EXOGENOUS FOLLICLE-STIMULATING HORMONE AND DEVELOPMENT OF HUMAN OVARIAN FOLLICLES

FOLLIKEL STIMULEREND HORMOON EN ONTWIKKELING VAN FOLLIKELS IN HET HUMANE OVARIUM TIJDENS GONADOTROFINE TOEDIENING

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Voor mijn ouders,
Jeanine, Karlijne en Annelotte

Contents

List of abbreviations

AD	androstenedione
BMI	body mass index
CHO	Chinese hamster ovary
DHEAS	dehydroepiandrosterone sulphate
E_2	17β-estradiol
FAI	free androgen index
F-G score	Ferriman/Gallwey score
FSH	follicle-stimulating hormone
GH	growth hormone
GnRH	gonadotropin releasing hormone
GnRH-a	gonadotropin releasing hormone-agonist
hCG	human chorionic gonadotropin
HMG	human menopausal gonadotropin
HPG	human pituitary gonadotropin
HX	hypophysectomized
IFMA	immunofluorimetric assay
IGD	isolated gonadotropin deficiency
IGF	insulin-like growth factor
im	intramuscular
INH	inhibin
IRMA	immunoradiometric assay
iv	intravenous
IVF	*in-vitro* fertilization
KS	Kallman syndrome
LH	luteinizing hormone
OHSS	ovarian hyperstimulation syndrome
PCOS	polycystic ovarian syndrome
pFSH	pure follicle-stimulating hormone
PMSG	pregnant mare serum gonadotropins
recFSH	recombinant follicle-stimulating hormone
rhFSH	human recombinant follicle-stimulating hormone
RIA	radioimmunoassay
sc	subcutaneous
SD	standard deviation
SEM	standard error of the mean
SHBG	sex-hormone-binding globulin
T	testosterone
TSH	thyroid stimulating hormone

1

Introduction

1.1 FOLLICLE-STIMULATING HORMONE

1.1.1 History

Vesalius (1515–1564) described the hypophysis as 'glandula pituitaria cerebri excipiens', 'the gland that extracts mucus from the brain'. Several centuries later, the anterior part of this small organ at the base of the hypothalamus embedded in the sella turcica appeared to produce specific gonad-stimulating substances. Injections of pituitary extracts in infantile rats (Smith & Engle, 1927) demonstrated that effects of hypogonadism were reversible. Similar effects on gonads were reported in animals in response to injection of urine of pregnant women (Aschheim & Zondek, 1927). These two German scientists described the pars anterior of the hypophysis as 'den Motor der Sexualfunktionen'. However, confusing results were obtained administering this urine to immature animals, achieving merely luteinization, whereas combining it with pituitary extracts also induced ovarian growth and ovulation (Engle, 1929). Simultaneously, the hypothesis of two gonad-influencing factors; Prolan A and Prolan B ('Follikelreifungshormon' and 'luteinisierendes hormon', respectively) was postulated (Zondek, 1930). It was found that Prolan B was not produced by the hypophysis but present in urine of pregnant women as an 'anterior pituitary-like hormone' (Collip et al., 1933). Luteinizing gonadotropins in urine of pregnant women were found to be of placental origin, whereas urine of climacteric women predominantly induced stimulation of follicle development in the ovaries (Leonard & Smith, 1934). Partial separation of two different gonadotropins was reported in extracts of the anterior lobe of the hypophysis (Fevold et al., 1933). One of these two gonadotropins was found to affect growth of ovarian follicles, whereas the second was capable of inducing luteinization ('gonad-stimulating hormone' = follicle-stimulating hormone [FSH] and luteinizing hormone [LH]). Wide clinical application of gonadotropins derived from human pituitary extracts and pregnant mare serum (PMSG) was hampered by scarcity of the original substrate. The clinical use was stopped following detection of anti-gonadotropin antibody formation (Leathum & Rakoff, 1948). Almost twenty years following isolation of gonadotropins from the pituitary gland, a novel way of extracting FSH-like substance from postmenopausal urine

was published (Borth et al., 1954). Gel-electroforesis of urine provided a new way to improve the purification and extraction process (Albrecht, 1956; Borth et al., 1957). This innovation in the production process of gonadotropins caused the wide introduction of these hormones as therapy for various disease states; primarily to induce follicle growth in infertile anovulatory women.

It is now possible to produce human recombinant FSH (rhFSH) through the transfection of Chinese hamster ovary cell lines with human α and β protein subunit genes (Keene et al., 1989). This technology provides the opportunity to modulate the chemical structure of FSH and develop FSH agonists as well as FSH antagonists (Boime, 1990). This may disclose a whole new era of clinical applications.

1.1.2 Structure and function

The group of pituitary glycoprotein hormones includes FSH, LH, thyroid-stimulating hormone (TSH) and placental human chorionic gonadotropin (hCG). The gonadotropins (molecular weight 28,000 for LH and 33,000 for FSH), produced by the anterior lobe of the pituitary gland are structured as a heterodimer of two noncovalently associated protein subunits, α and β, encoded by separate genes located on different chromosomes (Boim et al., 1992) in the human. The structure of the α subunits of all pituitary glycoproteins is identical. However, the β chains are unique and after linkage to the α chain determine specific hormone function (Fauser & Hsueh, 1989). Modification of glycosylation (carbohydrate additions to proteins) during the intracellular translational and posttranslational period appears to be the basis for heterogeneity of FSH molecules with respect to biochemical structure and biological potencies (Chappel et al., 1983). Differences in carbohydrate moieties of the glycoprotein (linkage of sialic acid/sulfate) cause differences in metabolic clearance and biologic activity (serum half-life as well as receptor affinity)(Boime et al., 1992). Changes in isoform distribution represent a potential way of modulating FSH biopotency (Fauser et al., 1989). The assembly and modification of glycoprotein subunits to produce functional heterodimers is a complex process which takes place in the endoplasmatic reticulum and the Golgi apparatus. Ultimately, the hormone is stored in secretory granules before secretion into the peripheral vessels. Finally, FSH and LH exert their effects by binding to specific membrane receptors on granulosa cells (Midgley et al., 1973), activating the second messenger (G protein and cAMP-system) and subsequently protein kinase A, resulting in stimulation of gene expression (Spiegel et al., 1985). Serum gonadotropin concentrations can be estimated using radioimmunoassays (RIA) containing radioactively-labelled antigens raised against specific parts of the dimers. Initially, clinical application of RIA was hampered by cross-reactivity (affinity

to α subunit as part of other glycoprotein dimers or to free-circulating biologically inactive α subunit). Presently, immunoassays have been improved and additional techniques for hormone assays are available: e.g. immunoradiometric assays (IRMA) and immunofluorimetric assays (IFMA). These tests use two highly specific monoclonal antibodies raised against amino-acid sequences of each of the glycoprotein subunits (Fauser et al., 1991). However, these "sandwich assays" (they enclose the glycoprotein from different sides) can still show cross-reactivity by attachment to non-specific epitopes. This illustrates some potential causes for discrepancies between results of immunological assay methods and actual biological activity. *In-vivo* bioassays serve to compare biological effects of gonadotropin preparations in a quantitative manner in animals. In the widely used Steelman-Pohley assay (Steelman & Pohley, 1953), 21 day-old female Sprague-Dawley rats are injected subcutaneously for 3 days and ovaries weighed on the 4th day. Disadvantages of this assay are that sensitivity is too low to detect small the amounts of FSH in serum, reproducibility is poor and the procedure is cumbersome (Fauser & Hsueh, 1988). However, the ability to take metabolic half-life of the hormone into account is an important advantage of the *in-vivo* test. Therefore, biopotencies of commercially available urinary gonadotropin preparations are still measured using the Steelman-Pooley assay. *In-vitro* bioassays were introduced in order to improve sensitivity and to develop easier tests with improved reproducibility. Various *in-vitro* cultures, using different animal or human target tissues and different end points, have been tested (Van Damme et al., 1974 [LH bioassay in mouse leydig cells]; Padmanabhan et al., 1987 [FSH bioassay in granulosa cells]; Dahl et al., 1989 [modified FSH bioassay in human granulosa cells]).

Circulating levels of the gonadotropins as measured at any given moment represent the balance between pituitary release and metabolic clearance. Following intravenous injection, the half-life of urinary FSH was demonstrated to be approximately 2 hours (Franchimont, 1971). FSH disappearance following hypophysectomy resulted in a fast part of the disappearance curve showing a half-life of approximately 4 hours, whereas the slow part demonstrated a half-life of approximately 70 hours (Yen et al., 1970). In contrast to FSH, LH was reported to have a rapid disappearance (half-life about 20 minutes for the fast part of the curve, about 230 minutes for the slow component, following hypophysectomy (Yen et al., 1968). Half-life of hCG varied between 6–9 hours during the first part of the curve and 24 to 37 hours during the second part (Franchimont & Burger, 1975). Serum half-life of intramuscularly administered hCG was determined to be approximately 32 hours, whereas after subcutaneous administration half-life was extended (38 ± 3 hours) (Saal et al., 1991). Following intramuscular injection of urinary FSH preparations, the half-life was estimated to be approximately 35 hours (Diczfaluzy & Harlin, 1988). Maximum serum

concentrations and area under the curve (AUC) appeared to be dose-dependent following a single injection, whereas time to reach maximum FSH serum levels was dose-independent.

The long half-life of purified follicle-stimulating hormone (pFSH) after intramuscular administration (>24 hours) induces accumulating serum FSH levels (Sharma et al., 1987; Mizunuma et al., 1990). In addition, pharmacokinetic data show gradual but distinct accumulation of circulating FSH in the majority of patients (Diczfaluzy & Harlin, 1988); steady state levels were demonstrated following approximately 4–5 days of daily pFSH administration in hypogonadotropic women (Mizunuma et al., 1990). Based on these data, computed simulation patterns of the time-concentration profile of serum FSH exhibited steady state levels following approximately 2 to 3 days after decreasing the gonadotropin doses. Half-life of recombinant human FSH appeared to be around 44 hours following a single intramuscular injection in hypogonadotropic female volunteers and was related to body weight (Mannaerts et al., 1993). Following weekly increasing administration of doses of rhFSH, steady state levels appeared after approximately 4 to 5 days (Section 2.3). Despite the unknown biochemical basis, significant differences between various lots of commercially available urinary gonadotropin preparations were reported concerning their ability to stimulate follicular development and ovulation in an *in-vivo* hamster ovulation bioassay (Cook et al., 1988).

1.2 REGULATION OF OVARIAN FUNCTION

1.2.1 Initiation of growth of primordial follicles

Approximately two million primordial follicles are present in the human ovaries at birth (Peters, 1979). At menarche not more than 400,000 to 500,000 are left (Hillier et al., 1981). These follicles are characterized by an oocyte in the prophase of the first meiotic division, surrounded by a single layer of granulosa cells. Transformation of granulosa cells from a flat to a cuboid shape and simultaneous oocyte growth leads to formation of a primary follicle (for review see Pache, 1993). This initiation of growth is thought to be a continuous process independent from gonadotropins (Hisaw, 1947; Peters et al., 1973), although some animal studies suggest that minute amounts of gonadotropins need to be present. The assumption that primordial follicles can develop in the absence of gonadotropins is based on observation of ovarian changes during childhood, and continued early folliculogenesis following hypophysectomy (Edwards et al., 1977). Secondary or preantral follicles are typified by an increase of granulosa cell layers (stratum granulosum). Formation of an antrum within the granulosa cell layers characterizes the advancement to the antral stage. The maturing follicle can

either continue its development or undergo atresia. The process of initiation of growth and subsequent atresia is responsible for the overall decrease in numbers of follicles present in the ovary. Apoptosis ('programmed cell death') appears to underlie this atretic process, occurring predominantly in antral follicles (Billig et al., 1993). The time elapsed between the primordial stage and development of a preovulatory follicle is not yet clearly defined in the human. Morphological studies (Gougeon, 1982) suggest that successive waves of growing follicles ensure a continuous process of folliculogenesis. The overall life-span of a maturing ovulatory follicle can be estimated by summation of: a) 60 days (until a size of 1 mm), b) 2 weeks (during the preceding luteal phase [until a size of 4 to 6 mm]), and c) 2 weeks (corresponding to the follicular phase of the ovulatory cycle [until a size of approximately 20 mm]) (Gougeon, 1982).

1.2.2 Granulosa and theca cell steroid production

Early investigations (Fevold, 1941) pointed towards the synergism of both gonadotropins for stimulation of ovarian estrogen production. The observation that two different ovarian cell types (the theca cells stimulated by LH, and the granulosa cells controlled by FSH) are involved in estrogen synthesis, was initially reported by Falck (1959). Subsequently, this hypothesis was confirmed using highly purified urinary FSH in a hypogonadotropic model; in rodents (immature rats [Eshkol et al., 1967] and in females [Couzinet et al., 1988]). Administering highly purified urinary FSH preparations (containing only minute amounts of LH) resulted in diminished estrogen production but normal follicle growth. The concept that both theca cells and granulosa cells are needed for adequate estrogen biosynthesis is referred to as the 'two-cell, two-gonadotropin' hypothesis.

FSH membrane receptors are exclusively present on granulosa cells (Menigish et al., 1991), whereas LH receptors can be observed on both theca (Rajaniemi & Vanha–Perttula, 1972) and granulosa cells (Erickson et al., 1979b). LH stimulates theca cells to convert intracellularly stored C27 steroids (cholesterol), by the cytochrome P450 side-chain cleavage enzyme complex (P450 SCC) to pregnenolone. This rate-limiting first conversion process is followed by two possible routes; a) the predominant pathway in the human (delta-4 pathway) leading to biosynthesis androstenedione (AD) (Ryan & Smith, 1965), and the delta-5 pathway resulting in formation of dehydroepiandrosterone (DHEAS) (Aakvaag, 1969) (for comprehensive review see Gore-Langton & Armstrong, 1988). AD will transverse the basal lamina and accumulate in the antral fluid of the developing follicle (McNatty & Baird, 1978). This provides granulosa cells with the substrate which can subsequently be converted by the cytochrome P450 aromatase enzyme complex in 17β-estradiol (E_2). Specific enzyme activities are limited to

different types of ovarian tissue (theca cells or granulosa cells). Estrogens in turn promote FSH receptor proliferation on granulosa cells, amplify aromatase activity and enhance the ability of FSH to induce LH receptors (Hillier et al., 1988). E_2 concentrations in pooled fluid obtained from large follicles appeared to be >1000-fold higher as compared to simultaneously pooled fluid from small follicles (< 8 mm) and levels correlate with follicle size (Westergaard et al., 1986). It was also shown that granulosa cell numbers correlate with follicular size and E_2 concentrations in follicular fluid (McNatty et al., 1976; McNatty et al., 1979a; McNatty et al., 1979b; McNatty et al., 1979c, Westergaard et al., 1986).

1.2.3 Estrogens and follicle development

The selection of a single dominant follicle is associated with increased aromatase activity and a subsequent rise in intrafollicular E_2 production (Erickson et al., 1979; Hillier et al., 1981). Confirmation that E_2 is produced by the dominant follilcle was provided by the observation of elevated E_2 levels in venous plasma draining the ovary bearing a follicle larger than 10 mm as compared to concentrations at the contralateral side (Baird & Fraser, 1975). In this way, the selected follicle is able to maintain a highly estrogenic local environment. Due to incremental E_2 levels in peripheral blood, negative feedback actions are exerted, leading to decreasing FSH serum levels and securing single dominant follicle development. Local actions of E_2 – as a potential autocrine regulator – are: a) increase of mitogenic activity (causing further granulosa cell proliferation and growth of the follicle) (Williams, 1940), b) inhibition of atresia (Richards, 1975), c) increment of FSH and induction of LH receptor content of granulosa cells (Rao et al., 1978), d) enhancement of FSH-induced aromatase activity, and e) stimulation of production of follicular fluid. This autocrine up-regulation by E_2 could underlie continued development of follicles in the presence of decreasing FSH serum levels. An increasing number of potential local modulators of FSH action, such as activin/inhibin (Miro & Hillier, 1992), insulin-like growth factor (IGF) and binding proteins (Fauser & Hsueh, 1988; Adashi et al., 1989) may play important roles with respect to intrafollicular estrogen synthesis. However, to a large extent these observations are based on *in-vitro* animal studies, and it is unclear as yet what their role is in human ovarian physiology.

Several pathophysiological conditions suggest that elevated E_2 concentrations are not mandatory for the induction of follicular growth (Rabinovici et al., 1991, chapter 2.2). More recently, a difference in *in-vitro* function of FSH and LH was delineated; FSH appeared to support steroid production as well as cell proliferation and LH solely stimulated steroidogenesis (Yong et al., 1992). An additional observation questioning the possibility of direct actions of estrogens include the absence of E_2

receptors on granulosa cells in the primate ovary (Hild-Petito et al., 1988). A local direct role of estrogens remains questionable and the need for coordinate action of both FSH and LH leading to estrogen production and follicle growth needs to be reexamined. The minimal requirements of LH to achieve steroidogenesis and FSH to stimulate follicle growth need further elucidation.

