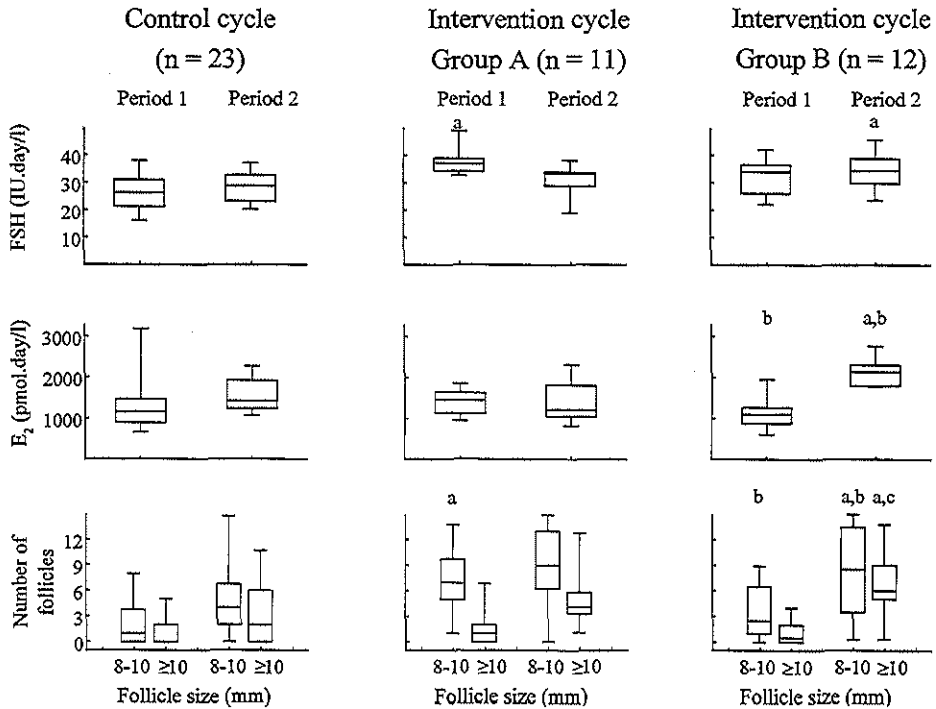

Figure 3.2.4
Area-under-curve (AUC) of FSH (*upper panel*), E_2 (*middle panel*) and total follicle number (*lower panel*) during 2 periods in the control cycle and intervention cycle in 23 regularly cycling women. Period 1 is defined as Day_{LH+14} until Day_{LH+19} and Period 2 from Day_{LH+19} until Day_{LH+24}. Boxes indicate 25th and 75th percentiles, with the horizontal line representing the median value. Whiskers span the range between the 5th and 95th percentile of the data.
[a] Significantly different ($P < 0.05$) comparing a similar period from the intervention cycle versus the control cycle.
[b] Significantly different ($P < 0.05$) comparing a similar period from Group B versus Group A.
[c] $P = 0.07$, comparing a similar period in Group B versus Group A.

ongoing growth to sizes beyond 10 mm in subsequent days. In group B, daily administration of 75 IU FSH from Day_{LH+19} until Day_{LH+23} did not result in a statistically significant increase ($P \geq 0.8$) in the mean number of follicles of 8 - 10 mm, as compared to the control cycle. However, in the following days an increasing number of follicles ≥ 10 mm was observed ($P < 0.05$), suggesting ongoing growth of these follicles.

Area Under the Curve (AUC) for FSH and E_2 and the total number of follicles during control and intervention cycles

The median AUC for FSH of Period 1 (from Day_{LH+14} until Day_{LH+19}) in the intervention cycle in Group A was significantly higher compared to the same period in the control cycle (median AUC Period 1: 37 [32 - 49] IU·day/l vs. 27 [17 - 34] IU·day/l, $P = 0.004$). The AUC for FSH of Period 2 in the intervention cycle in Group B (Day_{LH+19} until Day_{LH+24}) was significantly higher compared to the control cycle (34 [23 - 46] IU·day/l and 28 [21 - 39] IU·day/l, respectively, $P = 0.002$) [Figure 3.2.4, *top panel*]. During the intervention cycle the median AUC's of Period 1 in Group A and Period 2 in Group B were not significantly different ($P = 0.15$) from each other.

Despite the short, but significant increase in E_2 levels on Day_{LH+15} and Day_{LH+16} in response to administration of FSH in Group A, the AUC for E_2 of Period 1 was not significantly different compared to the control cycle (1440 [940 - 1853] pmol·day/l vs. 1162 [678 - 3604] pmol·day/l; $P = 0.33$). In contrast, in Group B a significantly higher AUC for E_2 was observed in Period 2 of the intervention cycle as compared to the control cycle; 2144 [1799 - 2799] pmol·day/l and 1361 [992 - 2463] pmol·day/l, respectively ($P < 0.01$). Comparing both groups during Period 2 in the intervention cycle, the AUC for E_2 in Group B was greater than Group A (median AUC's 2144 [1799 - 2799] pmol·day/l versus 1223 [818 - 2423] pmol·day/l, respectively, $P < 0.01$).

After administration of 375 IU FSH on Day_{LH+14} in Group A, a significant increase of small (8 - 10 mm) follicles was observed in Period 1 compared to a similar period in the control cycle (6 [1 - 22] follicles vs. 1 [0 - 8] follicle; $P < 0.01$) (Figure 3.2.4, *bottom panel*). After daily administration of 75 IU FSH in Group B, a statistically significant increased number of small (8 - 10 mm) follicles, as well as an increased number of follicles ≥ 10 mm was observed as compared to the control cycle during Period 2 (Figure 3.2.4, *bottom panel, right graph*) ($P = 0.02$ and $P < 0.01$, respectively). The latter was reflected by an increase in number of follicles sized 12 - 15 mm (mean: 0.9 [range 0 - 3] and 2.9 [range: 0 - 9] in control and intervention cycle, respectively, $P = 0.02$) and follicles sized > 15 mm (mean: 0.7 [range: 0 - 3] and 1.4 [range: 0 - 4], respectively, $P < 0.02$). Furthermore, during Period 2 a higher number of follicles > 10 mm was observed in Group B as compared to Group A (mean 7.4 and 4.3, respectively [$P = 0.05$]).

3.2.4 Discussion

At the onset of the menstrual cycle a cohort of small (2 - 5 mm) antral follicles is present in the ovaries (Gougeon, 1996). This cohort will continue to grow in response to stimulation by FSH (Gougeon and Testart, 1990), a process referred to as follicle recruitment (Hodgen, 1982). The FSH threshold concept has been proposed in the late 1970's by Brown (Brown, 1978) on the basis of serum estrogen levels secreted by developing follicles, and more recently has been substantiated by others by sonographical assessment of follicle growth (van Weissenbruch *et al.*, 1993; van der Meer *et al.*, 1994). According to the FSH threshold concept, increasing FSH concentrations should surpass a distinct level in order to initiate the final gonadotrophin dependent phase of follicle growth. Some researchers suggest selection of a follicle destined to gain dominance to occur during this period (McNatty *et al.*, 1983; Chikazawa *et al.*, 1986; Gougeon and Testart, 1990), although direct evidence to support this idea is lacking. Moreover, it has been hypothesized that follicles exhibit different degrees of FSH sensitivity at the time of recruitment. The follicle with the highest sensitivity will benefit most from increasing FSH levels and will subsequently gain dominance (Scheele and Schoemaker, 1996).

As opposed to ovarian hyperstimulation for *in vitro* fertilisation, the aim of induction of ovulation is to stimulate single dominant follicle development and ovulation. FSH levels are elevated above the threshold by daily administration of gonadotrophins. Conventional treatment protocols are often complicated by the occurrence of multiple follicle development, which may result in ovarian hyperstimulation syndrome or multiple pregnancies. It has been proposed that multiple follicle development is induced by elevating FSH concentrations far above the threshold (Schoemaker *et al.*, 1993). By starting with a lower dose of gonadotrophins and stepwise small increments, chances of inducing monofollicular growth should increase with a concomitant reduction of complications. This regimen is referred to as the "low-dose, step up" protocol (Polson *et al.*, 1987; White *et al.*, 1996). However, these stimulation protocols are characterised by FSH concentrations remaining above the threshold throughout the follicular phase, which may interfere with single dominant follicle selection. Indeed, it has been demonstrated that the extent of accumulation of FSH in the late follicular phase determines the magnitude of the ovarian response (Ben-Rafael *et al.*, 1986).

FSH concentrations reach a maximum in the early follicular phase of the normal menstrual cycle and decrease thereafter. The significance of this timely decrease in FSH to ensure monofollicular development has been demonstrated previously in primates (Zeleznik, 1981; Zeleznik and Kubik, 1986) as well as in the human (van Santbrink *et al.*, 1995b). These findings indicate that the dominant follicle requires less FSH to continue its development, probably due

to induction of locally acting factors, such as various growth factors or the induction of LH receptors, that enhance FSH sensitivity (Erickson, 1996). It is less likely that factors interfering with FSH receptor binding and activation are involved in this regulation (Schipper *et al.*, 1997). Although the dominant follicle continues its maturation, decreasing FSH concentrations fall below the threshold level of less mature follicles of the recruited cohort. Consequently, these follicles cannot sustain their growth and become atretic. On the basis of these findings the FSH window concept has been proposed, stressing the significance of the (limited) duration of FSH elevation above the threshold level rather than the height of the elevation of FSH for single dominant follicle selection (Fauser, 1994a; Fauser and van Heusden, 1997). In line with the FSH window concept, application of a decremental dose regimen for safe and effective gonadotrophin induction of ovulation - mimicking the dynamics of normal follicular phase FSH levels - has proven successful (van Santbrink *et al.*, 1995a; van Santbrink and Fauser, 1997).

The present study, administering exogenous FSH in regularly cycling women, demonstrates that a distinct but short increase in FSH levels in the early follicular phase around the onset of menses induces an increased growth of small follicles during subsequent days. However, dominant follicle growth (beyond a size of 10 mm) is not affected. In contrast, a slight, but extended elevation of FSH levels during the mid to late follicular phase - effectively preventing the physiological decrease in FSH concentrations - did result in the development of multiple dominant follicles. The present results correspond partly with conclusions drawn from a recent study (Lolis *et al.*, 1995). A single injection of 450 IU FSH to ovulatory women (suffering from unexplained infertility) on cycle day 2, and additional FSH administration (1 - 3 ampules daily) during subsequent days resulted in an increased number of preovulatory follicles, dependent on the dose of FSH administered. The authors concluded that supraphysiological FSH levels in the early follicular phase are a prerequisite for the induction of multiple follicle development, although the number of preovulatory follicles was determined by the height of FSH levels during later stages of the follicular phase. Observations from the present study show that multiple dominant follicle development can be induced by a minor interference with late follicular phase FSH levels only. This is further supported by previous observations from our group, suggesting that the magnitude of late follicular phase decrease in endogenous serum FSH levels determines dominant follicle development and related E_2 production (van Santbrink *et al.*, 1995b). In the present study, analysis of the number and size of follicles just before ovulation is of limited significance, because exogenous FSH was administered for a fixed number of days relative to the preceding LH surge, resulting in a variable number of days without exogenous FSH before ovulation.

The more than 2-fold increase in FSH concentrations (after administration

of 375 IU FSH) in the early follicular phase can be regarded as well above the threshold level. Moreover, observed maximum levels may even be an underestimation of actual maximum concentrations because serum FSH was assessed 24 h after injection. It has been reported previously that a single injection of FSH in normal men resulted in maximum FSH levels 6 - 8 h thereafter, with a 30 - 50% decrease after 24 h (Mizunuma *et al.*, 1990). Although the same applies for FSH administered in Group B, FSH concentrations in this group should be considered less above the threshold level compared to Group A. Distinct differences in dominant follicle growth comparing both groups stress the significance of timing and duration of the FSH elevation, as opposed to the FSH concentration *per se*. It may be proposed that recruited follicles increase their sensitivity to FSH during the course of their development (Zeleznik and Kubik, 1986; Hall *et al.*, 1991), supposedly due to the induction of a variety of autocrine factors (Giudice, 1992; Findlay, 1993; Erickson, 1996; van Dessel *et al.*, 1996a).

Induction of the aromatase enzyme during follicle recruitment has been proposed to determine selection of the dominant follicle (Hillier *et al.*, 1981; Baird, 1987; Erickson, 1996). Indeed, E_2 concentrations in serum increase abruptly from the day a dominant follicle can be visualised by TVS (Pache *et al.*, 1990; van Santbrink *et al.*, 1995b;). Moreover, high E_2 concentrations in the antral fluid of follicles beyond 10 mm in size indicate that aromatase activity is particularly expressed in dominant follicles (van Dessel *et al.*, 1996b). The present study shows that a short surge of supraphysiological concentrations of FSH is capable of stimulating aromatase activity in small antral follicles. However, E_2 production is not sustained even though FSH levels after the surge are similar compared to normal follicular phase concentrations. It may therefore be disputed whether induction of aromatase activity in small antral follicles is a determining factor for dominant follicle selection rather than a feature of the dominant follicle after selection.

Results from the present study are supportive of the FSH window concept, which stresses the importance of a limited duration of elevated FSH levels above the threshold, as opposed to the magnitude of FSH increase for follicle selection and dominance. The significance of a timely decrease in FSH levels to ensure single dominant follicle development may be relevant for gonadotrophin induction of ovulation protocols (Fauser and van Heusden, 1997). As shown recently, ovulation induction applying a decremental dose regimen ("step down" protocol) may result more frequently in mono ovulatory cycles and reduced chances for complications (van Santbrink and Fauser, 1997). Findings from the present study may also provide a basis for further investigation regarding follicle maturation arrest in polycystic ovary syndrome patients. Furthermore, these results support observations on follicle development during and after the pill-free interval in oral contraceptive pill users. During this period, FSH levels may

reach normal follicular phase concentrations, sufficient to initiate follicle recruitment (Fauser and van Heusden, 1997). Extension of the pill-free interval may thus result in dominant follicle development despite decreasing FSH concentrations due to reinitiation of pill intake (Killick, 1989). In conclusion, elevation of FSH levels high above the threshold level for a short period of time in the early follicular phase does not increase the number of dominant follicles. When a decrease in FSH is prevented in the late follicular phase augmented sensitivity for FSH allows for several follicles to gain dominance. Follicle dominance may not be dependent on FSH levels as rigidly as previously assumed. These observations support the FSH window concept and may exhibit clinical implications for ovulation induction treatment and oral contraceptive use.

3.3 Lack of correlation between maximum early follicular phase serum FSH levels and menstrual cycle characteristics in women under the age of 35

3.3.1 Introduction

In women approaching menopause, endocrine and menstrual cycle changes indicate altered ovarian function. The most consistent endocrine finding is elevated early follicular phase serum follicle-stimulating hormone (FSH) levels, wich are not accompanied by a rise in luteinizing hormone (LH) (Sherman *et al.*, 1976; Ahmed Ebbiary *et al.*, 1994a). This gradual increase in FSH concentrations may result from decreased endocrine feedback signals through diminished production of oestradiol (E_2) and inhibins (Sherman and Korenman, 1975; Klein *et al.*, 1996a) by the decreasing follicular pool (Richardson *et al.*, 1987; Faddy *et al.*, 1992). Recently, the number of antral follicles in the early follicular phase as assessed by ultrasound (Reuss *et al.*, 1996), and peripheral levels of one of the inhibins, inhibin-B (Klein *et al.*, 1996d), have been found to decrease with advancing age, which may reflect a reduction of the number of recruited follicles. The remaining reproductive potential has been referred to as "ovarian reserve" (Scott and Hofmann, 1995). Evaluation of ovarian reserve prior to initiation of ovarian stimulation by measuring basal or stimulated FSH concentrations in the early follicular phase may provide prognostic information regarding chances for success of infertility treatment (Navot *et al.*, 1987; Scott *et al.*, 1989; Fanchin *et al.*, 1994; Scott and Hofmann, 1995; Hansen *et al.*, 1996; Kim *et al.*, 1997).

Throughout reproductive life, increased FSH concentrations during the luteo-follicular transition of the menstrual cycle stimulate growth of a cohort of small antral follicles (Hall *et al.*, 1992). According to the threshold concept, the intercycle rise in FSH should surpass a distinct level in order to recruit this

cohort of follicles (Brown, 1978). Later during the follicular phase, one of the recruited follicles will gain dominance and eventually ovulate (Hodgen *et al.*, 1985). Concomitant with the presence of a dominant follicle, serum E_2 levels increase whereas FSH concentrations decrease (van Santbrink *et al.*, 1995b). Apart from E_2, inhibin-B secreted in the early follicular phase by the cohort of recruited growing follicles may also be involved in the negative feedback regulating FSH secretion (Groome *et al.*, 1996).

A recent study from our group showed distinct (up to 2.5-fold) differences in maximum serum FSH levels in a well-defined group of young women presenting with normal ovarian function (van Santbrink *et al.*, 1995b). This observation may be related to distinct differences in the FSH threshold level due to different intra-ovarian modification of FSH action by growth factors (Seifer *et al.*, 1995; Fauser and van Heusden, 1997). Therefore, high FSH concentrations may not necessarily indicate decreased ovarian reserve. The aim of the present study is to examine to what extent the variation in endogenous FSH stimulation of the ovary is related to menstrual cycle characteristics in young normo-ovulatory women.

3.3.2 Materials and Methods

Subjects and study design

This study was approved by the local Ethics Review Committee and written informed consent was obtained from all participants. Volunteers were acquired through advertisement in a local newspaper and paid for their participation. A total of 39 regularly cycling women entered the study. Inclusion criteria for this study were: age 20 - 35 years, a history of regular menstrual cycles for at least 6 months with cycle lengths between 26 and 31 days, normal body weight (body mass index [= weight divided by the square of body length] between 19 and 24 kg/m^2), no medical or hormonal treatment during at least 3 months prior to the study and no previous history of infertility.

Daily blood withdrawal was performed between 13.00 and 17.00 h, starting several days before the expected onset of menses until the next ovulation. Transvaginal sonography (TVS) of the ovaries was performed every other day from the initiation of the study until sonographically assessed ovulation (disappearance or > 50% decrease in size of largest follicle, if > 15 mm), as described previously (Pache *et al.*, 1990; van Santbrink *et al.*, 1995b). Six or 7 days after ovulation a blood sample was taken to assess midluteal progesterone (P) levels.