1.2.4 Dynamics of follicle growth and selection during the menstrual cycle: The FSH-'threshold'/'window' concept

Initially, developmental characteristics of the dominant follicle were documented by sequential scanning throughout the normal cycle using transabdominal ultrasound (O'Herlihy et al. 1980, Queenan et al. 1980, Kerin et al. 1981). Under optimal conditions, a varying number of small follicles will be visible at a size of 6 to 8 mm in the early follicular phase (day 3 to 5). In a longitudinal study using transvaginal ultrasound in regularly menstruating women, it was possible to discriminate the dominant follicle by its size (>9 mm) (Pache et al., 1990; van Santbrink et al., 1995). Furthermore, serum E_2 levels increased significantly only after a dominant follicle could be discriminated by sonography (Fauser et al., 1993c, van Santbrink et al., 1995). These data correspond with *in-vitro* findings (Erickson et al., 1979a, Hillier et al., 1981) demonstrating significant induction of aromatase activity only in granulosa cells obtained from follicles beyond a size of 6–8 mm. Cross-sectional (non-dynamic) morphological studies (Chikazawa et al., 1986, McNatty et al., 1983) assume that selection starts at approximately 4 to 8 mm in follicular size. Nevertheless, *in-vitro* granulosa cell studies indicate that there is a distinct moment at which up-regulation of granulosa cell function may be observed. Beyond a diameter of 9 mm, the mean diameter of the selected follicle will increase by approximately 1 to 2 mm daily (Ylöstalo et al., 1979; van Santbrink et al., 1995a), until ovulation at approximately 18–26 mm (Wetzels 1983; Leerentveld et al., 1984). Remaining antral follicles from the recruited pool will lack sufficient stimulation by FSH (due to decreasing concentrations during the late follicluar phase) and become atretic.

Although mechanisms by which selection of a single dominant follicle occurs during the follicular fase of the normal cycle remain to be elucidated, the 'FSH-threshold' concept initially put forward by Brown (Brown, 1978) has gained wide support. Brown developed this concept as a clinician, based on observation of ovarian response (as monitored by urinary estrogen excretion) following administration of exogenous gonadotropins. The narrow range of requirements for FSH to initiate cyclic ovarian function was emphasized. His conclusions may be summarized as follows: there exists a threshold requirement of FSH below which follicular growth does not occur.

If this threshold is surpassed by increasing serum FSH concentrations (by approximately 10 to 30%), follicles start to grow. Recently, new insight has been gained concerning regulatory mechanisms during folliculogenesis (Schoemaker et al., 1993; Fauser et al., 1993b). Minimal serum FSH levels initiating dominant follicle growth (serum E_2 increment) in PCOS patients appeared to be the mean of serum FSH levels around the moment of presumed selection (Weissenbruch et al., 1993). Based on previous observations (Chikazawa et al., 1986), the day of presumed selection was based on retrograde extrapolation, as the day at which the first follicle that reached 12 mm had been approximately 5 mm in size (presumed daily follicle growth of 2 mm). Minimal serum FSH levels to induce dominant follicle growth ('above threshold-dose') during gonadotropin administration in infertile PCOS patients were determined using an intravenous administration system (van der Meer et al., 1994). The 'FSH-threshold' theory was modified to a 'Three pillar model' (referring to three different stages of follicle development) leading to dominant follicle selection (Scheele, 1994).

It was postulated that it takes approximately 80 days for a resting primordial follicle to develop up to ovulatory size (Gougeon et al., 1980). Under normal conditions, the formation of FSH receptors is initiated in small preantral follicles (Menigish et al., 1991). Only if the 'FSH-threshold' is surpassed – which takes place during the luteo-follicular transition of the normal menstrual cycle – a restricted number (cohort) of follicles will be rescued from atresia, gain gonadotropin dependence and continue to grow (recruitment). The longer FSH serum concentrations are above the threshold levels (i.e. the wider the 'FSH-window'), the more follicles will continue their development (Zeleznik & Hillier, 1984). In normally cycling women, this FSH-window is restricted to allow ongoing growth of a limited number of recruited follicles. Diminished stimulation of non-dominant follicles by relatively low FSH levels plays an important role in selection of a single dominant follicle (Fritz et al., 1982; Hodgen, 1982; Goodman & Hodgen, 1983; Zeleznik & Hillier, 1984). The leading follicle demonstrates a significant decrease in FSH dependency (due to local up-regulation) which underlies ongoing growth despite decremental FSH concentration (as described in 1.2.2). Enlargement of the dominant follicle is also accompanied by accumulation of LH receptors (Yong et al., 1992).

Various aspects of recruitment and selection of the dominant follicle have been substantiated *in-vivo* in the monkey (Hodgen, 1982; Zeleznik & Kubik, 1986) and in sheep (Baird, 1983). A proportion of these observations have also been confirmed in humans. These studies have convincingly demonstrated that: 1) luteectomy results in a rapid rise in serum FSH which in turn induces follicle recruitment and normal follicle development, suggesting indeed that follicles are ready to be recruited once the FSH threshold is surpassed (Dizerega & Hodgen 1981). 2) Anti-estrogen antibodies administered during the early follicular phase (causing incremental

serum FSH levels) induce multiple follicular development (Zeleznik et al., 1985) which emphasizes the importance of decreasing FSH levels for selection of a single dominant follicle. 3) Electrocautery of the largest follicle during the midfollicular phase in the monkey, and observed postponement of a new LH surge by approximately 14 days, indicates that a non-dominant follicle could not regain dominance (Dizerega & Hodgen, 1982). 4) In GnRH-a pretreated macaque-monkeys demonstrating estrogenic response to a combined LH and FSH preparation, a subsequent infusion of decreasing (12.5%/day) quantities of FSH revealed further maturation of the dominant follicle, whereas growth of non-dominant follicles was not supported (Zeleznik & Kubik, 1986). In addition, a step-down human menopausal gonadotropin (HMG) protocol in the animal model showed better synchronization of follicular rupture, and reduced susceptibility to delayed ovulations (Abbasi et al., 1987). This study indicates that if the FSH-window is restricted, this subsequently leads to a more homogeneous pool of recruited follicles and a reduced period of ovulations. In 3 hypogonadotropic infertile women, continued growth of the dominant follicle was observed during decremental supply of intravenously administered FSH (10% reduction daily) (Glasier et al., 1988). It was stated that FSH is above threshold levels (the 'FSH-gate') during a limited period (Baird, 1987). Whereas 'FSH threshold' indicates the minimal dose to support follicle growth, the number of days during which FSH is above the threshold (length of the 'FSH-window') will determine the number of growing follicles (for review see Fauser et al., 1993b; Fauser, 1994a). Consequently, multiple ovulation can occur if serum FSH concentration is above threshold levels for an extended period of time.

1.3 GONADOTROPIN INDUCTION OF OVULATION

1.3.1 History

Initially, gonadotropin preparations were extracted from serum of pregnant mares (Pregnant Mare Serum Gonadotropin [PMSG]) (Cole & Hart, 1930). Their use in anovulatory women gave rise to non-reproducible and unsatisfactory results. Due to the observation that if ovulation occurred in PMSG cycles it was only in those accompanied by a spontaneous LH surge, a combination of PMSG and human chorionic gonadotropin (hCG) was advocated. This approach appeared to be successful (Hamblen & Davies 1945, Finkler 1949). However, due to the inability to monitor ovarian response to medication, outcome was still unsatisfactory. In addition, repeated PMSG injections induced formation of antibodies against these non-primate gonadotropins. During the mid-fifties, the clinical use of gonadotropins as treatment of anovulatory infertility was questioned (Palmer, 1957). The first pregnancy following administration of human pituitary gonadotropins (HPG)

was established in a hypogonadotropic woman (Gemzell, 1958). Moreover, no anti-hormone activity was measured. A new method of extracting gonadotropin from urine of menopausal women (Albrecht, 1956) made extensive clinical application possible. Some years later, the first pregnancy was reported using 'menotropins' in a woman with hyperprolactinaemia/ hypogonadism syndrome (Lunenfeld et al., 1960). In Australia until 1981, almost 50% of the 1056 HPG-treated patients conceived during 4008 cycles, whereas urinary preparations revealed similar success rates (Kovacs et al., 1984). However, HPG administration was stopped in 1984, due to the association of HPG with a slow-acting virus causing encephalitis (Jacob-Kreutzfeld disease) (Frasier & Foley, 1994).

1.3.2 Indications for treatment

During the pioneering years of gonadotropin therapy, medication was administered to a heterogeneous group of infertile women with oligo-amenorrhoea (Rabau et al., 1967; Caspi et al., 1974; Webb-Wilson & Arronet, 1977). Initially gonadotropin therapy was expected to be the suitable replacement in patients suffering infertility and primary or secondary amenorrhea due to hypogonadotropic hypogonadism (World Health Organization (WHO) classification group I [Lunenfeld & Insler, 1974]). At present pulsatile intravenous administration of GnRH is preferred in this patient group.

The major indication for treatment with gonadotropins nowadays is infertility due to menstrual disturbances classified as WHO group II (women with low to normal gonadotropin levels and normal estrogen concentrations). Clomiphene citrate (Greenblatt et al., 1961) is considered treatment of first choice in this group with normogonadotropic amenorrhoea (MacGregor et al., 1968; Gorlitzky et al., 1972; Garcia et al., 1977) including patients suffering from polycystic ovary syndrome (PCOS) (Yen et al., 1970) because of reasons of efficacy, clinical results and an acceptable complication rate. Prospective randomized studies comparing clomiphene citrate and gonadotropins have not been performed (Tal, 1985). Moreover, clomiphene citrate might have detrimental effects on oocyte quality (Regan et al., 1990) due to pharmacologically augmented LH levels (Wu, 1977) and subsequently increased serum testosterone concentrations. Approximately 20 to 30 per cent of clomiphene-citrate-treated patients do not ovulate (MacGregor et al., 1968, Garcia et al., 1977), mostly due to PCOS (Adams et al., 1986). Gonadotropin therapy in PCOS patients leads to a higher incidence of complications than in patients suffering from other causes of anovulation (Gemzell & Roos, 1966; Wang & Gemzell, 1980). However, the clinical profile of clomiphene-resistant anovulatory infertility is poorly defined and many of the above mentioned conclusions are not based on well-designed

prospective studies. Although defining this syndrome is a subject of discussion (Fauser et al., 1993a, Balling-Smith & Franks, 1993), it may be hypothesized that in PCOS patients a local intra-ovarian dysregulation causes disturbance in selection of the dominant follicle (Pache et al., 1992b; Pache et al., 1993; Fauser, 1994a). Next to indication for treatment, clinical outcome of therapy is determined by many other factors (Fauser, 1993c) which will be discussed later. In PCOS ovarian follicles mature normally to the stage of development where significant aromatase activity is induced under normal conditions (presumably 6 to 8 mm) (Erickson et al., 1979; Hillier et al., 1981; Pache et al., 1992b, Fauser, 1994b). Increment of serum FSH by administering exogenous gonadotropins can induce sufficient aromatase activity, and concomitant follicular growth. Based on extensive previous study (Pache et al., 1993), PCOS was defined in the studies presented in this thesis as a pathophysiological feature present in women with infertility and oligo/amenorrhea, scoring positive in at least three out of four of the following criteria: 1) obesity, 2) hirsutism, 3) elevated androgen levels (free androgen index [FAI]; testosterone [T] X 100 / sex-hormone-binding globulin) and/or dehydroepiandrosterone sulphate (DHEAS), and 4) polycystic appearance of ovaries by sonography (Pache et al., 1991b). In non-galactorrheic hyperprolactinemic women not responding to bromocryptine (Friesen & Tolis, 1977), application of gonadotropins alone may also be a good alternative (Farine et al., 1982). In patients with anovulation and hypergonadotropic hypogonadism (WHO group III criteria) success rates of gonadotropin induction of ovulation are low (Check et al., 1990; Johnson & Peterson, 1979).

Well-established indications for the use of gonadotropins other than for treatment of anovulation are limited. However, gonadotropins are administered for improvement of cervical mucus quality (Check, 1980), luteal phase defect (Zimmerman et al., 1982), induction of multiple follicles prior to intra-uterine insemination, and as an empirical therapy for infertility with unknown cause (Fauser, 1993c).

1.3.3 Dose regimens

Fixed or variable dose regimens of gonadotropin administration have been used to induce ovulation in infertile oligo- or amenorrheic women. The variable dosage has been used more commonly, permitting variation in the daily gonadotropin dosage and the duration of administration. During fixed administration, gonadotropins were injected on predetermined days based on expected steroidogenic response (Butler, 1969). Although success of this economic dose regimen was comparable to variable schemes (Thompson & Hansen, 1970), an enhanced complication rate induced the decline in clinical use. Furthermore, improvement of monitoring techniques (Schoot et al., 1992d)

made it possible to adjust the dose according to the observed ovarian response. Many dose schedules have been advocated to improve efficacy and reduce complications, as can be seen in the table below.

The largest and most significant study (Thompson & Hansen, 1970) revealed that: 1) the success rate of different regimens was comparable, 2) hCG (500 to 10,000 IU) had to be administered 1 to 2 days subsequent to arrest of HMG stimulation, 3) the variable step-up regimen was used most often, probably due to reasons of efficacy. Although there is some difference in sensitivity to gonadotropins between cycles (Rosemberg & Nwe, 1968; Brown et al., 1969), this is less variable than differences between patients (Marshall et al., 1969).

At present, 'step-up' regimens are based on admistration of increasing amounts of gonadotropins until ovarian response is considered to be 'sufficient' (one large follicle or a significant rise in serum estrogen

Major dose regimens of HMG and hCG through im injections

FIXED REGIMENS	
– Single large dose followed by hCG on day 11	Marshall et al., 1969 Seddon, 1970
– Daily HMG, starting hCG 2 or 3 days after stopping HMG	Marshall et al., 1969
– Daily HMG, last 2 or 3 days hCG (overlapping)	Thompson & Hansen, 1970
– Daily HMG (alone)	Thompson & Hansen, 1970
– HMG on alternate days (e.g. day 1, 4, 8)	Radwanska et al., 1980
VARIABLE REGIMENS	
– Descending doses of gonadotropins (hCG: (non)-overlapping)	Thompson & Hansen, 1970
– Ascending doses of gonadotropins (hCG: (non)-overlapping)	Bertrand et al., 1971 Kurachi et al., 1985

concentration [Polson et al., 1987]). A slower and less pronounced increase of gonadotropin doses is referred to as the 'low-dose, step-up' regimen (weekly changes in dose, increments no more than ½ ampule [= 37.5 IU]/day). The method has been shown to improve treatment outcome (Seibel et al., 1985; Polson et al., 1987, Buvat et al., 1989; Shoham et al., 1991; Hamilton-Fairly et al., 1991; Meldrum, 1991). Different results (incidence of ovarian hyperstimulation syndrome [OHSS] as high as 21%) were reported in other studies using the low-dose protocol (Hull, 1991; Herman et al., 1993). It was already stressed by Brown (Brown, 1978) in his paper describing the 'FSH-threshold' concept that 'maturing follicles are producing estrogens within 2–3 days after the threshold has been reached which, under normal conditions, prevents overshoot of FSH through negative feedback mechanisms'. In contrast to the follicular phase of the normal menstrual cycle, FSH serum levels gradually increase during 'step-up' regimens in gonadotropin induction of ovulation. In the monkey model, it has been convincingly shown that interference with decreasing FSH serum levels elicits multiple follicle development (Zeleznik et al., 1985). Preliminary clinical experience with decreasing doses of gonadotropins – step-down regimens'– for induction of ovulation (Mizunima et al., 1991; Fauser et al., 1993b; Fauser et al., 1994c; van Santbrink et al., 1995b) are promising.

1.3.4 HMG versus pFSH

Two urinary gonadotropin preparations are commercially available; human menopausal gonadotropins (HMG [Humegon^R and Pergonal^R]), and purified FSH (pFSH [Metrodin^R]). HMG preparations contain an equivalent amount of 75 IU FSH and 75 IU LH *in-vivo* bioactivity. A successive supplementary purification step substantially decreased LH-like activity leading to a novel commercial pFSH preparation. Besides obtaining a more purified product, the rationale of developing a pFSH preparation was two-fold. Firstly, ovulation induction using gonadotropins in patients with elevated endogenous LH serum levels could – on theoretical grounds – preferably be performed without a surplus of exogenously administered LH. Secondly, FSH alone was believed to increase folliculogenesis (Schoemaker et al., 1978). Furthermore, it was speculated that LH in gonadotropin preparations could be responsible for the high incidence of treatment complications in patients with elevated serum LH levels (Raj et al., 1977; McFaul et al., 1990). However, other studies (Jacobson & Marshall, 1969; Louwerens, 1969) have indicated that the effectiveness of gonadotropin preparations and the occurrence of OHSS were not dependent on the LH/FSH ratio (Lanzone et al., 1987). pFSH administration did result in decreased LH levels as compared to HMG in PCOS (Anderson et al., 1989). Until now clinical studies have failed to demonstrate clear advantages of pFSH over HMG (Seibel et al.,

1985; Venturoli et al., 1986; McFaul et al., 1990; Tanbo et al., 1990; Sagle et al., 1991). However, it cannot be excluded at this stage that studies with larger patient numbers would disclose significant differences in clinical outcome due to different LH content in gonadotropin preparations.

1.3.5 Adjuvant medication

Initially, uncontrolled studies in PCOS patients (Fleming et al., 1985; Charbonnel et al., 1987; Hedon et al., 1991), suggested that the success rate of gonadotropin treatment could be improved and the complication rate reduced, if GnRH agonist medication was added. Improvement of treatment results was associated with down-regulation of elevated endogenous LH levels, as well as prevention of a premature LH surge (Dodson et al., 1985). It was demonstrated that continuous suppression by a GnRH agonist during consecutive gonadotropin-induced cycles resulted in lower estrogen production, although folliculogenesis was unchanged (Remorgida et al., 1989). Elevated LH concentrations – often seen in PCOS – may adversely affect follicular development, and were suggested to have detrimental effects on oocyte quality (Stanger & Yovich, 1985; Regan et al., 1990). Rather few randomized, controlled clinical trials, each of them using limited numbers of patients, have been published (Hompes et al., 1986; Dodson et al., 1987; Fleming & Coutts, 1988; Dodson et al., 1989; Bachus et al., 1990; Homburg et al., 1990). A beneficial effect of adjuvant treatment with GnRH agonist in gonadotropin ovulation induction on the pregnancy rate could be demonstrated only by some authors (Fleming & Coutts, 1988). In the remaining trials (Hompes et al., 1986; Dodson et al., 1987; Dodson et al., 1989; Bachus et al., 1990; Homburg et al., 1990), clear advantages of additional GnRH agonist medication could not be established. A meta-analysis combining the results of these five trials demonstrated a significant difference in favor of GnRH-agonist co-administration with respect to the pregnancy rate only (Schoot et al., 1992c). A large meta-analysis assessing the efficacy of adjuvant GnRH agonist for *in-vitro* fertilization (IVF) and gamete intrafallopian transfer showed favourable results of this adjuvant therapy (Hughes et al., 1992). Effects of GnRH agonists co-treatment on the luteal phase are unclear (Bachus et al., 1990). Differences in length and amplitude of the progesterone rise during the luteal phase as compared to regular cycles were reported following induction of ovulation using gonadotropins and GnRH agonist in PCOS patients (Donderwinkel et al., 1993). Luteal support (hCG or progestins) is advised to prevent GnRH agonist-induced luteolytic effects (Bentick et al., 1988). However, convincing data indicating that luteal support improves pregnancy rate following induction of ovulation are lacking.

Growth hormone (GH) -dependent generation of intra-ovarian IGF-I and

the subsequent local potentiation of gonadotropin-dependent ovarian function was observed in rats (Adashi et al., 1985; Davoren & Hsueh, 1986; Jia et al., 1986). GH may affect human ovarian function directly, or through augmented hepatic production of IGF-I, or by increased intra-ovarian production of IGF-I. Exact mechanisms of action, however, remain unclear. mRNA for IGF-I in human granulosa cells appeared to be absent (El Roey et al., 1993). Comparing E_2 changes in normal cycling women and in patients undergoing controlled ovarian hyperstimulation, differences appeared to be independent of respective GH levels. (Stone & Marrs, 1991). Complementary roles of GH and FSH in patients with panhypopituitarism were evident (Blumenfeld & Lunenfeld, 1989). In addition, in a patient with IGF-I deficiency secondary to GH receptor abnormality, normal follicle development was demonstrated and a rise of estrogens occurred after gonadotropin administration (Dor et al., 1992). Initially, adjuvant GH treatment during gonadotropin-induced cycles in women with low response to gonadotropin therapy suggested a role for the use of GH in induction of ovulation (Homburg et al., 1988; Ibrahim et al., 1991). More recently, larger studies have failed to show additional effects of GH to enhance ovarian response during gonadotropin induction of ovulation (Jacobs et al., 1991).

Less often, co-treatment may include dexamethasone in the case of adrenal hyperandrogenemia or bromocriptine in the case of hyperprolactinemia.

1.3.6 Monitoring treatment response

Treatment outcome has improved substantially over the years, mainly because of improved treatment monitoring and the introduction of new dosage regimens. The purpose of monitoring induction of ovulation is two-fold: 1) to prevent complications and risks, and 2) to improve efficacy of therapy. During the first years of gonadotropin treatment surveillance of ovarian response to gonadotropins administration was limited. The incidence of complications was described almost 10 years after its introduction (Neuwirth et al., 1965). Initially, Gemzell and co-workers used bimanual palpation of the ovaries, cervical mucus changes and vaginal cytology during their first successful series (Gemzell & Roos, 1966). Cervical mucus changes served as a fast but questionable *in-vivo* bioassay for estrogenic response (Igarashi & Matsumoto, 1957; Insler, 1972), whereas pregnanediol excretion in urine was an indicator of successful ovulation (or luteinization). Assay procedures for the measurement of urinary estrogens (estriol, estrone and estradiol) (Brown, 1956) were time-consuming, which precluded their use in daily clinical decision making. In a decade with emerging complications, clinical investigators (Hancock et al., 1970; Crooke et al., 1971) observed a correlation between the increment of excreted steroids and complications of gonadotropin therapy. Due to lack of sensitivity, the clinical use of steroid

measurements to predict (and prevent) complications was not generally accepted (Taymor et al., 1972). The availability of a RIA for the measurement of E_2 serum concentrations (Robertson & Steele, 1972) replaced the cumbersome assay of urinary estrogen excretion (Brown et al., 1968). Comparing urinary estrogens and two serum E_2 RIAs no differences were found (Tredway et al., 1974). Clinical significance – more than timing of hCG administration – of the new RIA for E_2 in serum was questioned (Wu, 1978). In a retrospective analysis, plasma E_2 levels at the day of hCG administration did not show a significant difference comparing patients with multiple and single ovulation (Wu, 1976). Prompt detection of rapid E_2 increase and rate of increment appeared to be clinically useful to increase pregnancy rates and diminish complications (Schwartz & Jewelewicz, 1981; Wilson et al., 1982; Pittaway & Wentz, 1983; Haning et al., 1983). Furthermore, application of maximum serum E_2 levels during gonadotropin induction of ovulation was generally accepted in an attempt to reduce complication rates (Marrs et al., 1980; Schwartz et al., 1981).

A linear correlation was found between between sonographical measurement of follicle size and serum E_2 concentrations during the late follicular phase of spontaneous (monofollicular) cycles (Freundl et al., 1981). The volume of the dominant ovary also showed a linear correlation with serum E_2 levels (Mango et al., 1988). However, in contrast to this correlation during the normal cycle (Ylostalo et al., 1979), this relationship was absent if multiple follicles developed during gonadotropin therapy (Cabau et al., 1976; Schmidt et al., 1981; Haning et al., 1982).

Following visualization of Graafian follicles in the ovary using gray-scale ultrasound (Kratochwil et al., 1973), real-time transabdominal sonography was developed (Hackeloer, 1974). Accuracy of measurement of diameter and volume of follicles was validated by laparoscopic puncture (Kerin et al., 1981; Leerentveld et al., 1984). Intra- and interobserver variations of sonographic measurement appeared to be within an acceptable range (Leerentveld, 1984; Eissa et al., 1985; Forman et al., 1991). The possible clinical role of real-time ultrasound during gonadotropin induction of ovulation was investigated (Hackeloër et al., 1977). This caused a further reduction of therapy-related complications (Bordt et al., 1986). The possible relationship between the number of small and medium-sized ovarian follicles at the moment of hCG administration and the incidence of OHSS have been investigated (Tal et al., 1985; Blankstein et al., 1987). In order to reduce risks of therapy, the size and number of secondary follicles have to be small at the moment of hCG administration.

It has been clearly established that sonographic monitoring of the ovarian changes using a transvaginal probe is superior to transabdominal scanning (Hull, 1989) due to: 1) a smaller distance from the probe to pelvic organs, 2) the use of high resolution of probes (5–7 mHz) and 3) an improvement of patient compliance and convenience (no full bladder warranted). Four main

parameters can be assessed in the ovary: a) volume, b) follicle number, c) follicle size and d) amount and density of stroma. Transvaginal sonographic assessment of endometrial growth in stimulated and unstimulated cycles demonstrated a correlation with both the diameter of the leading follicle as well as with serum E_2 level (Randall et al., 1989). Whereas transabdominal scanning provided accurate information on large follicles only, the higher resolution of transvaginal sonography enables the systematic investigation of changes in the number of small and medium-sized follicles (>2 mm) during the early follicular phase (this thesis, section 3.2). Sonography of the ovaries during induction of ovulation can provide data concerning ovarian follicular growth, next to timing of hCG injection based on size of the dominant follicle (Sallam et al., 1982; Hoffman et al., 1985; Venturoli et al., 1986; Lanzone et al., 1987; Mango et al., 1988; McFaul et al., 1990). Comparison of these studies investigating follicular response during gonadotropin-induced cycles is hampered by methodologic differences (patient selection, dose regimens used).

1.3.7 Treatment outcome

Due to differences in patient selection (age, weight, anovulatory disorder, parity, previous medication, definition of clomiphene resistance), applied dose regimens, monitoring of ovarian response or adjuvant treatment, it is difficult to compare results of different reports. A potential selection of the infertile population with a poor history in infertility treatment in research clinics (academic hospitals) may also bias treatment results. Moreover, the lack of cumulative pregnancy rates calculated using lifetime analysis (Lamb & Cruz, 1972), can also generate a bias in the estimation of treatment efficacy. In the first North American review of different gonadotropin dose regimens (634 patients during 1538 treatment cycles), ovulation was demonstrated in 68% of all cycles, whereas pregnancy rate was 12% (Thompson & Hansen, 1970). Reported pregnancy rates ranged between 23% (Buttler, 1969) and 82% (Healy et al., 1980). In addition, an average cumulative pregnancy rate of 44% following approximately 3 cycles was reported (Lunenfeld et al., 1985). Cumulative conception rate differed between 90% in hypogonadotropic women (Lunenfeld et al., 1985) compared to below 40% in PCOS patients (Dor et al., 1980). Treatment outcome seems to show a tendency to improve during the past 20 years (Diczfalusy & Harlin, 1988).

Abortion occurs in approximately 20–40% of pregnancies following gonadotropin induction of ovulation. Recently a hypothesis was proposed concerning detrimental effects of tonic high LH levels, causing premature onset of the last meiotic division of the oocyte which is considered to have a negative impact on fertilization (Homburg et al., 1990). This could serve as

an explanation for the relatively high incidence of spontaneous abortion in PCOS patients (Regan et al., 1990). Furthermore, obesity and elevated LH levels were also considered as factors significantly decreasing success rates of gonadotropin treatment in PCOS patients (Hamilton-Fairley et al., 1991). With regard to hypergonadotropic patients with elevated FSH serum concentrations, decreased estrogen production can be useful in predicting low ovarian response (Toner et al., 1991).

1.3.8 Complications

Two major complications of gonadotropin therapy include OHSS and multiple pregnancies. The origin of both complications can be found in sustained stimulation of multiple follicles. The most common complication of gonadotropin treatment is multiple gestation. Initial studies reported multiple pregnancies in up to 30 – 50% of stimulated cycles (Gemzell & Ross, 1966), and up to nine viable fetuses (Carey, 1976). At present, the incidence of multiple gestation has decreased to 10–35% (Schwartz et al., 1981). Obstetrical problems and perinatal outcome of multiple pregnancies and their social consequences warrant careful monitoring of treatment cycles (Petrikovsky et al., 1989). Selective reduction of the number of fetuses seems to reduce the incidence of immature and premature birth, and decreases maternal complications (Boulot et al., 1993).

OHSS has been classified based on the severity of the signs, symptoms and laboratory findings in 6 categories (Rabau et al., 1967). Sonographic measurement of the ovaries (mean diameter) has been added to this traditional classification (Golan et al., 1989). Moderate ovarian hyperstimulation involves enlargement of ovaries (<5 cm) and minor laboratory disturbances, whereas a moderate grade OHSS is characterized by abdominal distension, nausea, vomiting and diarrhea, with ovaries usually between 5–12 cm. Severe OHSS is a life-threatening disease, demonstrating greatly enlarged ovaries (> 12 cm in diameter) accompanied by pleura effusion, ascites, oliguria, electrolyte imbalance, hemoconcentration and thromboembolic phenomena (Moses et al., 1965; Merkus et al., 1990). An acute fluid shift is caused by an increase of capillary permeability, allowing fluid to escape in the third space. Acute fluid accumulation in the peritoneal and pleural cavities leads to hypovolemia, hemoconcentration and increased osmolarity. Reduced renal perfusion results in oliguria, azotemia, hyperkalemia and acidosis. Increased hemo-concentration may lead to thrombosis and thromboembolism. Treatment of severe OHSS should include correction of the fluid shifts, whereas abdominal paracentesis can relieve respiratory distress. In addition, prostaglandin synthetase inhibitors have been successfully used to modify changes due to OHSS. The use of antihistamines is not generally accepted.

The incidence of OHSS depends on the applied dose regimen and monitoring technique. Furthermore, incidence is increased in case of pregnancy, suggesting the involvement of hCG (Rabau et al., 1967; Golan et al., 1989). The overall incidence of mild OHSS varies between 8 and 23% of treatment cycles. Moderate OHSS was observed up to 7% whereas severe OHSS was reported in less than 2% of gonadotropin-induction cycles (Schenker & Weinstein, 1978). The ability of ultrasound to visualize the ovaries allows more precise assessment of ovarian enlargement and free fluid (ascites) leading to early detection of OHSS (McArdle et al., 1983). Mild OHSS is probably more common than previously suggested and should be accepted as a frequent consequence of gonadotropin-stimulated ovarian activity. The risk of developing OHSS can be calculated in conception and non-conception cycles in hypogonadotropic and PCOS patients (Haning et al., 1984). Patients at risk should be stimulated using less gonadotropins. The 'young, asthenic, pregnant PCOS patient with previous complaints due to hyperstimulation by gonadotropins, demonstrating the typical necklace ovaries at ultrasound and obtaining hCG as luteal support' emerges to be risking development of OHSS (Navot et al., 1988). The development of large numbers of small or medium-sized follicles at the moment of hCG administration may underlie complications of treatment (Tal et al., 1985; Blankstein et al., 1987). As previously described, pFSH or co-treatment with GnRH agonists did not alter the incidence of OHSS.

1.4 OUTLINE OF THE THESIS

The **first objective** of this study was to provide a review on previous and current knowledge regarding FSH regulation of ovarian function (Chapter 1).

The **second objective** was to obtain data concerning ovarian folliculogenesis and steroidogenesis in hypogonadotropic female volunteers during human recombinant FSH administration (Chapter 2).

The **third objective** was to investigate in PCOS patients the role of decreasing doses of exogenous gonadotropins (following presumed selection of the dominant follicle) on serum hormone concentrations and follicle growth (Chapter 3).

2

Ovarian response following human recombinant FSH administration in hypogonadotropic female volunteers

2.1 BACKGROUND HUMAN RECOMBINANT FSH

Commercially available preparations of urinary gonadotropins have been successfully used since the 1960's in the treatment of infertility. However, only 1–5% of the total protein content of HMG preparations actually exhibits FSH activity. This FSH shows a high degree of structural heterogeneity due to the presence of different glycoforms as shown by isohormone profiling (Storring et al., 1992). In urinary preparations varying amounts of LH and HCG are present (Harlin et al., 1986). Although the protein contaminants, as well as LH and HCG, have never been shown to be harmful, only FSH is needed for clinical use.

Other disadvantages of urinary preparations are the theoretical risk of contamination and the limited supply of urine of postmenopausal women to purify the product.