Hormone assays

Blood samples were centrifuged within 2 h after withdrawal and serum was stored at - 20° C until assayed. FSH and LH levels were assessed by

immunoradiometric assays. Part of the samples (from 16 subjects) was assayed using the assay obtained from Medgenix (Fleurus, Belgium), as described previously (Fauser *et al.*, 1991). Samples from the remaining 23 subjects were assayed with the Amerlite (Ortho-Clinical Diagnostics, Amersham, U.K.) assay. Comparative analysis of both assays revealed no significant differences between the results obtained using both methods (Amerlite = 1.00 x Medgenix - 0.02 U/l; $r = 0.93$, n = 20). Intra- and interassay coefficients of variation were < 3% and < 8% for FSH, and < 5% and < 15% for LH, respectively. E_2 levels were assessed by radioimmunoassay (RIA) provided by Diagnostic Products Corporation (Los Angeles, CA), with intra- and interassay coefficients of variation < 15% and < 18%, respectively (van Santbrink *et al.*, 1995b). Mid-luteal P was determined by RIA as described before (de Jong *et al.*, 1974), with intra- and interassay coefficients of variation being < 16% and < 17%, respectively. Inhibin-A and -B levels were measured using an enzyme-linked immunosorbent assay obtained from Serotec (Brooks University, Oxford, UK) as described earlier (Groome *et al.*, 1996). Intra- and interassay coefficients of variation were < 9% and < 15%, for both assays respectively. Inhibin-A and -B levels were measured in serum samples obtained every other day, taking the day of maximum FSH levels (FSH_{max}) as reference. Dependent on individual cycle length these samples had been collected between 8 days prior to day of FSH_{max} and 8 days thereafter. In all hormone assays each sample was tested in duplicate, with all samples from one subject in the same assay.

Data analysis

Results are presented as mean ± SD if normally distributed, and as median and range if distributed otherwise. Correlation coefficients given are Spearman's or Pearson's, dependent on the distribution. *P* values are two sided with 0.05 taken as limit for statistical significance. Day of dominance is defined as the day at which the dominant follicle was 10 mm (assessed by TVS), and was determined by extrapolating the linear growth curve of the preovulatory follicle backward until the day on which the diameter of this follicle was 10 mm, as described previously (Pache *et al.*, 1990; van Santbrink *et al.*, 1995b). Day of E_2 rise was estimated using piece-wise linear regression relating $\log(E_2)$ to cycle day as described previously (van Santbrink *et al.*, 1995b). Maximum FSH concentration was defined as the highest level of FSH during the follicular phase, disregarding the post-nadir increase prior to the LH-peak.

3.3.3 Results

Cycle characteristics

The mean age of the subjects studied was 28 ± 4 (SD) years. All 39 subjects were ovulatory during the studied cycle as demonstrated by TVS and by elevated mid-

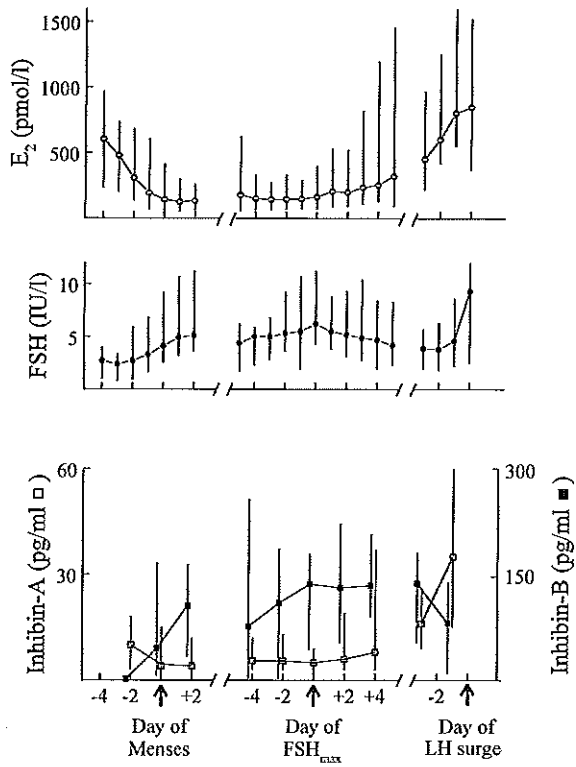

Figure 3.3.1
FSH (*upper panel*), E_2 (*middle panel*) and inhibin-A and inhibin-B (*lower panel*) serum concentrations during the follicular phase in 39 young, normo-ovulatory women. Data are presented as median values with bars spanning the 5th to 95th percentile. The time scale on the x-axis is split into days around the onset of menses (Day of Menses = cycle day 1), around the day of maximum FSH concentrations (Day of FSH_{max}) and days prior to ovulation (Day of LH surge).

luteal P levels (41 ± 14 nmol/l). Median cycle length was 28 days (range: 24 to 31 days). In 5 subjects the study cycle was of shorter duration than expected on the basis of cycle history. The mean duration of the follicular phase was 15 ± 3 days, and of the luteal phase 13 ± 2 days. In this population, age did not correlate significantly with cycle length, nor with the length of the follicular phase (r = -0.08, P = 0.61 and r = -0.29, P = 0.08, respectively) (data not shown).

Hormone levels

Median levels and range of FSH, E_2 and inhibin-A and -B during the follicular phase are depicted in Figure 3.3.1. The median FSH concentration on cycle day 3 (CD 3) was 5.1 (3.6 - 11.2) IU/l, and did not correlate with age (r = 0.19, P = 0.24). During the follicular phase, FSH levels reached a maximum level of median 6.2 (4.3 - 11.2) IU/l on CD 6 (2 - 15). The distribution of the FSH concentrations on CD 3 and on the day of maximum FSH concentration

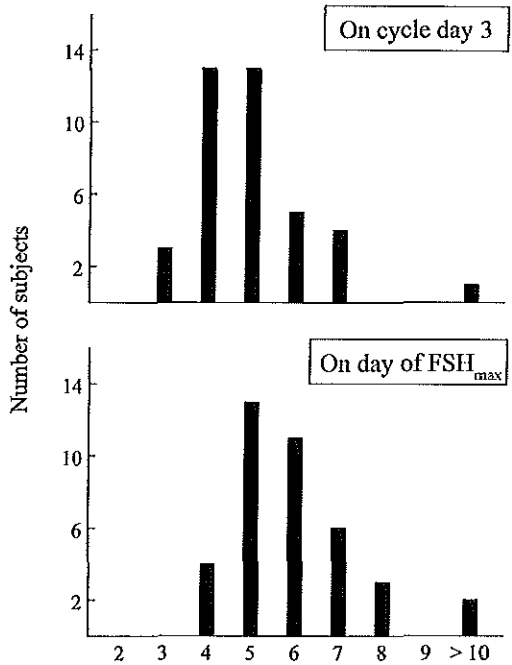

Figure 3.3.2
Distribution of FSH concentrations on cycle day 3 (*upper panel*) and on the day of maximum FSH concentration (*lower panel*) on the basis of daily blood sampling during the follicular phase in 39 young, normo-ovulatory women.

(FSH_{max}) is depicted in Figure 3.3.2. Maximum FSH levels did not correlate with age, cycle length or duration of the follicular phase (Figure 3.3.3).

On CD 1 the serum E_2 concentration was 140 (70 - 420) pmol/l and remained at this level, until CD 8 (4 - 15) when a loglinear increase occurred, as calculated with piece-wise linear regression. The cycle day at which the E_2 concentration rose correlated well with the cycle day at which follicle dominance (Day_{dom}) was assessed by TVS (r = 0.70, P < 0.01, data not shown). The correlation between day of FSH_{max} and day of E_2 rise was weak (r = 0.34, P = 0.03, data not shown), while the correlation between day of FSH_{max} and Day_{dom} was not significant (r = 0.28, P = 0.08, data not shown). Neither the day of E_2 rise, nor the Day_{dom} correlated with the FSH_{max} concentration (Figure 3.3.4). After the day of E_2 rise, E_2 levels increased with a doubling time of 3.9 (2.1 - 5.4) days until a median preovulatory level of 847 (370 - 1530) pmol/l was reached.

Inhibin-A and inhibin-B levels on CD 1 were 7 (3 - 18) pg/l and 40 (1 - 140) pg/l, respectively. Inhibin-A remained at this low level until CD 9 (3 - 12), when levels started to increase until 35 (15 - 88) pg/l at the day of LH surge. The cycle day of the inhibin-A increase correlated well with the day of E_2 rise (r = 0.56, P

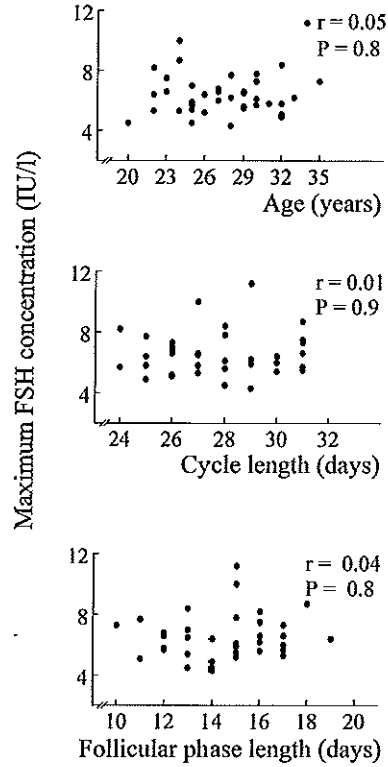

Figure 3.3.3
Distribution of maximum follicular phase FSH serum concentrations related to age (*upper panel*), cycle length (*middle panel*) and length of the follicular phase (*lower panel*) in 39 young, normo-ovulatory women. Correlation coefficients *r* are Spearman's.