To achieve production of a human FSH dimer, genomic clones containing the complete FSH α and β subunit coding sequences were transfected into Chinese hamster ovary (CHO) cells (Boime et al., 1990). Subsequently stable cell lines synthesizing the FSH dimer were selected (Keene et al., 1989). CHO cells appeared able to assemble the subunits and to glycosylate the molecule. Due to the capacity of CHO cell lines to grow in a serum-free medium, high purity of the glycoprotein product was ensured. Selected cell lines demonstrated a high number of plasmid copies which remained well-integrated during culturing of the cells for a period of three months. In addition, the isohormone profile remained stable during this interval. The biological activity of recombinant human FSH was ascertained using rat granulosa cell cultures and intact immature rats (Galway et al., 1990; Mannaerts et al., 1991). Human recFSH appears to be very similar to urinary and pituitary FSH although recFSH shows minor differences in the structure of the carbohydrate side chains and contains more basic isohormones than the hormone from both natural sources (Kloosterboer, 1994). This does not imply that recFSH differs from FSH produced by women of reproductive age

because the higher amount of acidic isohormones in urinary FSH may reflect selective metabolism by the kidney, whereas pituitary FSH has primarily been derived from old-age pituitaries. The more basic isohormone profile of recFSH and its stronger receptor binding complex/ signal transduction may explain its higher potency compared to FSH in normal cycling women. Furthermore, the shorter half-life of basic recFSH explains the significantly lower serum FSH levels (Out et al., 1995).

The absence of intrinsic LH activity of recFSH provides an opportunity to study separate roles of FSH and LH during folliculogenesis and subsequent steroid production. Initial studies were performed in hypogonadotropic patients incapable of spontaneous endogenous gonadotropin synthesis and secretion, because of the reduced risks in the case of FSH-antibody formation.

2.2 HUMAN RECOMBINANT FSH INDUCES GROWTH OF PREOVULATORY FOLLICLES WITHOUT CONCOMITANT INCREASE IN ANDROGEN AND ESTROGEN BIOSYNTHESIS IN A WOMAN WITH ISOLATED GONADOTROPIN DEFICIENCY

2.2.1 Introduction

Chinese hamster ovary (CHO) cells transfected with FSH subunit genes are capable of secreting the intact FSH dimer (Keene et al., 1989). Moreover, the biological activity of human recombinant FSH (rhFSH) was ascertained using rat granulosa cell cultures and intact immature rats (Galway et al., 1990; Mannaerts et al., 1991). Commercially available preparations of purified urinary gonadotropins may have varying molecular composition (Harlin et al., 1986), and all available preparations contain varying degrees of LH. Although rhFSH also consists of a mixture of FSH isohormones, bioactivity appears identical to pituitary FSH, without LH bioactivity (Mannaerts et al., 1991a). The hypothesis of gonadotropin synergism, introduced almost half a century ago (Fevold, 1941) emphasized the importance of an interplay between LH and FSH for normal estrogen production and follicular development. This concept was supported by observations in infantile mice after injection of pure urinary FSH (Eshkol & Lunenfeld, 1967). The observed increase in ovarian weight in combination with absent uterine growth, suggested that FSH alone is incapable of initiating estrogen production but still leads to follicular development. Moreover, the administration of purified FSH in hypogonadotropic women resulted in normal follicular development and low serum estrogen levels (Couzinet et al., 1988). An ongoing study using rhFSH in hypogonadotropic subjects, to assess safety and pharmacokinetical characteristics of the drug, provided an unique opportunity to explore whether LH exposure is essential for the induction of adequate estrogen production and subsequent follicle growth.

2.2.2 Patient and methods

A healthy female (age; 39 years, weight; 53 kg) suffering from primary amenorrhea due to isolated congenital gonadotropin deficiency, volunteered to enter an open phase I clinical trial with rhFSH to assess its tolerance, safety, pharmacokinetic and pharmacodynamic properties. Serum levels were 0.37 IU/L for LH and 1.2 IU/L for FSH. The study was approved by the Ethics Review Committee of the Erasmus University/Dijkzigt Hospital and informed consent was obtained. Wish for procreation was absent. Three successful gonadotropin-induced pregnancies (one twin pregnancy) had been established previously. Physical examination and routine urine/serum examinations were normal. Autoimmunity was excluded by anti-nuclear (ANA) and specific anti-FSH (anti-Org 32489) antibody assays. She refrained from estrogen replacement therapy 10 days prior to this study.

rhFSH (Org 32489; Organon International BV, Oss, The Netherlands) was administered in a daily dose of 75 IU (standardized according to the Steelman–Pohley *in-vivo* FSH bioassay [Mannaerts et al., 1991]) im for 7 days, followed by 150 IU/day during week 2. Medication was discontinued according to protocol criteria, because one ovarian follicle was found to exceed a diameter of 14 mm on day 13. On day 19, multiple follicles (n=6; 12-18 mm) were observed. After informed consent was obtained 3 follicles (13, 15, and 18 mm diameter) were punctured (no oocytes were obtained, fluid was stored separately) and 10,000 IU human chorionic gonadotropin (hCG) (Pregnyl^R; Organon International BV) was administered im.

Blood withdrawal and pelvic sonography on alternate days was continued for 3 weeks following the last rhFSH injection. Transvaginal sonography, using a 5 mHz transducer (Model 1550; Philips Medical Systems, Eindhoven, The Netherlands), was performed as published previously (Pache et al., 1990). Endometrial thickness was measured between the two parallel (opposite) hyperechogenic myometrium–endometrium interfaces. The largest distance, measured in the sagittal plane, representing two layers of endometrium, was recorded. For reference values, fluid was obtained from 118 individual non-dominant follicles (3–9 mm diameter) between cycle day 2–12 in 16 regularly cycling women (control) (Pache et al., 1992a), and from 7 dominant follicles (13–24 mm diameter), between cycle day 10 and 12, from 7 regularly cycling women. Serum and follicular fluid was centrifuged and stored at –20°C. Immunoreactive LH and FSH serum levels were assessed in one assay using a immunoradiometric assay (IRMA) kit (Delfia, Pharmacia, Woerden, The Netherlands). Data are expressed in terms of MRC 78/549 for FSH and MRC 80/552 for LH. Intra-assay coefficients of variation were less than 4.8% for FSH and less than 4.7% for LH. E_2 levels in serum and follicular fluid were estimated by RIA (Diagnostics Products Corp. Los Angeles, CA) (Pache et al., 1992a; Fauser et al., 1991a).

Progesterone (P) was measured in serum by RIA using an antibody against 11-α-hydroxyprogesterone-hemisuccinate bovine serum albumin complex. Serum and follicular androstenedione (AD) levels were measured using the antiserum described by Frölich and coworkers (Frölich et al., 1976), after extraction with diethyl ether. Intra-assay coefficients of variation were less than 5% for E_2, and less than 7% for AD. The anti-FSH antibody assay used labelled Org 32489 as a ligand and mouse antibodies raised against Org 32489 as a reference (detection limit: 0.5 pmol/L). ANA was measured using an immunofluorescence technique in a Hep-2 cell line (Bio-Lab, Amersfoort, The Netherlands).

2.2.3 Results

Baseline hormonal parameters showed confirmation of the hypogonadotropic hypogonadal state (FSH; 1.2 IU/L, LH; 0.37 IU/L, E_2; 63 pmol/L). Thyroid-stimulating hormone, prolactin and cortisol levels were within normal limits (data not shown). Sonography before rhFSH administration showed follicles at a size below 4 mm in each ovary (Fig. 1). Follicular development started after the daily dosage was increased from 1 to 2 ampules rhFSH on day 7. Following the last rhFSH injection (day 12), multiple ovarian follicles increased in size (maximum 22 mm). After puncture, two follicles of more than 14 mm remained. Following hCG injection, follicles decreased gradually in size. Endometrial thickness increased by 2 mm during the treatment period (from 4 to 6 mm) (Fig. 1).

A rise in serum FSH levels was observed during rhFSH administration with a maximum concentration of 8.5 IU/L (Fig. 1), followed by a decrease up to 1.3 IU/L on the day of hCG administration. Serum LH concentrations varied between 0.09 and 0.38 IU/L before hCG administration, and no systemic changes occurred. Serum E_2 levels showed a gradual increase to a maximum of 236 pmol/L on day 15 (day of maximum follicular size) (Fig. 2a). Serum P showed no elevation following hCG administration (data not shown). Fluid obtained from the largest follicle (18 mm) revealed an E_2 concentration of 9,400 pmol/L, and AD was 675 nmol/L. The remaining two follicles (15 and 13 mm) showed E_2 and AD concentrations of 3,800 pmol/L, 160 nmol/L and 3,100 pmol/L, 115 nmol/L respectively (Fig. 2b). Concentrations of both FSH and LH were below detection limits in follicular fluid (data not shown). In normal small (3–9 mm) follicles (n=118) the median E_2 level was 91 (range 1.5–7,000) x 1,000 pmol/L, and AD levels 3,160 (330–9,580) nmol/L. In normal large (13–24 mm) follicles (n=7) E_2 levels were 14,563 (10,657–20,446) x 1,000 pmol/L, and AD levels 3000 (620–5,480) nmol/L.

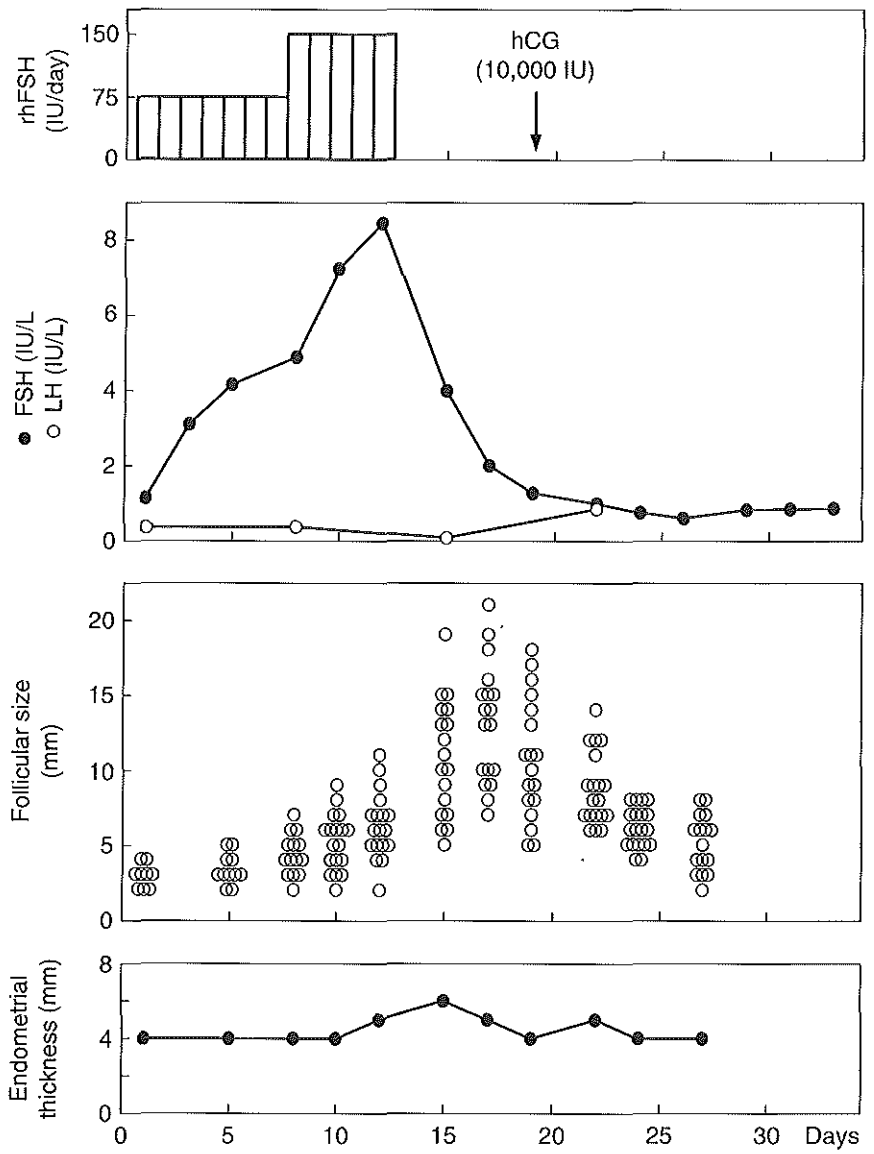

Figure 1 rhFSH and hCG dose regimens administered to a patient with isolated gonadotropin deficiency, serum FSH (IU/L) and LH (IU/L) levels in the upper two panels. Diameters (mm) of separate follicles as determined by vaginal sonography for both ovaries, and sonographic estimation of endometrial thickness (mm) in the lower two panels.

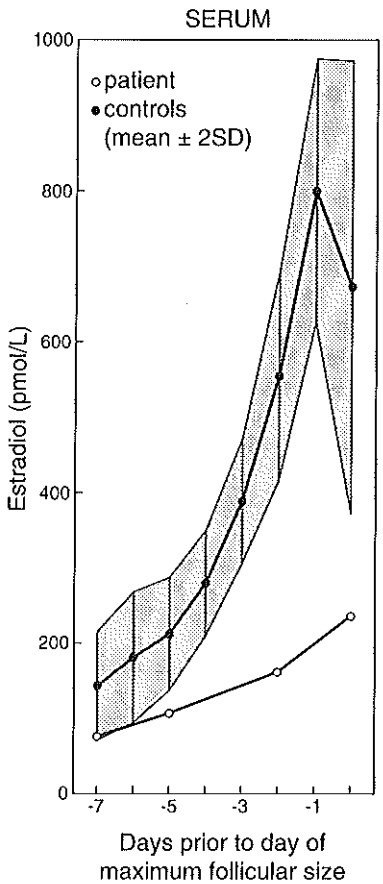

Figure 2a Serum estradiol (pmol/L) concentrations of a patient with isolated gonadotropin deficiency following rhFSH administration prior to the day where ovarian follicles reach their maximum size (day 17). As a reference, daily serum estradiol (pmol/L) levels (mean ± 2SD) are shown in seven normally cycling women up to the day of LH peak.

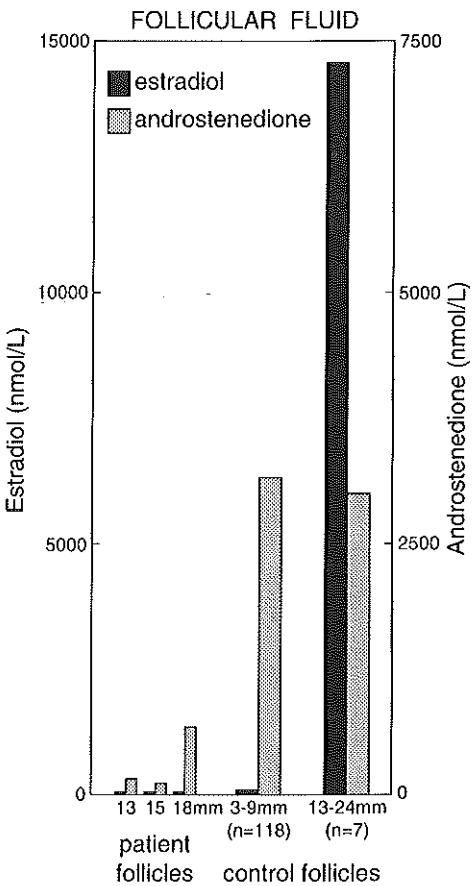

Figure 2b Estradiol (E_2)(nmol/L) and androstenedione (AD)(nmol/L) follicular fluid concentrations in three separate follicles (13, 15, 18 mm diameter) in a patient with isolated gonadotropin deficiency after rhFSH administration. Median E_2 and AD concentrations in small (3–9 mm) follicles (n=118), and large (13-24 mm) follicles (n=7).

2.2.4 Discussion

This study represents the first data on rhFSH administration in the human. No adverse effects and no anti-rhFSH antibody formation was observed. Development of follicles occurred following administration of increasing amounts of rhFSH, similar to that observed following exogenous gonadotropins used for induction of ovulation. Following the last injection of rhFSH follicular development continued towards pre-ovulatory sizes whereas FSH concentrations decreased, in keeping with previous observations (Schoot et al., 1992a) suggesting that growth of dominant follicles may be less dependent on circulating FSH concentrations.

Based on previous studies in the rat it is generally believed that estrogens are essential for normal follicular development. Recent clinical observations suggest that this concept may need to be revised for the human (Chappel et al., 1991). For instance, ovarian follicular development could be induced in a woman with inadequate estrogen and androgen biosynthesis due to 17α- hydroxylase deficiency (Rabinovici et al., 1991). In addition, it appears that normal follicular growth can be induced using purified urinary FSH preparations (Couzinet et al., 1988). In the present study, in sharp contrast to normal follicular development, E_2 levels remained low. Observed levels around day 15 are similar to early follicular phase E_2 concentrations in normal cycles (Schoot et al., 1992a)(Fig. 2a). The minor increase in endometrial thickness is also in favor of minimal estrogen bioactivity. E_2 and AD concentrations in 3 aspirated large follicles appeared to be extremely low, as compared to concentrations in small and large follicles in controls (Fig. 2b). This indicates that ovarian follicles are incapable of producing sufficient amounts of AD in the presence of minute amounts of LH (below 0.38 IU/L). The subsequent inability of normal estrogen production within follicles is in keeping with the two-cell two-gonadotropin hypothesis, indicating that FSH-induced granulosa cell aromatase activity can only lead to augmented E_2 production if a sufficient amount of the aromatase substrate AD is available. This underlines the concept of a LH threshold for sufficient estrogen production. It was surprising, indeed, that the mitogenic activity induced by FSH (i.e. proliferation of granulosa cells (Rao et al., 1978) did take place in a local environment with extremely low estrogen concentrations. This observation points to a differential regulation by FSH of steroidogenic and mitogenic granulosa cell activity. Direct effects of local estrogens on oocyte development are unknown.

Various reasons could explain the absence of a rise in serum P level following the injection of hCG. It may be hypothesized, based on *in-vitro* observations (Kessel et al., 1985), that physiological FSH levels in combination with low local E_2 concentrations, as observed in the present subject, were insufficient for the induction of LH receptors, and that these follicles were therefore not responsive to hCG. The possibility that declining

FSH serum levels caused a decrease in number of LH receptors (Jia & Hsueh, 1984) on the day of hCG administration cannot be ruled out. In addition, remaining follicles may have been to small for adequate hCG response.

In summary, data presented in this study indicate the significance of sufficient amounts of LH – next to FSH – for adequate E_2 production by ovarian follicles and further suggest differential regulation of mitogenic and steroidogenic granulosa cell activity by FSH. Since FSH alone – in the absence of LH – can induce growth of preovulatory follicles, it seems questionable whether estrogen biosynthesis is mandatory for follicular development in the human. For induction of ovulation, however, sufficient estrogen concentrations seem important for production of cervical mucus and normal endometrial function.

2.3 HUMAN RECOMBINANT FSH AND OVARIAN RESPONSE IN GONADOTROPIN-DEFICIENT WOMEN

2.3.1 Introduction

Advances in recombinant DNA engineering resulted in secretion of the intact human follicle-stimulating hormone (FSH) dimer by Chinese hamster ovary cells (Keene et al., 1989). Subsequently, animal studies showed that recombinant human FSH (rhFSH) lacks intrinsic luteinizing hormone (LH) activity and exhibits a high specific FSH bioactivity as compared to urinary gonadotropin preparations (Mannaerts et al., 1991). In order to assess safety and pharmacokinetical properties of this compound, initially a single dose study was undertaken in hypogonadotropic female and male volunteers (Mannaerts et al., 1993). Administration appeared to be safe and no anti-rhFSH formation occurred. Multiple-dose rhFSH administration in hypogonadotropic females provides the opportunity to study the effects of FSH alone – without the presence of endogenous or exogenous LH – on granulosa cell steroid and immunoreactive inhibin (INH) production and development of ovarian follicles. Information obtained may add to recent contentions based on case histories indicating that: a) LH is needed to provide the substrate for appropriate estrogen biosynthesis (Schoot et al., 1992b) confirming that the classical two-cell two-gonadotropin concept (Fevold 1941) is operational in the human, and b) follicles may fully mature without a concomitant increase in estrogen levels (Schoot et al., 1992b; Rabinovici et al., 1991) suggesting differential regulation of steroidogenic and mitogenic activity by FSH. This multicenter study describes pharmacodynamic effects of daily injections (with weekly increments) of rhFSH in 7 hypogonadotropic females.

2.3.2 Materials and methods

Subjects and Study Design: Seven gonadotropin-deficient, but otherwise healthy female volunteers, participated in this multicenter study. The study protocol was approved by the local ethics review committees and written informed consent was obtained from all participants. Four subjects suffered from congenital isolated gonadotropin deficiency (IGD) (n=3) or Kallman syndrome (KS) (n=1), whereas the remaining volunteers (n=3) were diagnosed as secondary panhypopituitarism (hypophysectomy = HX) due to surgical removal of a nonmalignant pituitary tumor (craniopharyngioma or adenoma). In the past, all 3 females with IGD received gonadotropins for induction of ovulation. All three showed a normal ovarian response and two conceived. No previous gonadotropin treatment had been given to the KS patient. Gonadotropins were administered in one of the females with previous neurosurgery resulting in a triplet pregnancy. For further clinical information of participating subjects see Table 1 (Results section). Autoimmunity was excluded by antinuclear antibody assays. All subjects refrained from oral estrogen replacement therapy (starting one week before injection up to one week after the last injection), while appropriate thyroid and glucocorticoid therapy (if required) was continued in the HX volunteers (pat# 6,7). All subjects received daily im injections of rhFSH (Org 32489; Organon Int. BV, Oss, The Netherlands) during a maximum period of 3 consecutive weeks in an increasing dose regimen (week 1; 75 IU daily [=1 ampule], week 2; 150 IU daily and week 3: 225 IU daily). 75 IU of the compound was dissolved in 0.5 ml of solvent (150 IU in 1 ml and 225 in 1.5 ml) and injected at 24-h intervals in the upper quadrant of the buttock. To reduce the risk of ovarian hyperstimulation, daily injections of rhFSH were discontinued when at least one ovarian follicle attained a mean diameter of 14 mm, and or serum estradiol (E_2) concentrations were above 1,200 pmol/L. Sonography (transabdominal or transvaginal, using 3.5–5 mHz probes) was performed every other day to monitor changes in endometrial thickness (Shoham et al., 1991) whereas growth of all individual ovarian follicles was measured (Pache et al., 1990). Blood samples were taken on alternate days prior to the moment of rhFSH injection (day 1, 3, 5, 8, 10, 12, 15, 17, 19). Additional blood sampling was performed during 3 weeks following discontinuation of rhFSH administration – or earlier if appropriate – using similar intervals (day 22, 24, 26, 29, 31, 33, 36, 40). Blood samples were centrifuged and serum was stored in 0.5 ml serovials at –20°C until assayed. Safety analysis included clinical observations i.e. blood pressure, heart rate and body temperature as well as laboratory assessments (urinalysis, blood biochemistry and hematology). Serum samples were analyzed for the presence of anti-rhFSH antibodies using a sensitive radioimmuno-precipitation assay and ^{125}I-recombinant FSH as a tracer, as published previously (Mannaerts et al., 1993).

Hormone assays: Immunoreactive FSH and LH was measured by an immunofluorometric assay (IFMA) using the time-resolved fluoroimmunoassay technique and reagent kits 1244-017 for human FSH and 1244-31 for human LH (Delfia: Pharmacia, Woerden, The Netherlands). These two-site assays employ a β-directed capturing monoclonal antibody (MCA) and an α-directed europium-labeled detection MCA. The assays were performed as described by the manufacturer using the Delfia instrumentation system and MultiCalc software (Pharmacia)(Mannaerts et al., 1993). FSH and LH immunoreactivity was expressed in terms of the 2nd International Reference Preparation (IRP) of pituitary FSH (code no. 78/549) and the 2nd International Standard (IS) for pituitary LH (code no. 80/552). The sensitivity of IFMA was 0.05 IU/L for both gonadotropins and the intra- and interassay coefficients of variation (CV) were below 4.8% and 4.3% for FSH, and 4.7% and 7.5% for LH, respectively. The cross-reactivity of the FSH kit with LH was <0.08% and of the LH kit with FSH <0.01%. Serum testosterone (T) and E_2 were assessed by radioimmunoassay (RIA) using a coat-a-count T RIA (reagent kit TKTT1 DPC, detection limit 0.27 nmol/L) and a double antibody E_2 RIA (reagent kit KE2D1 DPC, detection limit 11.6 pmol/L, Diagnostic Products Corporation, Los Angeles, CA). The intra-assay and interassay CV's were <9% and 13% for the T assay and <4% and 5% for the E_2 assays, respectively. In addition, intra- and interassay CV for the androstenedione (AD) RIA (Diagnostic Products Corporation, Los Angeles, CA) were less than 8 and 10%, respectively. Serum immunoreactive INH levels were measured by RIA as previously described (Lahlou et al., 1993) using an antiserum (No.1989) raised against purified bovine 31 kDa INH. Purified bovine 31 kDa INH iodinated by the lactoperoxidase method was used as a tracer. The standard was a pool of human follicular fluid (280 U/mL) which was calibrated against a rete testis standard preparation of defined bioactivity. The immunoactivity of 0.121 U follicular fluid was equipotent to 1 ng recombinant human INH (Biotech Australia, specific *in-vitro* bioactivity 51,060 U/μg protein using World Health Organization (WHO) standard 86/690 as the standard). The recombinant α-subunit of human INH exhibited complete cross-reactivity in this assay system. The standard pool, which was diluted in plasma from castrated subjects, provided dose responses parallel to the plasma dilution curves. The sensitivity of the assay was 30 U/L and the intra-assay and interassay CV's were <10 %.

Data analysis: Comparison of baseline and maximum hormone levels, and time-interval to maximum immunoreactive INH and E_2 elevation was performed by means of the Wilcoxon's test. Changes in serum FSH concentrations were analyzed in all subjects (n=7). Due to the absence of follicle development, and absent increase in E_2 and immunoreactive INH serum concentrations in 2 HX volunteers ([#6,7] presumably due to early

ovarian failure), analysis of ovarian response was restricted to the remaining 5 subjects. Data are presented as mean ± standard deviation (SD), or median and range. Differences were considered to be statistically significant if $P < 0.05$.

2.3.3 Results

Age and BMI (weight/height^2) of all volunteers was 37.7 ± 6 years and 23.8 ± 2.5 kg/m^2, respectively (Table 1). Four women received rhFSH (#2,5,6,7) for 3 weeks whereas 3 women had to stop earlier because of ongoing follicle growth (subject #1 day 12; subject #3 day 16; subject #4 day 18). None of the women exceeded the upper limit of serum E_2 (>1,200 pmol/L).

Table 1 Individual clinical and endocrine characteristics of 7 hypogonadotropic female volunteers – due to isolated gonadotropin deficiency (IGD: n=3; #1–3), Kallman syndrome (KS: #4) or secondary panhypopituitarism (HX: n=3; #5–7) – participating in this multiple-dose study using rhFSH.

Volunteer	*Diagnosis*	*Age*	*BMI*	*Previous HMG response*	*T4*	*Cortisol*
		(years)	*(kg/m^2)*	*(+/–)*	*(nmol/L)*	*(nmol/L)*
1	IGD	39	20.4	+	NA	471
2	IGD	39	23.9	+	89	190
3	IGD	38	23.6	+	93	430
4	KS	25	24.2	NT	160	384
5	HX	42	28.6	+	NA	498
6	HX	45	22.0	NT	207	1200*
7	HX	36	23.9	NT	230	536

IGD = isolated gonadotropin deficiency
KS = Kallman syndrome
HX = secondary panhypopituitarism due to hypophysectomy
NT = no previous gonadotropin treatment
NA = not available
*above normal limits due to medication

Table 2 Potential theca cell stimulation and maximum androgen response due to multiple-dose rhFSH administration in hypogonadotropic female volunteers.

Volunteer	*LH (IU/L)*		*AD (nmol/L)*		*T (nmol/L)*	
	baseline	*maximum*	*baseline*	*maximum*	*baseline*	*maximum*
1	0.37	0.38	4.23	4.14	0.36	0.43
2	0.06	0.08	3.31	3.26	0.55	0.64
3	0.23	0.47	2.05	1.92	0.57	0.50
4	<0.05	0.05	7.76	4.73	0.89	0.50
5	0.09	0.13	1.16	1.95	<0.38	0.52
6	0.06	0.08	0.20	0.14	<0.38	<0.38
7	<0.05	0.13	0.08	0.27	<0.38	<0.38

Median [range] serum concentration of FSH and LH prior to rhFSH administration in the overall group was 0.25 [<0.05–1.15] IU/L and 0.06 [<0.05–0.37] U/L, respectively. Serum AD concentrations appeared to be lower in the group with previous surgery (0.20 [0.08–1.16] nmol/L) as compared to the gonadotropin deficient volunteers (2.68 [2.05–7.76] nmol/L);p=0.07)(Table 2). Baseline levels of serum T were not significantly different (p=0.06) in the HX group (<0.38 nmol/L) as compared to IGD women (0.56 [0.36-0.89] nmol/L). Initial E_2 serum levels did not differ between both groups (21.9 [<5.1–37.7] pmol/L for IGD women versus: 5.1 [<5.1–7.6] pmol/L) for the HX group (Table 3). Initial immunoreactive INH serum levels were low (IGD-group; 31 [<30–149] U/L, HX-group; 30 [<30–89] U/L).

As a result of similar daily dosages during one week, changes in serum concentrations FSH appeared to stabilize after approximately 5 days (Fig 3). On the fifth day – following four injections of 75 IU rhFSH – steady state serum FSH levels in 7 subjects were measured ranging between 1.5 and 4.2 (median: 3.3) IU/L. Steady state concentrations of serum FSH on the fifth day of week 2 (150 IU/day;n=7) varied between 4.0 and 8.5 (median 7.2) IU/L. Maximum FSH concentrations ranged between 5.5 and 11.8 (median 8.1;n=7) IU/L (Table 3). Decline to baseline FSH levels occurred within 7 to 13 days after cessation of administration (Fig 3). Maximum serum LH concentration during the period of rhFSH administration remained low (0.13 [0.05–0.47] IU/L) (Table 2). Peak AD concentrations during rhFSH administration tended to

Table 3 Granulosa cell immunoreactive INH and E_2 production and follicle development in response to rhFSH administration in 7 hypogonadotropic female volunteers.

Volunteer	*FSH (IU/L)*		*INH (U/L)*		*E_2 (nmol/L)*		*Follicle number*[a]		
							<8	*8–13*	*>14*
	baseline	*maximum*	*baseline*	*maximum*	*baseline*	*maximum*	*(mm)*	*(mm)*	*(mm)*
1	1.15	8.5	<30	659	32.3	210	0	8	9
2	0.56	11.8	<30	581	<5.1	77	0	2	1
3	1.07	10.1	31	659	37.7	140	0	4	1
4	0.25	7.1	149	388	11.6	49	–[b]	–[b]	1[b]
5	0.07	8.3	<30	993	7.6	112	20	3	0
6	0.10	8.8	30	69	14.3	18	2	0	0
7	<0.05	9.9	89	143	2.3	49	0	0	0

[a]: number of ovarian follicles on the day of maximum rise in E_2 serum concentrations
[b]: measured by abdominal ultrasound

be higher in the volunteers with IGD (3.70 [1.92–4.73] nmol/L), as compared to the HX group (0.27 [0.14–1.95] nmol/L). No statistically significant difference (p=0.14) was observed between baseline and maximum AD levels. For serum T concentrations no difference between both groups or between baseline and maximum levels were observed (p=0.5) (Table 2). Maximum serum E_2 concentrations were reached (112 [18–210] pmol/L;n=5) on day 19 [15–24]. Maximum serum immunoreactive INH concentrations were 659 [388–993] U/L on day 15 [12–22]. In all five volunteers with ovarian follicular development, the day of immunoreactive INH increase appeared to be significantly earlier as compared to onset of E_2 increase (p=0.04). In four of five subjects, immunoreactive INH dropped sharply just after discontinuation of rhFSH (Fig. 3). Initial transvaginal scanning of the ovaries revealed small follicles (<8mm) in all the females of the IGD/KS group and one woman in the HX group (#5). No small follicles were observed in the remaining 2 HX women (#6,7). The presence of a single large ovarian follicle (at least 1 follicle ≥ 15mm in diameter) was observed by ultrasound in 4 volunteers with IGD or KS (#1,2,3,4) during the second or third week of administration. Moreover, one woman showed development of more than 5 large follicles (#1). Follicular growth in these patients with one single large ovarian follicle appeared to be 2.1 ± 0.9 mm/day.