< 0.01; data not shown). Maximum inhibin-B levels were 170 (94 - 310) pg/l on CD 7 (1 - 14), decreasing thereafter to 77 (10 - 149) pg/l on CD 9 (2 -15). The cycle day of maximum inhibin-B correlated significantly with the day of FSH_{max} ($r = 0.53$, $P < 0.01$; data not shown), whereas the maximum inhibin-B concentration was neither correlated with FSH_{max} ($r = 0.05$, $P = 0.80$), nor with age ($r = -0.13$, $P = 0.47$, Figure 3.3.5).

Follicle growth

On CD 3 the mean number of small (2 - 10 mm) antral follicles was 11 (4 - 21) for both ovaries. The number of follicles in the early follicular phase was not correlated with FSH_{max} ($r = -0.10$, $P = 0.70$), nor with age ($r = -0.11$, $P = 0.58$) (Figure 3.3.6). The number of antral follicles on CD 3, day of FSH_{max} (13 [6 - 20]) or day of maximum inhibin-B (13 [6 - 22]) was not correlated to inhibin-B concentrations on these respective days ($r = -0.20$, $r = 0.07$ and $r = 0.25$, respectively; data not shown). However, the sum of the surface of each antral follicle present on a particular day (considered representative for the total inhibin-B producing capacity), was significantly correlated with the inhibin-B concentration ($r = 0.40$, $P < 0.01$) (data not shown).

After Day_{dom} (CD 7 [4 - 13]), the dominant follicle displayed a mean linear increase in size of 1.5 ± 0.4 mm/day, until a mean preovulatory size of 20.5 ±

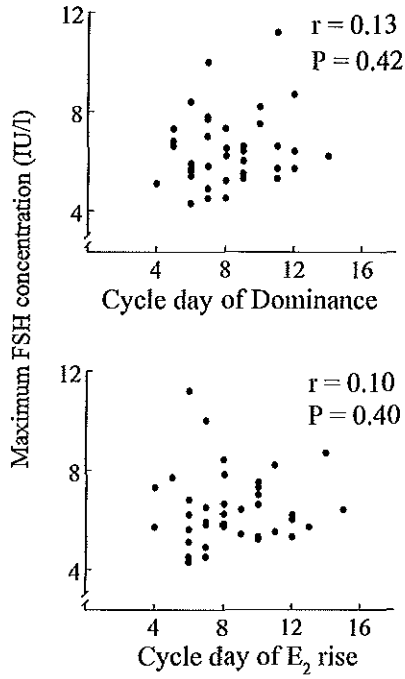

Figure 3.3.4
Distribution of follicular phase maximum serum FSH concentrations related to cycle day of dominance (as assessed by TVS) (*upper panel*) and cycle day of E_2 rise (*lower panel*) in 39 young, normo-ovulatory women. Correlation coefficients *r* are Spearman's.

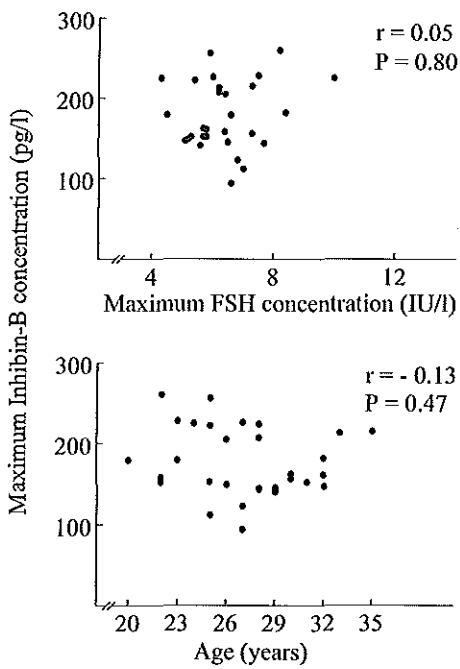

Figure 3.3.5
Distribution of follicular phase maximum serum inhibin-B concentrations related to maximum FSH concentration (*upper panel*) and age (*lower panel*) in 39 young, normo-ovulatory women. Correlation coefficients *r* are Spearman's.

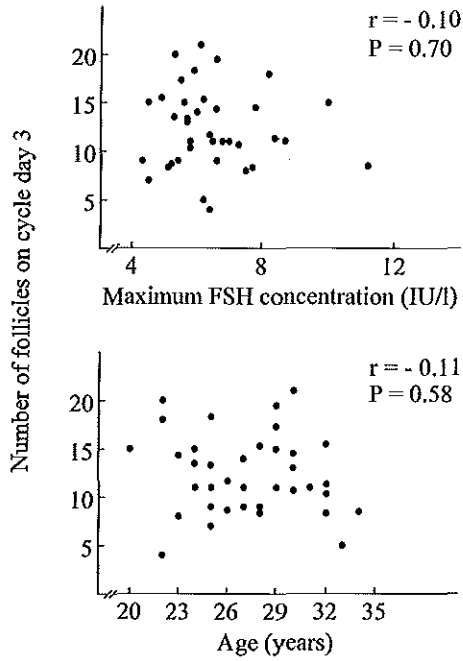

Figure 3.3.6
Distribution of the number of small antral follicles (2 - 10 mm) observed in both ovaries on cycle day 3 by transvaginal ultrasound related to maximum FSH concentration (*upper panel*) and age (*lower panel*) in 39 young, normo-ovulatory women. Correlation coefficients *r* are Spearman's.

3.0 mm. The growth rate of the dominant follicle did not correlate significantly with the doubling time of E_2 (r = - 0.13, P = 0.4), nor with the doubling time of inhibin-A (r = -0.3, P = 0.19; data not shown). Both the growth rate of the dominant follicle and the preovulatory size were not correlated to FSH_{max} (r = 0.1, P = 0.72 and r = -0.1, P = 0.45, respectively; data not shown).

Despite the lack of correlation between the absolute hormone concentrations, the data indicate a sequential order in the occurrence of day of FSH_{max} (median CD 6), day of maximum inhibin-B (median CD 7), Day_{dom} (median CD 7), day of E_2 rise (median CD 8) and day of inhibin-A rise (median CD 9) (Figure 3.3.7). The day of FSH_{max} occurred earlier in the cycle compared to maximum inhibin-B ($P < 0.01$), whereas maximum inhibin-B concentration preceded Day_{dom} ($P < 0.01$). No difference was found between day of selection, E_2 rise and inhibin-A rise. The sequence of events as represented in Figure 3.3.7 is confirmed by a mean slope of 0.89 ± 0.57 days ($P < 0.01$) from day FSH_{max} until day of inhibin-A rise. Disregarding the days of FSH_{max} and maximum inhibin-B in the analysis revealed a mean slope of 0.46 ± 1.35 days (P = 0.1).

3.3.4 Discussion

In the years prior to menopause early follicular phase serum FSH levels start to rise concomitant with changes in menstrual pattern (Treloar *et al.*, 1967; Ahmed Ebbiary *et al.*, 1994b; Reame *et al.*, 1996). The diminished response of the ovary

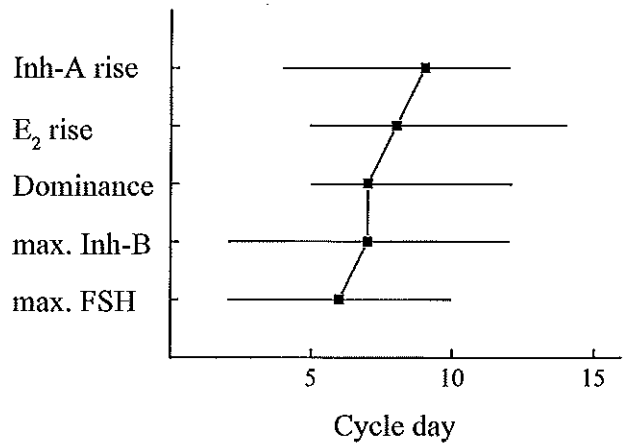

Figure 3.3.7
Cycle day at which maximum serum FSH concentration, maximum serum inhibin-B concentration, follicle dominance (as assessed by TVS), E_2 concentration increase and inhibin-A concentration increase occurred during the follicular phase in 39 young, normo-ovulatory women. Data are presented as median values with bars spanning the 5th to 95th percentile.

to FSH stimulation with advancing age is generally attributed to the decrease in quantity and quality of the pool of recruitable follicles (Richardson *et al.*, 1987; Meldrum, 1993). Eventually the follicular pool is exhausted and endocrine feedback mechanisms in the ovarian-hypothalamic-pituitary axis become disrupted, resulting in an increase in gonadotrophin concentrations to post-menopausal levels. It is assumed that, in the years preceding menopause, serum FSH levels indirectly reflect a woman's residual ovarian function, i.e. the ovarian reserve (Scott and Hofmann, 1995). Indeed, increased early follicular phase FSH concentrations in women of advanced reproductive age (> 35 years) have been associated with decreased success of infertility therapy (Muasher *et al.*, 1988; Scott *et al.*, 1989; Ebrahim *et al.*, 1993; Cahill *et al.*, 1994; Hansen *et al.*, 1996). Assessment of ovarian reserve prior to infertility treatment by measuring FSH serum levels on cycle day 3 has been put forward as a good predictor of treatment outcome.