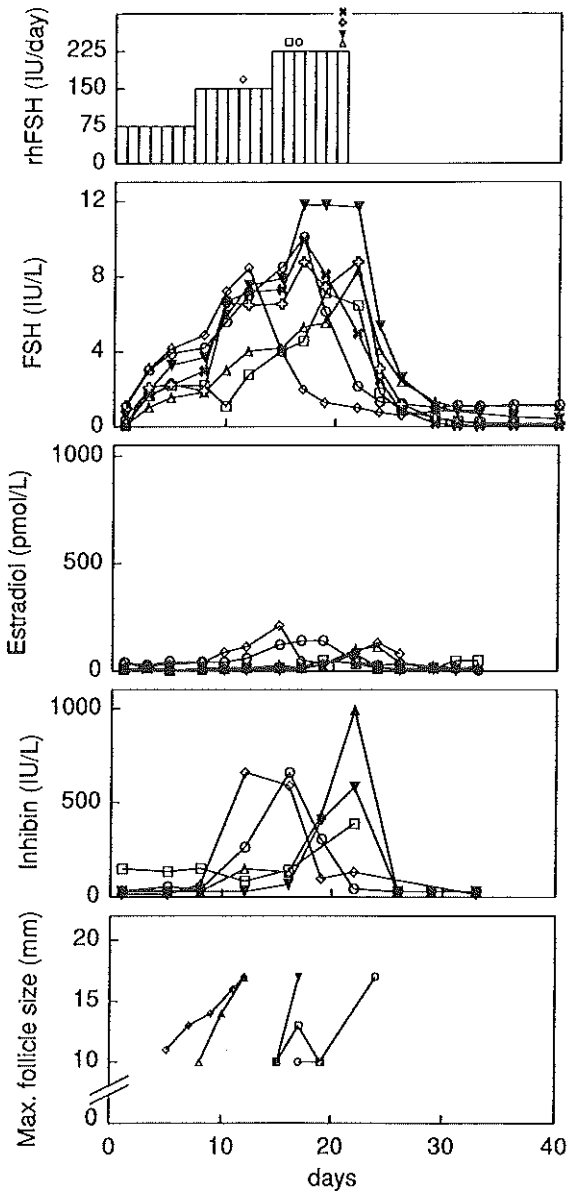

Figure 3 Daily dosage of rhFSH (upper panel; cessation of further rhFSH administration in 3 subjects is indicated) and individual serum FSH (IU/L), estradiol (pmol/L) and immunoreactive Inhibin (U/L) levels in 7 hypogonadotropic female volunteers participating in a multiple-dose study. Changes in sonographic size of the largest ovarian follicle (above 10 mm) during rhFSH administration are indicated in the lowest panel.

2.3.4 Discussion

This study deals with multiple-dose rhFSH administration in 7 hypogonadotropic female volunteers, either due to the selective decrease of gonadotropin biosynthesis (IGD, or KS) or hypophysectomy (HX). The absence of endogenous and exogenous LH allows us to study the effects of FSH alone on ovarian steroid and INH production and follicle development. A discrepancy between E_2 response and follicle growth of three of these women has recently been reported as case histories (Schoot et al., 1992b; Shoham et al., 1993). To examine the pharmacokinetical properties of rhFSH following im administration, a single-dose (300 IU rhFSH) study has been published recently concerning 8 hypogonadotropic females (Mannaerts et al., 1993). Highest FSH levels were observed after 27 ± 5h and $t_{1/2}$ was 44 ± 14 h.

From the present study it is clear that rhFSH exhibits no intrinsic LH activity since no rise in serum LH nor AD or T concentrations (Table 2) occurred. rhFSH is administered im daily, with weekly increments (from 1 to 3 amp [equivalent to 75 IU]/day). The observed steady-state serum FSH levels around 5 days is in agreement with the calculated half-life based on previous bolus studies. Maximum FSH levels as observed in the present study (median 8.8 IU/L) are in the same order of magnitude as compared to perimenstrual concentrations in spontaneous cycles (Fauser et al., 1993a) and maximum serum FSH levels during gonadotropin induction of ovulation according to a step-down dose regimen (Schoot et al., 1992a). FSH stimulation resulted in an immunoreactive INH rise (median 659 U/L) after 15 days which was similar to regularly cycling women (McLachlan et al., 1987). In contrast, only a minimal increase in E_2 (median 112 pmol/L) concentrations could be observed following 19 days of rhFSH administration. This discrepancy in hormone biosynthesis by granulosa cells can be explained by the absence of androgen substrate availability mandatory for adequate estrogen production. Normal immunoreactive INH rise provides proof of normal granulosa cell function. Immunoreactive INH is used as a marker for granulosa cell function in this study, but it should be realized that considerable doubt has been raised (Miro & F Hillier, 1992) recently concerning its biological relevance. It should also be emphasized that 2 patients (#6,7) have been excluded from analysis of ovarian response, since an increase in serum immunoreactive INH levels and follicle growth as monitored by ultrasound was absent – presumably due to early (age 36 and 45 years) menopause – in these women. To further study the potential significance of androgen substrate availability, subjects were divided into groups based on absent or intact adrenal function. Indeed, a difference in AD and T concentrations was observed comparing 4 individuals suffering from IGD/KS versus 3 women that underwent hypophysectomy. However, no difference was observed with regard to maximum E_2 responses following rhFSH stimulation. In addition, no correlation between serum AD and E_2

concentrations could be observed. This may come as no surprise, since AD concentrations in follicle fluid (representing the amount of substrate available for aromatization within ovarian follicles) are 1,000-fold higher (Pache et al., 1992a) and therefore minor differences in serum AD levels may not influence local concentrations. Another intriguing observation is that the observed increase (doubling of baseline levels) in immunoreactive INH starts on day 12, whereas the minor rise in E_2 concentrations begins thereafter (day 15). This difference is statistically significant ($p=0.04$). It may be hypothesized that immunoreactive INH produced by granulosa cells may in turn act as a paracrine regulator to stimulate theca cell androgen production which in turn provides some substrate for E_2 synthesis by granulosa cells (Hsueh et al., 1986; Hillier et al., 1991). A similar mechanism could be speculated for changes in the intra-ovarian insulin-like growth factor (IGF) system, since it has been shown that IGF-1 is a potent stimulator of androgen synthesis by theca cells in culture (Hernandez et al., 1988; Magoffin et al., 1990; Bergh et al., 1993).

The present study clearly extends previous observations indicating that high intrafollicular E_2 concentrations may not be a conditio-sine-qua-non for ongoing maturation of follicles. A discrepancy between serum E_2 levels and follicle development has been observed comparing urinary FSH and human menopausal gonadotropin (HMG; with similar LH and FSH activity)(Couzinet et al., 1988) and combining urinary FSH with long-term gonadotropin releasing hormone agonist cotreatment (Remorgida et al., 1989). In addition, a case history has been published dealing with a patient with 17α hydroxylase deficiency and therefore incapable of producing estrogens (Rabinovici et al., 1991). Exogenous gonadotropins were able to induce growth of follicles up to the pre-ovulatory stage in this patient and oocytes could be fertilized *in-vitro*. Puncture of 3 follicles (13, 15, 18 mm in diameter) following rhFSH administration in subject #1 has shown that both E_2 and AD concentrations within the follicles were indeed extremely low as compared to intrafollicular levels in the late follicular phase under normal conditions (Schoot et al., 1992b; Pache et al., 1992a). In the present study 4 subjects exhibited extensive growth of at least 1 large follicle, whereas the number of medium-sized follicles also increased during rhFSH administration. However, in one patient (#5), follicle growth was disrupted even after an increase of daily dose of rhFSH. Although we feel that the physiological significance of this finding should be interpreted with great care, this study clearly demonstrates that follicles can be stimulated to growth to the pre-ovulatory size without a concomitant rise in E_2 production. The assumption that FSH actions at the follicular level are to a great extent dependent on local up-regulation by E_2 is mainly based on *in-vitro* animal studies (Kessel et al., 1985) and could not be operative *in-vivo* in the human. In fact, so far there is doubt about the presence of E_2 receptors within the human ovary, whereas androgen receptors have been clearly demonstrated by immunocyto-

chemistry (Straus, 1992). Although there is accumulating evidence that estrogens are not mandatory for follicle development in the human, it is well established that under normal conditions estrogen levels are strongly correlated with follicle size (Templeton et al., 1986). In line with these observations, it is believed that disturbed estrogen production is responsible for cessation of follicle maturation in polycystic ovary syndrome patients (Franks, 1989). It may be speculated that under normal conditions next to FSH other estrogen-associated factors (such as changes in the inhibin or IGF system) are responsible for further stimulation of follicle growth, and therefore the rise in estrogens is associated with, but not causally related to, follicle development.

It may be concluded from the present study that: 1) rhFSH exhibits no intrinsic LH activity since a rise in serum androgen concentrations was absent. 2) rhFSH stimulation in hypogonadotropic women resulted in an immunoreactive INH rise which was similar to normal, whereas in contrast only a minor increase in E_2 concentrations could be observed. This indicates normal granulosa cell function and insufficient availability of androgens as substrate for aromatization. 3) Despite the minimal estrogen increase, ovarian follicles developed normally to the pre-ovulatory stage, confirming a dissociation between mitogenic and steroidogenic activity of FSH.

3

Gonadotropin induction of ovulation according to a decremental dose regimen in polycystic ovary syndrome patients

3.1 BACKGROUND STEP-DOWN CONCEPT

A considerable proportion of patients who suffer from infertility and anovulation exhibit insufficient response to anti-estrogen medication. Urinary gonadotropins represent an effective second-line treatment for this group of patients. However, the incidence of complications caused by gonadotropin treatment in anovulatory patients is a major concern (see also sections 1.3.7 and 1.3.8). Due to direct stimulation of the ovaries, development of multiple follicles may lead to multiple pregnancies and ovarian hyperstimulation syndrome (Blankstein et al., 1987). In as much as 50% of patients, complications (chiefly multiple pregnancies, ovarian hyperstimulation or early pregnancy wastage) do occur. Consequently, there is a clear need to test new concepts concerning regulation of follicle growth, and improve treatment outcome. Next to doses administered, numerous additional factors may affect the outcome of gonadotropin therapy, such as: 1) indication for treatment, 2) patient diagnosis and underlying endocrine abnormalities, 3) age and body weight of the patient, 4) previous medication, 5) gonadotropin medication used (HMG or pFSH), 6) adjuvant medication, 7) monitoring of ovarian response and 8) luteal support (for review see Fauser, 1993c). For a more detailed discussion see section 1.3 of this thesis.

The objective of increasing the dosage of gonadotropins during conventional 'step-up' regimens for ovulation induction is to attain and continue the minimal dose required to initiate ovarian response. It was concluded that step-up dose regimens should be individualized: initial daily intramuscular doses of 1 or 2 ampules of gonadotropins, with dose increments of 1 ampule per day every 3 days in case of insufficient response. In practice, this means that if estrogen levels in serum rise or if a dominant follicle is observed by sonography, the 'effective' dose is sustained until ovulation is triggered by administering hCG. These step-up dose regimens have since

been applied by most clinicians (Thompsen & Hansen, 1970). Through the improvement of monitoring of ovarian response (sonography and hormone assays) (Schoot et al., 1992c) success rates have increased, but the incidence of complications remains relatively high.

In the mid-1980's a new method of stimulation was introduced, referred to as the 'low-dose, step-up' regimen (Seibel et al., 1985). The initial dose was low (½ to 1 ampule/day), with no dose adjustment for 1–2 weeks. Subsequent dose increments (½ ampule/day) were applied at weekly intervals if necessary. This empirical method was based on the concept of the 'FSH-threshold' for ovarian stimulation as described by Brown (section 1.2.4.) (Brown, 1978). It was hypothesized that the ovarian requirement for FSH operates in a narrow range. Only a minor increase of administered FSH resulting in serum levels just above the threshold would induce normal follicle growth, whereas a further increase would cause excessive stimulation (Scheele et al., 1993). This minimal requirement of FSH could initiate growth of a synchronized 'recruited' cohort of follicles (Hodgen, 1982). Recently – with the use of intravenous gonadotropin administration and rapid FSH serum measurement – the concept of an FSH-threshold requirement for follicle growth has been further substantiated (Weissenbruch et al., 1993; van der Meer et al., 1994). Serum FSH concentrations below an empirical FSH threshold resulted in an absent estrogen rise whereas an increment of serum FSH level induced a serum E_2 rise and follicle growth. Combined results of 6 different studies using low-dose step-up regimens indicate an overall pregnancy rate of 15% per cycle, 11% multiple pregnancies and a 39% miscarriage rate (Hull, 1991).

The step-down principle for gonadotropin induction of ovulation is based on newly developed insights into the process of selection and growth of the dominant ovarian follicle in the normal cycle (for a review see Fauser et al., 1993; Fauser, 1994b). Key physiological considerations related to normal follicle development that may help to understand the ovarian response to exogenous gonadotropins under patho-physiological conditions are:

1) A minimum stimulation of FSH above the threshold is needed to rescue follicles from atresia during the luteo-follicular transition (follicle recruitment) (Hodgen, 1982).
2) Decrease of serum FSH during the follicular phase is essential for development of a single dominant follicle and atresia of the remaining follicles from the recruited cohort (Zeleznik & Kubik, 1986).
3) The dominant follicle continues to grow despite decreasing FSH presumably due to local (auto- or paracrine) up-regulation (section 1.2.4).

Various aspects of recruitment and dominant follicle selection have been substantiated *in-vivo* in the monkey model (DiZerega & Hodgen, 1980; Zeleznik, 1981; Zeleznik & Kubik, 1986; Abbasi et al., 1987) and some of these observations have also been confirmed in the human (Glasier et al.,

1989; Gougeon & Testart, 1990). If follicles reach a certain stage of development at a size of approximately 2–5 mm, they undergo atresia unless FSH levels are elevated (Gougeon, 1993). The number of recruited follicles is correlated to the duration of the period with serum FSH levels above threshold values (width of the FSH-gate) (Baird, 1987). Appropriate FSH stimulation will initiate granulosa cell activity and stimulate estrogen production. Normal follicles will gain dominance above 8–10 mm and produce significant amounts of E_2 only during further development until ovulation (Erickson et al., 1979). The remaining follicles will go into atresia, in part due to negative feedback actions of ovarian steroids on FSH release (Hillier, 1981; Baird, 1987). It may be postulated that FSH induction of aromatase activity is enhanced by local factors only in the follicle destined to gain dominance (Fauser et al., 1993). Due to (estrogen-mediated) up-regulation, growth of the dominant follicle continues even with decreasing FSH serum levels, i.e. the FSH threshold of the dominant follicle decreases (Scheele, 1994). In contrast, unaltered doses of exogenous gonadotropins following detection of the dominant follicle (as is the case in step-up regimens) can unintentionally disturb the selection process, and therefore stimulate continuous growth of non-dominant follicles. Continued recruitment and follicular growth following ongoing gonadotropin administration agrees with the 'FSH - gate' theory by Baird (Baird, 1987). This continued stimulation of the ovaries is intended during hyperstimulation prior to IVF, deliberately aiming at disruption of the selection process and resulting in multiple follicle development (Hughes et al., 1992). The premises of this step-down dose regimen are:

a) The initial dose has to be sufficient to surpass the threshold.
b) FSH has to be kept above the threshold level for a certain period of time (the FSH-'window').
c) The decreasing levels of serum FSH levels during late follicular phase need to be sufficient to support dominant follicle growth.

It is yet to be determined what can be gained from surpassing the threshold after an extended period of time, as is the case in the low-dose 'step-up' regimens. Studies in the rat demonstrate no significant change in the proportion of healthy follicles following bolus administration of gonadotropins compared with previous spontaneous cycles (Hirschfield, 1989). It should also be emphasized that the relatively long half-life of FSH (30–40 h; [Diczfaluzy & Harlin, 1988; Mizunuma et al., 1990; Mannaerts et al., 1993]) leads to steady-state levels of serum FSH following 5–7 days if similar daily doses are administered intramuscularly. Hence, FSH accumulates in serum and reaches maximum levels in the late follicular phase, contrary to normal circumstances. Mechanisms ensuring monofollicular development under normal conditions may be overruled and follicles may be rescued from atresia if serum FSH levels are elevated in the late follicular phase (Zeleznik et al., 1985). In addition, during the course of prolonged elevated FSH levels,

additional follicles may be recruited and undergo further maturation, resulting in a more heterogeneous group of mature follicles and an extended period of ovulation (Abbasi et al., 1987). Indeed, the magnitude of response during ovarian hyperstimulation for IVF appeared to be dependent on the magnitude of late follicular phase FSH accumulation (Ben Rafael et al., 1986). Low-dose step-up regimens may partly overcome these shortcomings since the FSH-threshold will be surpassed to a minor extent and the FSH-window may not be as wide. However, it is not clear what can be gained from an extended period of time before the FSH threshold is reached (can the ovary be sensitized?) and still obtain maximum FSH levels in the late follicular phase.

The first aim of our studies was to investigate the influence of decreasing doses of gonadotropins following the moment of 'presumed selection' in PCOS patients. The objective was to compare ovarian parameters (sonographic and endocrine data) during a step-down dose regimen with the effects of a continued (fixed) gonadotropin stimulation regimen for induction of ovulation (section 3.2). A retrospective study compared endocrine and sonographic parameters in PCOS patients exhibiting monofollicle development with the follicular phase of the normal menstrual cycle (section 3.3). Furthermore, endocrine background data are provided to substantiate ovarian sensitivity to gonadotropins in order to determine individual threshold dosage and to improve timing and adjustment of doses during a step-down dose regimen (section 3.4).