Results from the present study show that in normo-ovulatory young women, FSH concentrations on cycle day 3 span a wide range between 3.6 and 11.2 IU/l. Furthermore, the maximum FSH concentrations during the follicular phase displayed a comparably wide range of 4.3 to 11.2 IU/l, although at a higher median level. For each individual FSH levels displayed an increasing trend from cycle day 1 onward until the day on which a maximum FSH concentration was reached with a median of cycle day 6 (range 2 to 15). FSH serum levels on cycle day 3 are clearly an under representation of maximum follicular phase serum levels. Owing to shortening of the follicular phase, this

discrepancy may be reduced in older women.

The observed large inter-individual difference in maximum FSH levels suggests a variation in ovarian sensitivity for FSH stimulation in normo-ovulatory women. However, this is not reflected in a wide variation in ovarian function, since relevant biological outcome parameters such as follicle development and E_2 production appear not to be related to the height of the maximum FSH concentration. It was not possible to demonstrate a significant correlation between early follicular phase FSH concentrations and chronological age or to confirm the relationship between increased FSH levels and a relative shortening of the menstrual cycle as a consequence of a diminished ovarian function, as has been observed in women approaching menopause (Treloar *et al.*, 1967). Furthermore, results from the present study do not indicate a significant correlation of the number of antral follicles during the early follicular phase with maximum FSH levels or with age, although it has been reported recently that the number of antral follicles during the follicular phase decreases with advancing age (Reuss *et al.*, 1996). An accelerated decrease in follicle number has been observed after the age of 37 years (Faddy *et al.*, 1992), which is beyond the age of the subjects in the present study.

Over 20 years ago it has been proposed that increasing FSH concentrations in women approaching menopause might be due to diminished inhibin production by the ovaries (Sherman *et al.*, 1976). More recently, with the advance of specific immunoassays for inhibin-A and inhibin-B, it has been shown that serum inhibin-B concentrations increase in the early follicular phase in response to increasing FSH concentrations, whereas inhibin-A concentrations increase at a later stage during the cycle, reaching a maximum concentration in the mid-luteal phase (Groome *et al.*, 1996). Apart from a negative feedback effect at the level of the pituitary regulating the secretion of FSH, the presence of inhibin-B in the follicular fluid of the largest, dominant follicle suggests a local, intra-ovarian role for this protein in follicle selection (Groome *et al.*, 1996). Recently it has been demonstrated that low (< 45 pg/ml) inhibin-B concentrations in the early follicular phase are related to a reduced response to ovulation induction and indicate decreased ovarian reserve, irrespective of age (Seifer *et al.*, 1997). Inhibin-B levels are believed to represent the size of the cohort of follicles recruited for further development. The observed lack of correlation between maximum FSH levels and maximum inhibin-B concentrations in the present study, suggests indirectly that the number of follicles recruited for further development is independent of the magnitude of stimulation by FSH.

Results from the present study and from a previous study by our group demonstrate large inter-individual variation in follicular phase FSH levels in normo-ovulatory young women (van Santbrink *et al.*, 1995b). Although all subjects showed monofollicular growth, apparently governed by a similar

pattern of hormonal events, as shown in Figure 3.3.7, differences in maximum FSH concentrations did not correlate with parameters characteristic of ovarian ageing, such as menstrual cycle changes, and endocrine and ultrasound observations. The height of the FSH level may represent differences in sensitivity of the ovary to FSH stimulation (i.e. the FSH threshold), which in turn may be determined by a variety of intra-ovarian factors (Fauser, 1996). The significance of a single serum FSH determination for assessment of ovarian ageing may be reduced because, since a large overlap exists between younger and older subjects. This may reflect physiological differences in the FSH threshold. Changes in early follicular phase serum FSH concentrations in a given individual over time could provide a more sensitive test of ovarian ageing (te Velde *et al.*, 1997). Differences in local enhancement and regulation of FSH action by intra-ovarian factors such as IGFs or the activin and inhibin system may be involved (Giudice *et al.*, 1996). This concept is strongly supported by recent observations showing differences in follicular fluid IGF-II levels as a function of day 3 serum FSH in women independent from age (Seifer *et al.*, 1995). In addition, individual differences in the circulating FSH isohormone profile, differing in metabolic clearance rate and *in vitro* or *in vivo* bioactivity, may also be important (Ulloa-Aguirre *et al.*, 1995). Finally, the possibility of individual differences in FSH receptors cannot be excluded.

The general perception is that high follicular phase serum FSH concentrations indicate advanced reproductive ageing and reduced outcome of infertility therapy. Data presented in the present study suggest that large individual differences in FSH occur in normo-ovulatory women under the age of 35 and that other factors not related to ovarian ageing, such as differences in ovarian sensitivity to FSH should also be considered.

Chapter IV: General discussion and conclusions

The principal objective of this thesis was to examine the potential significance of local, intra-ovarian modulation of FSH action. Previous clinical and *in vitro* studies indicate that local (growth) factors play and important role in the modulation of FSH action. Disturbed folliculogenesis, such as in normogonadotrophic anovulation or PCOS, may be caused by local disregulation of FSH action, rather than by defective granulosa cells *per se*. Modulation of FSH action may involve both stimulatory and inhibitory factors. Some of these factors modify FSH activity at the level of the FSH receptor. Other factors, more specifically growth factors, act through their own receptors and pathways, thus modulating FSH-activated responses at an intracellular level. The ultimate response in the ovary to stimulation with FSH is a result of the combined stimulatory and inhibitory actions of FSH and a variety of local factors.

The inhibition of cellular responses by serum components has been an important obstacle for the development of reliable *in vitro* bioassays for FSH and other glycoprotein hormones (Jia and Hsueh, 1986b; Wang, 1988; Monaco *et al.*, 1989; Persani *et al.*, 1993). Since it is unknown which serum factors act directly on the FSH receptor and which via other receptors, we aimed at a bioassay system with which these factors could be distinguished. Whereas others have used specific FSH receptor binding assays, employing specific assay conditions such as hypotonic buffers and fractioning of serum, we have used cAMP production by cultured cells as the end point. Measurement of cAMP production as an immediate parameter of FSH receptor activation excludes the influence of factors which affect FSH-induced responses at an intracellular level. The Chinese hamster ovary cells, stably transfected with the human FSH receptor, provide a suitable model for this purpose. Using this assay we have demonstrated that human serum and follicular fluid contain a low and constant level of FSH receptor activation inhibitors. Moreover, the inhibitory effect of serum and follicular fluid from patients with normogonadotrophic anovulation was not elevated as compared to normal controls. On the basis of these results, the inhibition of FSH by factors interfering with FSH receptor binding and activation can be excluded as an important mechanism by which FSH-induced follicle development is disturbed.

This conclusion seems to contradict some previous reports, which demonstrated specific inhibition of FSH receptor binding by compounds from serum and follicular fluid. However, whereas FSH receptor binding inhibitors have been examined in isolated form after purification and concentration (Lee *et al.*, 1990; Lee *et al.*, 1993; Alouf *et al.*, 1997), in our studies nearly physiological concentrations of virtually all serum factors together were measured. Results obtained indicate that the total inhibitory effect of serum

factors is relatively independent from the concentration of these factors. It appears that serum may act as a buffer towards the inhibitory factors, even when these are present in nearly physiological concentrations. This concept was further demonstrated by the use of protamine as a FSH receptor activation inhibitor. Although not a naturally occurring inhibitory substance in the ovary by itself, protamine displays specific FSH inhibitory effects *in vitro*. Addition of serum attenuated the inhibitory effect of protamine completely. It is hypothesized, that peptides and protein factors with proven FSH receptor binding inhibitory activity in isolated and purified form, behave in a similar fashion when added together with serum proteins. Unfortunately it has not yet been possible to examine such inhibitors using this approach. Altogether, the present results illustrate the difficulty in the interpretation of data obtained from the use of (partially) purified factors under specific *in vitro* conditions and the extrapolation of such data to physiological conditions.

Local enhancement of FSH action by growth factors probably plays a crucial role in stimulation of follicle development and dominant follicle selection (Scheele and Schoemaker, 1996; Fauser and van Heusden, 1997). In order to study these growth factors, the most relevant *in vitro* model is provided by cultured human granulosa cells. Whereas human granulosa cells are easily isolated and cultured from follicular fluid obtained from patients undergoing IVF, these cells appear to have lost FSH responsiveness. Furthermore, these cells are derived from preovulatory follicles and may have undergone (partial) luteinization, due to administration of hCG prior to puncture. Therefore these granulosa cells may not be representative for the cell population of developing follicles, prior to dominant follicle selection. A more relevant model, using granulosa cells obtained from developing follicles in naturally cycling ovaries, was difficult to develop, partially due to practical difficulties and insufficient availability of tissue. On the limited occasions that sufficient human granulosa cells could be isolated, the cells displayed a poor morphological and functional quality *in vitro*. Moreover, cultured cells responded only moderately to stimulation with FSH. Apparently the isolation procedure affects the normal functional properties of cells. *In situ*, granulosa cells are part of an intercellular network, the microenvironment in which auto- and paracrine factors exert their regulatory effects. When cells are transferred to culture conditions, these structures are disrupted and adaptation may require induction of other regulatory mechanisms. It has been suggested that the expression of differentiated function and growth-related processes are inversely controlled in cultured granulosa cells (Orly *et al.*, 1980).

A valuable alternative option for *in vitro* studies of local regulation of follicle development is provided by cultures of intact (pre)antral follicles (Nayudu and Osborn, 1992; Roy and Treacy, 1993; Abir *et al.*, 1997; Oktay *et al.*, 1997b). The integrity of the intercellular architecture allows the local regulatory

mechanisms to be studied more appropriately. A multitude of factors is present at the same time in the physiological environment of the follicle, each with its own specific antagonistic or synergistic activity. Interactions exist between different growth factors, such as the inhibins and activins with the IGF system (Matzuk *et al.*, 1996; Li *et al.*, 1998). Furthermore, the activity of a specific growth factor itself may be subject to local regulation or fine-tuning, such as in the IGF/IGFBP/protease system (Holly and Hughes, 1994). The coexistence of various auto- and paracrine loops around the cells provide a reliable, and also a flexible regulatory system. Some factors modulate FSH-induced pathways directly within the cell. Others are induced by FSH, and mediate an effect on the cell which is independent from FSH-induced pathways. Altogether, the action of growth factors is "context dependent" (Sporn and Roberts, 1988). In other words, a specific combination and concentration of growth factors provides a useful message to the target cell, similar to words in a sentence. Misspelling or deletion of one or more factors (letters or words) may still be interpreted as a message; however, chances for failure increase.

Another option to study regulation of follicle development, specifically the interactions of FSH with growth factors, is the use of transgenic or knockout animals. In these animals the genes, encoding a specific growth factor may be over-expressed or inactivated. However, extrapolation of data obtained from *in vitro* culture models and transgenic and knockout animals to human physiology is not always directly applicable (Strohman, 1994). The obligatory role of the IGF system for normal ovarian function has been demonstrated in mice with an *Igf1* gene null mutation (Baker *et al.*, 1996). In the knockout animals follicles develop normally until the preantral stage. Final follicle development and ovulation does not occur, despite high doses of administered gonadotrophins. These observations suggest an obligatory role of IGF-I for normal folliculogenesis. However, it has been reported that in a human female with Laron-type dwarfism (an autosomal recessive growth hormone deficiency, resulting in extremely low IGF-I concentrations) normal follicle growth could be induced by exogenous gonadotrophins, suggesting IGF-I not to be essential for ovarian function in humans (Dor *et al.*, 1992a). A disadvantage of the knockout mouse model is illustrated by the fact that, despite the importance of IGFs for the regulation of growth in many tissues and organs, the development of the animal as a whole is only mildly affected. This might indicate that, within the multitude of factors involved in auto- and paracrine regulation, some of these factors compensate for the absence of another. Therefore, a single factor or single gene knockout animal may not provide sufficient information. A "conditional transgenic model" in which genes in specific cell types and at specific times could be knocked out or activated would be more appropriate (see for review: Cohen-Tannoudji and Babinet, 1998).

As has been suggested previously, the growth of a single dominant follicle continues, despite decreasing FSH concentrations, due to intrafollicular enhancement of FSH action (van Santbrink *et al.*, 1995a). This enhancement is considered the result of induction and action of growth factors (Fauser and van Heusden, 1997). Whereas the dominant follicle continues its development, the remaining follicles respond to falling FSH levels by undergoing atresia. The significance of the duration of FSH elevation above the threshold, rather than the level of elevation of FSH for single dominant follicle selection is stressed in the proposed FSH window concept. This concept has been substantiated by the endocrinological and ultrasound data from our *in vivo* studies. The main conclusion is that elevating FSH levels high above the threshold level for a relatively short period of time in the early follicular phase does not increase the number of dominant follicles. In contrast, prevention of the physiological decrease of FSH in the late follicular phase allows several follicles to gain dominance. The FSH window concept provides the basis for recently developed ovulation induction protocols (van Santbrink, 1998). This so-called "step-down" regimen is more in correspondence with normal physiology than conventional protocols. Undesired complications, such as multiple follicle development and ovarian hyperstimulation syndrome can thus be reduced. On the other hand, the realisation that increasing FSH concentrations during the later stages of the follicular phase results in multiple dominant follicle growth, may form the basis of more efficient and effective treatment regimens for ovarian hyperstimulation for IVF.

Measurement of early follicular phase FSH concentration and inhibin-B concentration have been suggested to indicate patients who are less likely to respond successfully to ovarian stimulation (Meldrum, 1993; Ahmed Ebbiary *et al.*, 1994b; Kim *et al.*, 1997). In women approaching menopause, the diminished production of E_2 and inhibin-B by the depleting ovarian follicle pool, is considered to represent ovarian ageing (Gosden and Faddy, 1994). As a result, early follicular phase FSH concentrations gradually increase with advancing age. An increase in circulating FSH levels is generally considered the most consistent endocrine manifestation of ovarian ageing (Meldrum, 1993; Ahmed Ebbiary *et al.*, 1994a; Klein *et al.*, 1996a; Klein *et al.*, 1996b; Klein *et al.*, 1996c). This is not only restricted to older women, since also in younger, regularly menstruating women increased concentrations of FSH in the early follicular phase have been associated with decreased fertility (Ahmed Ebbiary *et al.*, 1994b). Considering the significance of local potentiation and modulation of FSH by growth factors, it is doubtful whether early follicular phase FSH is representative for ovarian function as such. Results from our study indicate large inter-individual differences in the FSH threshold level, as reflected by the maximum FSH concentration during the follicular phase. In 39 ovulatory women under the age of 35, no correlations could be demonstrated between

maximum FSH levels and age, cycle length or inhibin-B production. Moreover, no correlations were demonstrated between the number of small follicles and age or maximum FSH levels. These results suggest that the use of FSH concentrations as a marker for ovarian ageing are to interpreted with caution. The large inter-individual differences in FSH levels may reflect differences in the function of ovarian factors which potentiate or diminish FSH action. The balance between all these factors, including FSH, ultimately determines ovarian sensitivity. Further research is required to find parameters which may serve as predictors of ovarian ageing. Longitudinal measurement of early follicular phase FSH may provide a more sensitive test. A relatively large increase in FSH levels over time within a given person could indicate more advanced ageing (Macklon and Fauser, 1998).

In summary, local modulation of FSH action by a variety of (growth) factors plays a decisive role in normal ovarian follicle development and dominant follicle selection. Disregulation of the action or expression of growth factors in the ovary may be the basis of follicle arrest in normogonadotrophic anovulation or PCOS, rather than increased levels of factors inhibiting FSH receptor activation. In order to study in detail the local regulation and factors involved, *in vitro* models are required in which the complex interactions of the various factors remain as intact as possible. Cultures of preantral human follicles may provide a valuable option. Currently, studies are underway assessing the expression and function of growth factors involved in follicle development using this model. The appreciation of local enhancement of FSH action by growth factors has led to the proposal of the FSH window concept. The validity and significance of the FSH window concept is supported by clinical data. On the basis of these observations new protocols for treatment of disturbed ovarian function, such as the "step-down" ovulation induction protocol have been developed. Further studies regarding extending the FSH window during controlled ovarian hyperstimulation for IVF are to be performed. The large differences in ovarian sensitivity to FSH between individuals, as reflected by differences in the FSH threshold level, is determined by the balance between ovarian factors which enhance or diminish the FSH signal. Such factors, which ultimately may determine ovarian response, may be involved in the process of ovarian ageing. Which parameters may prove useful for prediction of ovarian ageing is the subject of future studies.

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Summary

Chapter I:
Early detailed anatomical descriptions of the human ovary with reference to the physiology of reproduction are found in 15th and 16th century literature. Recent developments in molecular biology and genetics have increased insights in the (patho)physiology of reproduction and have provided the basis for the treatment of reproductive disorders. A central role in the regulation of ovarian follicle development is performed by FSH. FSH activity is subject to modulation by factors interfering with the interaction of FSH with its receptor, or by factors modulating FSH-induced responses at an intracellular level. Data from the literature indicate the presence of specific FSH receptor inhibitors in serum and follicular fluid. In addition, a variety of systemic and locally produced growth factors, such as the IGFs, inhibins and activins, are involved in the modulation of FSH activity.

In patients suffering from normogonadotrophic anovulation, the disturbed regulation of follicle development is caused by a local disregulation of FSH activity. Whether this is due to specific FSH receptor inhibitors or a disregulation of locally acting growth factors is the subject of the present thesis. The principal study objectives were to examine whether: 1) specific FSH receptor inhibitors are present in elevated concentrations in patients with normogonadotrophic anovulation compared to normal controls, and 2) the development of ovarian follicles is determined by locally acting growth factors, which could potentiate FSH activity. This has been examined using *in vitro* cell cultures and by serum hormone assessment and ovarian ultrasound examination during the normal menstrual cycle in volunteers.

Chapter II:
In order to study FSH stimulation of granulosa cells, a well defined and validated *in vitro* culture system is indispensable. Initially, granulosa-lutein cells from pooled follicular aspirates were stimulated *in vitro* with FSH and LH. Cells were responsive to LH and displayed responsiveness to FSH only after 7 days of preincubation. The results indicate that the used follicular cells represent a granulosa-lutein cell population with diminished responsiveness to FSH. Therefore, one has to be cautious with interpretation of data obtained through this widely used culture system for study of local regulation of follicular development.

Granulosa cells not stimulated with exogenous gonadotrophins were isolated from developing follicles from ovaries in patients undergoing elective surgery for benign gynaecological conditions. In culture the granulosa cells displayed a large variation in aromatase activity after stimulation with FSH. This could partially be attributed to the limited availability and the variable quality of the excised

tissue. In order to apply this method for *in vitro* studies of granulosa cell function and setting up an *in vitro* bioassay, a higher quantity and quality of ovarian tissue need to be obtained.