3.2 GROWTH PATTERNS OF OVARIAN FOLLICLES DURING INDUCTION OF OVULATION WITH DECREASING DOSES OF HUMAN MENOPAUSAL GONADOTROPIN FOLLOWING PRESUMED SELECTION IN PCOS PATIENTS

3.2.1 Introduction

During the follicular phase of the normal menstrual cycle follicle-stimulating hormone (FSH) serum levels decrease gradually (Ross et al., 1969; Fritz & Speroff, 1982; Hodgen, 1982). Mechanisms underlying the process of selection of the dominant follicle and subsequent monofollicular growth are largely unknown, but diminished stimulation of non-dominant follicles by relatively low FSH levels might play – next to intra-ovarian mechanisms – important roles (Fritz & Speroff, 1982, Hodgen, 1982). Moreover, it appears that growth of the dominant follicle is less dependent on stimulation by FSH, possibly because of autocrine effects of high intrafollicular estrogen concentrations (Hsueh, 1986). Indeed, it was shown in the monkey that follicles can continue to mature in the presence of FSH concentrations unable to support the growth of less mature follicles (Zeleznik & Kubik, 1986). In addition, a step-down human menopausal gonadotropin (HMG) protocol

showed better synchronization of follicular rupture, with reduced chances for delayed ovulations (Abbasi et al., 1987). If ovarian function is stimulated by exogenous gonadotropins for induction of ovulation, increasing dose regimens are commonly used (Diamond & Wentz, 1986) and attempts have recently been described to induce monofollicular growth by low-dose incremental dose regimens (Buvat et al., 1989). It may be hypothesized that increasing FSH levels during these treatment schedules unintentionally disturb the selection process, and therefore stimulate continuous growth of non-dominant follicles. Induction of multiple follicular development may underlay complications of treatment such as multiple pregnancy and ovarian hyperstimulation (Blankstein et al., 1987). It is surprising, indeed, that as yet no systematic effort has been made to test this hypothesis in the human. The estimated FSH serum profile during the step-down dose regimen has been reported recently, (Mizunuma et al., 1990) and preliminary clinical observations suggest the decremental dose regimen to be an alternative method for induction of ovulation in polycystic ovary syndrome (PCOS) patients (Mizunuma et al., 1991). Using repeated transvaginal sonography, the dominant follicle could be visualized at a size exceeding 9 mm in normal ovaries (Pache et al., 1990). The main objective of the present study was to investigate whether decreasing the HMG dose after presumed selection (based on sonographic criteria) combined with gonadotropin-releasing hormone (GnRH) analog treatment in PCOS patients, would diminish subsequent growth of non-dominant follicles, and still lead to ovulation. In addition, serum hormone profiles and growth patterns of ovarian follicles were compared with those obtained after a fixed HMG dose schedule, and in regularly cycling women.

3.2.2 Materials and methods

Subjects: Fourteen infertile patients (infertility duration; 4.9 ± 2.0 ($\pm$ SD) years) diagnosed as PCOS entered the study. Nine patients were amenorrheic, and 5 oligomenorrheic (cycle length 42.8 ± 3.1 days). Their mean age was 27.6 ± 2.2 yrs, and mean body mass index (BMI) was 28.9 ± 5.6 kg/m^2. They were all clomiphene resistant, defined as anovulation (diagnosed by basal body temperature, vaginal ovarian sonography, or progesterone estimates) during at least 3 months of 150 mg/d for 5 subsequent days of clomiphene citrate. PCOS was defined – according to strict clinical and biochemical criteria as published previously (Fauser et al., 1991) as women presenting with infertility and oligo/amenorrhea, scoring positive in at least three out of four of the following criteria: 1) Obesity (BMI > 26 kg/m^2), 2) Hirsutism (Ferriman & Gallwey score > 8), 3) Elevated androgen levels (free androgen index (FAI; testosterone (T) X 100 / sex hormone binding globulin) ≥ 5, and/ or dehydroepiandrosterone sulphate (DHEAS) ≥ 10 µmol/L), 4) Polycystic

appearance of ovaries by sonography (Pache et al., 1991b). Mean luteinizing hormone (LH) levels were 7.9 ± 2.1 IU/L. In all these patients thyroid-stimulating hormone levels and prolactin concentrations were within normal limits (data not shown). Seven regularly cycling women served as controls. Clinical and endocrine characteristics of PCOS patients and control women participating in this study are indicated in Table 4.

Study protocol: This study was approved by the Ethics Review Committee of the Erasmus University/Dijkzigt Hospital and informed consent was obtained from all participating women. In all PCOS patients gonadotropin therapy was combined with and preceded by GnRH agonist treatment to prevent premature luteinization, as described by Dodson and colleagues (Dodson et al., 1987). Buserelin (Hoechst, Amsterdam, The Netherlands) was administered at a daily dose of 3 × 400 µg intranasally (in), starting at the first day of a progestagen withdrawal bleeding. Three weeks later HMG (Humegon®; Organon, Oss, The Netherlands) was added at a daily dose of 225 IU (= 3 ampules of 75 IU each) im for 2 days. All HMG administered during this study was derived from the same batch (#890421-018), to exclude the possibility of batch differences in bioavailable gonadotropins (Harlin et al., 1986; Cook et al., 1988). Starting at the first day of HMG administration, daily blood withdrawal (just preceding

Table 4 Clinical and endocrine characteristics of participating women presenting with PCOS (using a fixed and a decreasing HMG dose regimen) and regularly cycling controls. (Data are given as mean ± SD or median [range]).

	PCOS (fixed dose)	*PCOS (decreasing dose)*	*Controls*
n	5	9	7
BMI (kg/m²)	30.3 ± 4.0	28.2 ± 6.3	21.7 ± 2.3
Amenorrhea	2	6	–[a]
Cycle length (days)	42.5 ± 5.5	43.0 ± 6.0	27.8 ± 2.0
F-G score	19.4 ± 8.5	23.6 ± 9.9	5.4 ± 1.0
LH (IU/L)	8.2 (3.7–12.5)	7.6 (4.4–18.8)	4.2 (2.4–6.4)
FAI	8.2 (3.5–22.1)	6.0 (2.6–16.5)	1.8 (1.0–2.0)
DHEAS (µmol/L)	7.0 ± 2.2	8.4 ± 1.0	–[b]

[a]: not applicable
[b]: not available

HMG injections, and 24 h following the previous injection) and vaginal sonography were performed until human chorionic gonadotropin (hCG) administration. After 2 days, HMG doses were decreased to 150 IU/day (= 2 amp) im in all patients. If at least 1 ovarian follicle exceeded a diameter of 9 mm, randomization for 2 different treatment regimens was performed. To exclude the possible influence of bodyweight on treatment response, randomization was preceded by stratification on bodyweight (BMI ≤ 26, or > 26). Daily doses of 150 IU were continued in the fixed-dose group (n=5) and the dose was diminished to 75 IU/d in the decreasing-dose group (n=9). Dosages remained unchanged in each group until at least one follicle attained a diameter of 18 mm or more. On that day buserelin and HMG medication was discontinued, and 10,000 IU of hCG (Pregnyl®; Organon, Oss, The Netherlands) was administered im. If more than 4 follicles exhibited a diameter of > 15 mm, further stimulation was canceled. If growth of follicles was absent during 7 subsequent days after randomization further medication was also withheld. Vaginal ovarian sonography was performed daily between 12:00 and 16:00 P.M. by the same observer (DCS), according to previously validated methods (Pache et al., 1990). A 5-MHz transvaginal transducer (Model 1550; Philips Medical Systems, Eindhoven, The Netherlands) was used. The diameter of small follicles was measured in 2 dimensions. If the mean of the longitudinal and anteroposterior diameter exceeded 7.0 mm, a diameter in the third lateral plane was added. Follicles were classified as small (mean size < 9 mm), medium-sized (mean size between 9 and 13 mm), and large (mean size ≥ 13 mm), based on observations indicating that women with ovarian hyperstimulation present with elevated numbers of intermediate follicles (Blankstein et al., 1987).

Hormone assays: After withdrawal, blood was centrifuged within 30 min, and serum was stored at –20°C until assayed. Immunoreactive FSH serum levels were determined using a commercially available immunoradiometric assay (IRMA) kit (Medgenix, Fleurus, Belgium) as described previously (Fauser et al., 1990). Data are expressed in terms of MRC 78/549 reference preparation, and intra- and interassay coefficients of variation were less than 3 and 8%, respectively. Estradiol (E_2) levels were estimated by radioimmunoassay (RIA) (Fauser et al., 1991b). Intra- and interassay coefficients of variation were 5 and 8%, respectively.

Data analysis: Endocrine and sonographic data related to the first day of HMG administration (day 1) were compared with onset of menses in regularly cycling women. Data obtained during the interval between randomization and hCG administration were compared with those in the period following presumed selection in the control group (Pache et al., 1990). Results are presented as mean ± SEM, unless otherwise stated. Comparison of hormone patterns, following randomization, between the two treatment regimens and controls, was performed using Repeated Measurements Analysis of

Variance. In this analysis, E_2 levels were logarithmically transformed to eliminate skew distributions. Sonographic observations were compared using Mann-Whitney's test or Wilcoxon's test. P-values given are two-sided, and 0.05 was considered the limit of statistical significance.

3.2.3 Results

Clinical and endocrine observations in participating PCOS women and normal controls are depicted in Table 4 (above). Dose regimens, serum profiles of FSH and E_2 levels, and growth patterns of ovarian follicles of two PCOS patients, one randomized for the decreasing and one for the fixed HMG dose regimen, are shown in Fig. 4. Two women, both in the decreasing dose regimen group, failed to show ongoing follicular development above 14 mm. Medication was discontinued in these patients and therefore information related to hCG administration could not be obtained (see Figs. 5, 6, and 7). Randomization (at least 1 follicle $\geq$ 9 mm) was performed on the fourth treatment day in 13 PCOS patients, and in the remaining woman on day 5. The interval between randomization and hCG injection was not significantly different comparing both treatment groups (3.6 $\pm$ 0.5 days for the fixed dose, and 4.7 $\pm$ 0.8 days for the decreasing-dose group). Proof of ovulation was obtained from all 12 patients who received hCG through sonography and mid-luteal progesterone estimates, which all exceeded 25 nmol/L (data not shown).

Hormone estimates: On the first day of HMG injection, after 3 weeks of buserelin treatment, mean FSH concentration (3.4 $\pm$ 0.4 IU/L) in the PCOS group (n=14) was significantly lower as compared to the control group (5.4 $\pm$ 0.7 IU/L) at the onset of menses, whereas mean serum E_2 levels did not differ (PCOS group; 115 $\pm$ 10 pmol/L, controls; 91 $\pm$ 7 pmol/L). FSH levels in the treatment group reached levels similar to the control group (5.2 $\pm$ 0.5 IU/L), within 1 day after starting HMG medication. During the first two days following the initiation of HMG administration, a clear increase in FSH levels was found (mean FSH at day 3: 7.6 $\pm$ 0.7 IU/L). At the day of randomization, a marked elevation in serum E_2 concentrations could be observed in PCOS patients (1844 $\pm$ 420 pmol/L), significantly ($p<0.001$) above normal concentrations (138 $\pm$ 15 pmol/L). In retrospect, no hormonal differences were present on the day of randomization, between both treatment groups (data not shown). Following randomization, mean serum FSH levels in the fixed-dose group remained unchanged, whereas a significant ($p < 0.001$) decline of mean FSH concentrations in the decreasing-dose group (mean daily decrease 0.45 $\pm$ 0.01 IU/L) was found. In controls, a significant ($p < 0.001$) decrease in FSH estimates could be observed during late follicular phase (mean daily decrease 0.36 $\pm$ 0.01 IU/L). Comparing the decrease of FSH

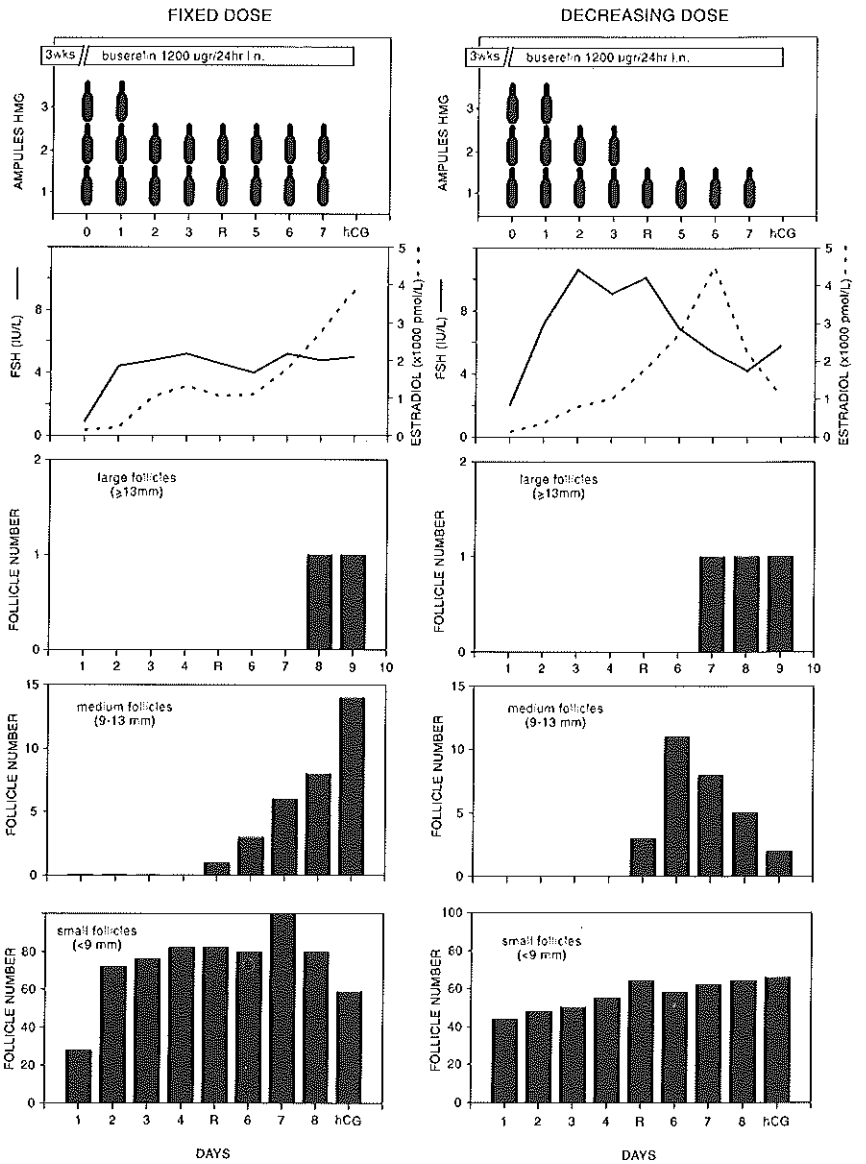

Figure 4 Buserelin and HMG medication schedule, daily FSH (IU/L) and E_2 (× 1000 pmol/L) serum levels, and numbers of large (≥ 13 mm), medium-sized (9–13 mm) and small (< 9 mm) ovarian follicles, as estimated by vaginal sonography, in a PCOS patient from the fixed-dose (left panel) and the decreasing (right panel) HMG dose group. R = day of randomization, chosen as day of presumed selection.

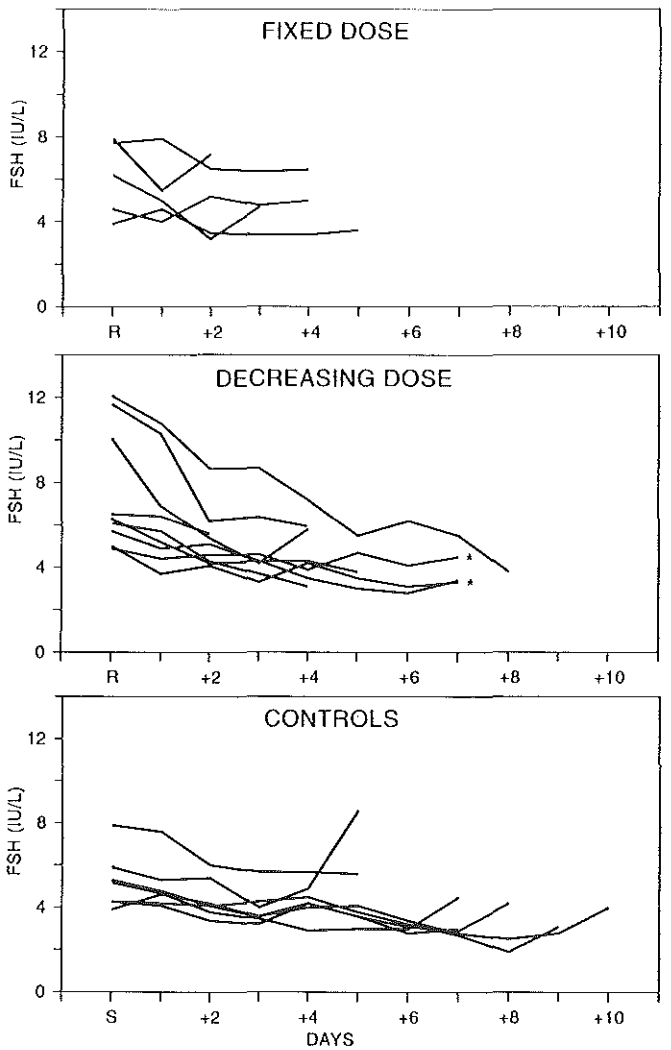

Figure 5 Individual serum FSH levels (IU/L) in the fixed HMG dose group (upper panel), the decreasing HMG dose group (middle panel), and normal controls (lower panel). R (randomization) in both treatment groups and S (selection) in controls represents the day of presumed selection of the dominant follicle. FSH levels are indicated until hCG injection in both treatment groups and until 1 day before LH surge in controls; * indicates: follicle maturation arrest and cancellation of treatment in 2 women in the decreasing-dose group.