Modulation of FSH by factors which act directly on the FSH receptor has been studied, using Chinese hamster ovary cells (CHO-F3B4), stably transfected with the human FSH receptor. CHO cells were incubated with human recombinant FSH (recFSH) and adenylate cyclase activity (cAMP) was measured. After addition of increasing amounts (up to 90% of the incubation volume) of hypogonadotrophic human serum (HS) the cAMP production was inhibited to approximately 40-60% of the control value. In contrast, FSH-stimulated oestradiol production in rat Sertoli cells was almost completely inhibited by addition of only 10% HS, suggesting that serum has more pronounced effects on events downstream from receptor activation. It is concluded that the inhibitory effects of serum on FSH-stimulated cAMP production in CHO-F3B4 cells are small, when compared to the inhibition of aromatase induction in rat Sertoli cells. Therefore, the CHO-F3B4 cells provide a useful model to measure inhibitory activity by serum or follicular fluid of FSH receptor activation.

In patients with normogonadotrophic anovulation, either with or without polycystic ovary syndrome (PCOS), factors interfering with FSH action may be involved in arrested follicle development. FSH-stimulated cAMP secretion in CHO-F3B4 cells was measured in the presence of serum or follicular fluid from regularly cycling women (n = 9) and anovulatory patients, without (n = 13) and with (n = 16) PCOS. The inhibition of FSH-stimulated cAMP production in the presence of serum samples from anovulatory patients, without or with PCOS, was similar to that from regularly cycling controls. Also follicular fluid samples obtained during the follicular phase in regularly cycling women, and follicular fluid samples from PCOS patients were tested. No differences in inhibition of FSH-stimulated cAMP production were found comparing PCOS follicles with size- and steroid content-matched follicles obtained during the normal follicular phase. The amount of inhibition of FSH receptor activation was similar in serum and follicular fluid. It is concluded that the inhibition of FSH receptor activation by proteins present in serum or follicular fluid may be of limited significance for normal and arrested follicle development.

Since inhibition of FSH receptor activation in the presence of HS appeared to be concentration independent at high concentrations of serum, serum constituents could act as a buffer against FSH receptor inhibitors. It was decided to validate this buffering activity of serum by using protamine, an inhibitor of G protein-coupled receptor activation. Addition of 10 μg/ml protamine inhibited FSH-stimulated cAMP production in CHO-F3B4 cells, whereas in the presence of HS this inhibitory effect was completely annihilated, leaving an inhibitory effect as observed in the presence of HS only. It is concluded that the

activity of an inhibitor depends on the concentration of serum proteins in the incubation media, which may act as a buffer. In a similar fashion, serum proteins could quench the inhibitory effects of naturally occurring inhibitory substances.

Chapter III:
According to the threshold concept, the FSH concentration need to surpass a distinct level in order to stimulate ovarian follicle growth. The window concept stresses the significance of a limited duration of elevated FSH levels above the threshold for single dominant follicle selection. In order to investigate effects on follicle growth of increased FSH levels - differing in duration and magnitude of elevation - during the follicular phase, a series of daily transvaginal sonography scans (TVS) of the ovaries and blood sampling (assayed for FSH and E_2) was performed during 2 consecutive cycles in normo-ovulatory women. After a control cycle, subjects were randomised and received either 375 IU urinary FSH SC as a single injection on the 14th day after the preceding LH peak (Day_{LH+14}) or 75 IU daily later in the follicular phase on 5 consecutive days, from Day_{LH+19} until Day_{LH+23}. Results indicate that a brief, but distinct elevation of FSH levels above the threshold in the early follicular phase does not affect dominant follicle development, although the number of small antral follicles did increase. In contrast, a moderate but continued elevation of FSH levels during the mid-late follicular phase (effectively preventing decremental FSH concentrations) does interfere with single dominant follicle selection and induces ongoing growth of multiple follicles. These findings substantiate the FSH window concept and support the notion of enhanced sensitivity of more mature follicles for stimulation by FSH.

It is hypothesized that the gradual increase in FSH concentrations in women approaching menopause results from the depletion of the ovarian follicular pool, a process referred to as "ovarian ageing". However, large individual differences in early follicular phase FSH levels, as observed in normo-ovulatory young women, suggest that relatively high serum FSH concentrations may not necessarily indicate ovarian ageing. In order to examine to what extent the variation in endogenous FSH stimulation of the ovary is related to menstrual cycle characteristics, serum levels of FSH, E_2 and inhibin-A and -B were measured throughout the follicular phase in 39 healthy volunteers in combination with ultrasound assessment of follicular growth. Median maximum follicular phase FSH was 6.2 IU/l with a 2.6-fold difference between lowest and highest values (range: 4.3 - 11.2 IU/l), and was observed on CD 6 (range: 2 - 15). The maximum FSH levels did not correlate with age, cycle length, or the maximum serum inhibin-B concentration. The mean number of small antral follicles in both ovaries on CD 3 did not correlate with age, nor with maximum FSH. These observations indicate a lack of correlation between maximum follicular phase serum FSH concentrations and parameters of ovarian ageing in women

under the age of 35. In addition, FSH levels assessed on cycle day 3 represent an underestimation of maximum early follicular phase FSH. Although FSH is crucial for the stimulation of follicle development, distinct individual differences in intra-ovarian modification of FSH action resulting in differences in the FSH threshold for stimulation of ovarian function may be operative.

Chapter IV:
In this chapter the reader is provided with an overview of the results and conclusions from the preceding studies. These findings are discussed in view of existing knowledge and of future perspectives.

Samenvatting

Hoofdstuk I:
Vroege anatomische beschrijvingen van het ovarium in relatie tot de fysiologie van de voortplanting worden aangetroffen in de 15de en 16de eeuw. Recente ontwikkelingen in de moleculaire biologie hebben de inzichten in de (patho)fysiologie van de voortplanting vergroot en vormen de basis voor de behandeling van fertiliteitstoornissen. Centraal in de regulatie van follikelontwikkeling in het ovarium staat het follikel-stimulerend hormoon (FSH). De activiteit van FSH wordt lokaal gereguleerd door factoren. In de literatuur worden verschillende factoren beschreven die binding en activering van de FSH receptor remmen. Daarnaast zijn een groot aantal groeifactoren bekend, zoals de IGF's, inhibine en activine, die via eigen receptoren de FSH activiteit moduleren op intracellulair niveau.

In patiënten met normogonadotrofe anovulatie wordt de verstoring van follikelontwikkeling veroorzaakt door lokale disregulatie van FSH. In hoeverre dit veroorzaakt wordt door specifieke FSH receptor remmers, dan wel door disregulatie van lokaal werkende groeifactoren is onduidelijk. In dit proefschrift worden twee hypotheses onderzocht: 1) bij patiënten met normogonadotrofe anovulatie bevinden zich in het serum en follikelvocht verhoogde concentraties van specifieke FSH receptor remmers; en 2) lokaal werkende groeifactoren die de werking van FSH versterken zijn van doorslaggevend belang voor normale follikelontwikkeling en selectie van een dominante follikel. Dit is onderzocht met behulp van *in vitro* celkweken en door middel van hormoonbepalingen in serum en echoscopische bestudering van follikelontwikkeling tijdens de normale menstruele cyclus.

Hoofdstuk II:
Om lokale modulatie van FSH *in vitro* te bestuderen, is gebruik gemaakt van gekweekte granulosa-luteine cellen geïsoleerd uit follikelpunctaten, verkregen tijdens IVF behandeling. Stimulatie van de cellen met gonadotrofinen toonde een hoge aromatase response van de cellen op stimulatie met LH en slechts geringe respons op FSH-stimulatie. Deze resultaten geven aan, dat dergelijke granulosa-luteine cellen mogelijk niet representatief zijn voor de celpopulatie in zich ontwikkelende follikels. Voor het *in vitro* bestuderen van lokale regulatie van FSH-activiteit blijkt dit model minder geschikt te zijn.

Granulosa celen werden geïsoleerd uit zich ontwikkelende follikels tijdens de normale menstruele cyclus uit ovaria van patiënten, die electief werden geopereerd. In kweek vertoonden de cellen een grote spreiding in aromatase respons na stimulatie met FSH. De grote verschillen in kwaliteit en vitaliteit van de geïsoleerde cellen en de beperkt beschikbare hoeveelheid van het geëxcideerde weefsel, belemmeren validering en toepassing van dit *in vitro* model voor de

bestudering van lokale modulatie van FSH activiteit.

Voor de studie naar factoren die direct aangrijpen op de FSH receptor is gebruik gemaakt van een Chinese hamster ovarium cellijn (CHO-F3B4), welke stabiel is getransfecteerd met de humane FSH receptor. Stimulatie van deze cellen met humaan recombinant FSH resulteert in een meetbare adenylate cyclase respons (cAMP). Na toevoeging van humaan hypogonadotroop serum (HS) in een concentratie van 90% v/v, daalde de cAMP productie door de CHO cellen tot maximaal 40-60% van de controle waarde. Daarentegen werd in gekweekte ratten Sertoli cellen een vrijwel complete remming van de FSH gestimuleerde E_2 productie waargenomen na toevoeging van slechts 10% HS. De resultaten duiden op een remming door factoren in serum van meer distaal, intracellulair, gelegen activering paden, in tegenstelling tot remming van de FSH receptor zelf. Met behulp van dit CHO model kunnen factoren, die specifiek de FSH receptor remmen, goed bestudeerd worden.

In patiënten lijdend aan normogonadotrofe anovulatie of polycysteus ovarium syndroom (PCOS), wordt de verstoorde follikelontwikkeling mogelijk veroorzaakt door factoren die aangrijpen op de FSH receptor en zo stimulatie van follikelgroei verhinderen. FSH gestimuleerde cAMP productie in CHO cellen in aanwezigheid van serum van controles met regulaire cyclus (n = 9) en van anovulatoire patiënten, zonder (n = 13) en met (n = 16) PCOS, toonde geen verschil in remming tussen deze groepen. Ook follikelvocht monsters uit normale en PCOS ovaria gaven geen verschil in remming van FSH receptor activering te zien. De mate van remming door serum was gelijk aan die door follikelvocht, ongeacht de follikelgrootte of steroid concentraties van het vocht. Deze resultaten duiden op een beperkte betekenis van de remming van FSH receptor activering in de (patho)fysiologie van follikelontwikkeling.

Het blijkt dat de mate van remming van FSH receptor activering *in vitro* bij hogere serumconcentraties (30 tot 90% v/v), onafhankelijk is van de serumconcentratie in het kweekmedium. De resultaten suggereren een buffering door serumeiwitten van FSH receptor remmers. Dit is getoetst met protamine, een remmer van activering van G eiwit-gekoppelde transmembraanreceptoren. Toevoeging van 10 µg/ml protamine remde de FSH-gestimuleerde cAMP productie in CHO-F3B4 cellen tot 5-10% van de controlewaarde. De protamine specifieke remming kon volledig worden opgeheven door toevoeging van HS. Daar dergelijke hoge serumconcentraties vergelijkbaar zijn met de fysiologische eiwitconcentraties in het ovarium zouden op deze wijze natuurlijk voorkomende FSH receptor remmers gebufferd kunnen worden.

Hoofdstuk III:

Volgens het "FSH drempel" concept is stijging van de FSH-concentratie boven een bepaalde drempelwaarde noodzakelijk om voortgaande follikelgroei te stimuleren. Het "FSH window" concept daarentegen legt nadruk op de

tijdsduur van de overschrijding van de drempelconcentratie. Door deze tijdsduur te beperken wordt voortgaande groei van slechts één dominante follikel gewaarborgd. De effecten van verhoogde FSH concentraties in de folliculaire fase – verschillend in duur en grootte van stijging – zijn bestudeerd in een groep van 23 jonge vrouwen met regulaire menstruele cyclus. Van de proefpersonen werd dagelijks serum E_2 en FSH concentraties bepaald en follikelgroei echoscopisch vervolgd. Metingen werden verricht in 2 opeenvolgende cycli. Na een controle cyclus werden de proefpersonen voor de tweede cyclus gerandomiseerd in 2 groepen. In behandelgroep A kregen de proefpersonen eenmalig 375 IE urinair FSH sc toegediend op de 14^{de} dag na de voorafgaande LH piek (Dag_{LH+14}). In behandelgroep B werd dagelijks 75 IE FSH toegediend gedurende 5 opeenvolgende dagen op een later tijdstip in de folliculaire fase, vanaf Dag_{LH+19} tot en met Dag_{LH+23}. De resultaten geven aan dat een korte, doch sterke stijging van de FSH spiegels boven de drempelwaarde vroeg in de folliculaire fase de dominante follikelgroei niet beïnvloedt, ondanks een toename in de groei van kleine follikels. Daarentegen resulteert een bescheiden maar langdurige verhoging van FSH spiegels tijdens de mid-folliculaire fase (waardoor de fysiologische daling van FSH wordt voorkomen) wèl in een toegenomen aantal dominante follikels. Deze bevindingen bevestigen het FSH window concept en ondersteunen de veronderstelling dat de gevoeligheid voor FSH toeneemt gedurende de ontwikkeling van de dominante follikel.

De geleidelijke stijging in FSH spiegels in vrouwen die de menopausale leeftijd naderen wordt geacht het gevolg te zijn van een afname van de follikelvoorraad in het ovarium, een proces wat geduid wordt als "ovarian ageing". Echter, in jonge, normo-ovulatoire vrouwen worden grote verschillen in de vroeg-folliculaire FSH spiegels aangetroffen. Dit zou erop kunnen wijzen dat hoge FSH spiegels niet noodzakelijkerwijs ovarian ageing aanduiden. In hoeverre de hoogte van de FSH concentratie in de folliculaire fase gerelateerd is aan karakteristieken van de menstruele cyclus, is onderzocht in een groep van 39 jonge vrijwilligsters. Gedurende de folliculaire fase werden dagelijks serum FSH, E_2 en inhibine-A en –B concentraties bepaald, in combinatie met echoscopische meting van follikelgroei. De mediaan van de maximale FSH spiegel in de folliculaire fase bedroeg 6.2 IU/l met een 2.6-voudig verschil tussen de hoogste en laagste gevonden spiegels (spreiding: 4.3 – 11.2 IU/l). Deze concentratie werd gemeten op cyclusdag 6 (spreiding: 2 – 15). De maximale FSH concentratie correleerde niet met leeftijd, cycluslengte of maximum inhibine-B concentratie. Het gemiddelde aantal kleine, antrale follikels op cyclusdag 3 correleerde evenmin met leeftijd of maximum FSH concentratie. Deze observaties duiden op een afwezigheid van correlatie tussen maximale FSH concentraties in de folliculaire fase en parameters voor ovarian ageing bij vrouwen onder de 35 jaar. Bovendien blijkt dat de FSH-concentratie in serum

op cyclusdag 3 een onderschatting geeft van de maximale FSH concentratie tijdens de folliculaire fase. Hoewel FSH van doorslaggevende betekenis is voor de normale follikelontwikkeling, is het zeer wel mogelijk dat de interindividuele verschillen in de FSH drempelwaarde bepaald worden door verschillen in intra-ovariële modificatie van FSH activiteit.

Hoofdstuk IV:
In dit hoofdstuk wordt ingegaan op de resultaten en conclusies van de voorafgaande studies. De bevindingen worden beschouwd tegen de achtergrond van de bestaande inzichten en geplaatst in het licht van toekomstig onderzoek.

Publications

Publications included in the present thesis:

Schipper, I., Fauser, B.C.J.M., van Gaver, E.B.O., Zarutskie, P.W. and Dahl, K.D. (1993) Development of a human granulose cell culture model with follicle-stimulating hormone responsiveness. *Human Reproduction,* **8**, 1380-1386.

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Schipper, I., Fauser, B.C.J.M., van Gaver, E.B.O., Zarutskie, P.W. and Dahl, K.D. (1992). Development of an in vitro model to investigate the effects of local factors on human granulosa cells. *74th Annual Meeting of the Endocrine Scociety, June 24-27, 1992, San Antonio, Texas.*

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Curriculum vitae auctoris

De schrijver van dit proefschrift, Izaäk (Jits) Schipper werd op 17 december 1964 geboren te Leiden. Tot zijn 11de groeide hij op te Ujung Pandang, Indonesië. Na terugkeer in Nederland doorliep hij de middelbare school en behaalde in 1983 aan het Revius Lyceum te Doorn het diploma Gymnasium b. In datzelfde jaar begon hij met de studie Geneeskunde aan de Erasmus Universiteit Rotterdam. Het artsexamen behaalde hij op 15 juni 1990. Reeds in de laatste fase van de studie werd het fundament gelegd voor het in dit proefschrift beschreven onderzoek en werd de belangstelling voor gynaecologie gewekt. Van september 1990 tot november 1991 vervulde hij de militaire dienstplicht in het militair hospitaal "Dr. A. Mathijssen" te Utrecht. Als artsassistent was hij werkzaam bij de afdeling Gynaecologie/Obstetrie (hoofd: Dr. Ph. Stoutenbeek) en na sluiting hiervan bij de afdeling Orthopedie (hoofd: Dr. A.A. van den Berg). In november 1991 begon hij met promotieonderzoek onder leiding van Prof. Dr. B.C.J.M. Fauser. Om basale en technische kennis hiervoor te verwerven verbleef hij gedurende 10 maanden te Seattle, USA, Department of Reproductive Endocrinology, VA Medical Center en University of Washington Medical School (Prof. W.J. Bremner en Dr. K.D. Dahl). Vanaf augustus 1992 was hij als OIO in dienst bij de Nederlandse Organisatie voor Wetenschappelijk Onderzoek en verrichtte hij de studies zoals beschreven in dit proefschrift bij het Instituut Endocrinologie & Voortplanting, Erasmus Universiteit Rotterdam (hoofd Prof. Dr. J.A. Grootegoed, begeleider en copromotor Dr. F.F.G. Rommerts) en bij de Sector Voortplantingsgeneeskunde, afdeling Verloskunde en Vrouwenziekten, Academisch Ziekenhuis Rotterdam (hoofd: Prof. Dr. B.C.J.M. Fauser). Na beëindiging van de praktische werkzaamheden voor dit onderzoek trad hij in maart 1997 in dienst als assistentgeneeskundige bij de afdeling Verloskunde en Vrouwenziekten, Academisch Ziekenhuis Rotterdam. Op 1 september 1998 begon hij als assistentgeneeskundige in opleiding tot gynaecoloog in het Zuiderziekenhuis te Rotterdam (opleider: Dr. M. van Lent). Sinds 1 september 1999 vervolgt hij zijn opleiding aan het Academisch Ziekenhuis Rotterdam (opleider: Prof. Dr. Th.J.M. Helmerhorst).

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