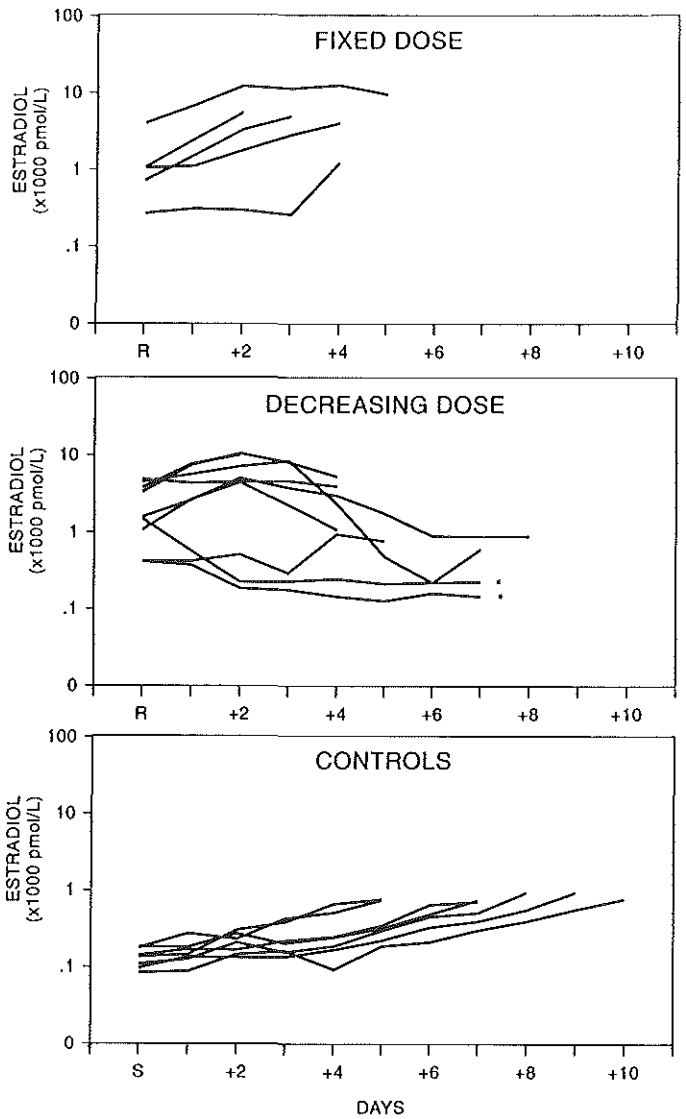

Figure 6 Individual serum estradiol levels (× 1000 pmol/L) in the fixed HMG dose group (upper panel), the decreasing HMG dose group (middle panel), and normal controls (lower panel). R (randomization) in both treatment groups and S (selection) in controls represents the day of presumed selection of the dominant follicle. E_2 levels are indicated until hCG injection in both treatment groups and until 1 day before LH surge in controls; * indicates: follicle maturation arrest and cancellation of treatment in 2 women in the decreasing-dose group.

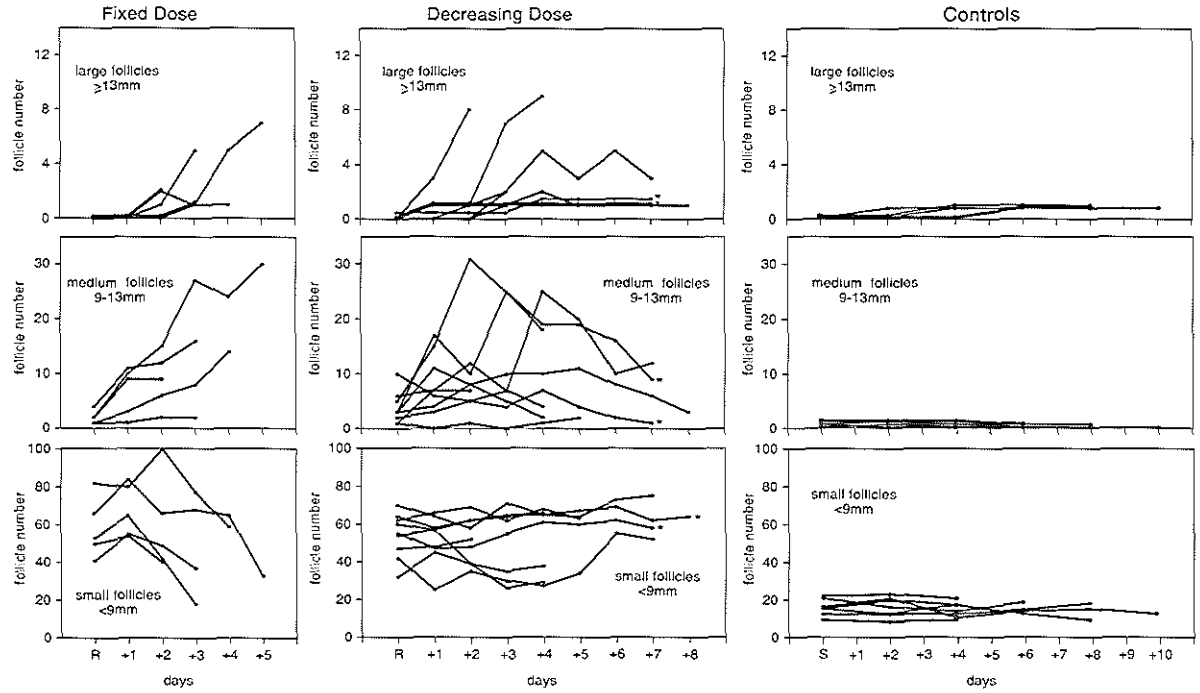

Figure 7 Individual numbers of large (≥ 13 mm; upper panel), medium-sized (9–13 mm; middle panel), and small (< 9 mm; lower panel) ovarian follicles in the fixed-dose (left panel), the decreasing HMG dose group (middle panel), and normal controls (right panel). R (randomization) in both treatment groups and S (selection) in controls represents the day of presumed selection of the dominant follicle; * indicates: follicle maturation arrest and cancellation of treatment in 2 women in the decreasing-dose group.

concentrations in the decreasing dose and control groups, no statistical difference was found (see Fig. 5). During the first two days following randomization, mean E_2 levels showed a similar increase in both treatment groups. Following the third day after randomization, mean E_2 concentrations decreased significantly ($p < 0.001$; average daily decrease: 26%) in the decreasing-dose group, whereas the E_2 levels in the fixed-dose group showed a significant increase ($p < 0.001$; average daily increase: 25%). A less pronounced, but significant increase was observed in the controls (see Fig. 6).

Ovarian sonography: Following 3 weeks of buserelin treatment, both ovaries of PCOS patients contained 36 ± 4.8 ($\pm$ SD) follicles (mean size 2.9 ± 0.3 mm). The mean total number of follicles increased towards the day of randomization to 55 ± 15, in the PCOS group. On the day of randomization, no significant differences existed in mean number of small (≤ 9 mm) and medium-sized (9–13 mm) follicles comparing both treatment groups (Fig. 7). Monitoring changes in ovarian follicles between randomization and the day of hCG administration in the fixed-dose group revealed a significant initial increase in the number of small follicles, followed by a significant ($p < 0.02$) decline towards hCG administration. In the decreasing-dose group, the mean number of small follicles remained unchanged between randomization and hCG administration. The mean number of medium-sized follicles showed an increase during the first three days following randomization in both treatment groups (follicle number increase in fixed-dose group; 9.2 ± 3.3, decreasing-dose group; 6.8 ± 3.2). In subsequent days up to hCG administration, a further increase of medium-sized follicles was seen in the fixed-dose group ($+3.3 \pm 1.4$), whereas a reduction was found in the decreasing-dose group (-3.0 ± 2.0). This difference, however, is just above the limit of statistical significance ($p < 0.07$). The number of large (≥ 13 mm) follicles (fixed-dose group; 3.1 ± 0.3, decreasing-dose group; 3.5 ± 0.2) was similar in both treatment groups. The mean number of follicles (7.4 ± 0.6) did not change throughout the follicular phase in control women (Pache et al. 1990).

3.2.4 Discussion

Based on initial observations (Thompson & Hansen, 1970; Taymor et al., 1967) it seems that decreasing HMG dose regimens were as successful as fixed-dose schedules for gonadotropin induction of ovulation. During single (Seddon, 1970) and intermittent (Radwanska et al., 1980) dose regimens complication rates were noted to be elevated and success rates to be reduced. For reasons of presumed safety and efficacy, the fixed-dose and step-up dose regimens were generally accepted for HMG therapy. Until today all efforts to improve treatment schedules have focussed on the use of

different preparations (i.e. purified FSH instead of HMG), and monitoring of ovarian response by sonography and estrogen estimates during incremental-dose regimens. Based on recently obtained insight in the regulation of ovarian function under normal conditions, it seems mandatory to reassess the possibility of reducing complication rates during induction of ovulation using decremental HMG dose regimens.

Previously, transabdominal monitoring of ovarian function during induction of ovulation was restricted by limited resolution, and systematic efforts to carefully monitor growth patterns of all ovarian follicles have been rare (Diamond & Wentz, 1986). Using vaginal sonography for the characterization of ovarian changes during the normal menstrual cycle (Pache et al., 1990), it could be established that: 1) follicles as small as 2mm in size can be visualized, 2) the number of follicles (roughly 8/ovary) remains unchanged during different phases of the cycle, and 3) the dominant follicle can be observed consistently if the follicle diameter exceeds 9 mm. Furthermore, this method has been applied to characterize polycystic changes of ovaries in women presenting with cycle abnormalities (Pache et al 1991b). Application of this technique also enables monitoring of changes in daily distribution of ovarian follicles during induction of ovulation by exogenous gonadotropins. Analysis of data was complicated by the inability to follow changes of individual follicles. An arbitrary classification of follicles into small, intermediate, and large was therefore required. GnRH analog treatment and subsequent pituitary desensitization was applied to minimize interference between administered HMG and endogenous gonadotropin secretion (Dodson et al., 1987). Indeed, FSH levels in the PCOS group after 3 weeks of buserelin treatment were below normal, with a rapid increase following initiation of HMG administration. Inter-individual variation in absorption and clearance of HMG (Diczfaluzy & Harlin, 1988; Fauser & Hsueh, 1989) may underlay the wide range of observed serum FSH levels in both treatment groups (Fig. 5). In the decreasing-dose group, FSH levels signficantly diminished after randomization, similar to FSH concentrations in the late follicular phase of the normal cycle and different from the FSH pattern in the fixed-dose group. It has to be realized that, in contrast to normal circumstances, FSH levels exhibit diurnal variation in both treatment groups because of single daily injections. Based on pharmacokinetic simulation programs Mizunuma et al. (1990) estimated that maximum levels raise about 30% above concentrations at the time of injection. Moreover, it is not known at present whether immunoactive FSH levels measured following exogenous HMG represent similar bioactivity (Ben-Rafael et al., 1986). Due to abnormal ovarian function in PCOS women with cycle abnormalities and clomiphene resistance, ovarian response was expected to show a wide variation, and to be different from controls. Endogenous luteinizing hormone (LH) concentrations, and the use of gonadotropin preparations with different LH/FSH ratios may also affect

ovarian responses. Far greater total numbers of ovarian follicles were noted, and especially the great number of medium-sized follicles (which never exceeded 1 in normal cycles; see Fig. 7) was remarkable. E_2 levels were similar at initiation of treatment as compared to controls, but at the day of presumed selection (4 days later) E_2 increments exceeded 10-fold. E_2 levels continued to rise in the fixed-dose group, whereas a significant decline of serum E_2 started 3 days after randomization in the decreasing-dose group. This may be related to the decrease in number of medium-sized follicles, suggesting that relatively low FSH levels cause maturation arrest of functional medium-sized follicles. In 2 patients from the decreasing-dose group the leading follicle showed arrested maturation and E_2 output remained low, suggesting that FSH levels may have been to low.

In summary, this study represents an attempt to systematically monitor ovarian response, by serum hormone estimates and vaginal sonography during gonadotropin induction. Obtained data suggest that a decreasing HMG dose regimen in combination with GnRH analog treatment may diminish unintended stimulation of medium-sized follicles. The observed decrease in circulating E_2 levels, concomitant with the reduction in number of medium-sized follicles in this group, further points to a functional decline in ovarian response. The ovarian hyperstimulation syndrome can be predicted by the observed number of intermediate-sized follicles, as shown by Blankstein and colleagues (Blankstein et al., 1987). Although the clinical relevance should be further tested by adjusting dose regimens in larger patient series, it may be suggested that the incidence of ovarian hyperstimulation (and presumably multiple pregnancies) can be reduced using decremental HMG dose regimens during gonadotropin induction.

3.3 GROWTH OF THE DOMINANT FOLLICLE IS SIMILAR TO NORMAL IN GONADOTROPIN-STIMULATED PCOS PATIENTS EXHIBITING MONOFOLLICULAR DEVELOPMENT DURING A DECREMENTAL DOSE REGIMEN

3.3.1 Introduction

Patients suffering from anovulatory infertility and PCOS are frequently resistant to clomiphene citrate treatment and need gonadotropins to induce ovulation (Wang & Gemzell, 1980). In these patients arrested development of ovarian follicles appears to be due to disturbed selection of the dominant follicle. This concept of normal early follicle development is based on observations regarding aromatase activity of cultured granulosa cells (Erickson et al., 1979), follicular fluid steroid hormone estimates (Pache et al., 1992b), and careful monitoring of ovarian function using transvaginal sonography (Pache et al., 1990; Pache et al., 1992a). It therefore seems of

special interest to further explore developmental characteristics of follicles after selection has taken place in PCOS due to a transient elevation of serum follicle stimulating hormone (FSH) concentrations subsequent to exogenous gonadotropin administration.

Assessment of growth rates of large ovarian follicles during multiple follicle development in gonadotropin-stimulated cycles is hampered by difficulties with recognizing individual follicles during successive sonographic investigations. Moreover – in sharp contrast to the late follicular phase during the normal menstrual cycle (Ylöstalo et al., 1979) – the correlation between serum estradiol (E_2) levels and follicle size has disappeared if growth of multiple large follicles is observed under these conditions (Mango et al., 1988). The aim of this study was to investigate the late follicular phase of PCOS patients exhibiting growth of one single dominant follicle following gonadotropin induction of ovulation using a decremental dose regimen and adjuvant gonadotropin releasing hormone (GnRH) agonist medication as previously described (Schoot et al., 1992a). Observations in regularly cycling women served as the control.

3.3.2 Materials and methods

Subjects and study protocol: Seven (from a total group of 35) gonadotropin-treated, infertile (duration; 4.7 ± 1.5 (± SD) years) PCOS patients were included from our infertility clinic, based on the presence of not more than 1 follicle ≥ 12mm diameter during gonadotropin induction of ovulation. Three patients were amenorrheic, and 4 oligomenorrheic (cycle length 44.7 ± 5.1 days). Their mean age was 29.4 ± 3.2 years, and body mass index (BMI) 29.1 ± 4.9 kg/m^2. They all failed to ovulate using clomiphene citrate up to 150 mg daily during five consecutive days. PCOS was defined according to strict clinical (BMI, Ferriman Gallwey score), endocrine (elevated dehydroepiandrosterone sulphate [DHEAS] serum levels [≥ 10 mmol/l], and high Free Androgen Index [FAI= testosterone x 100/ sex hormone binding globulin {SHBG} ≥ 5]) criteria and polycystic appearance of ovaries by transvaginal sonography as published previously (Fauser et al., 1991). Mean luteinizing hormone (LH) levels were 7.9 ± 2.1 IU/l, whereas FSH concentrations were 4.7 ± 1.4 IU/l. Sonographic and endocrine data of seven healthy regularly cycling women (C) (mean age 28.0 ± 1.2 years, BMI; 21.7 ± 2.3 kg/m^2, and cycle length; 28 ± 2 days) recruited through advertisement served as the control.

This study was approved by the Ethics Review Committee of the Erasmus University/Dijkzigt Hospital and informed consent was obtained from all participating women. In all PCOS patients gonadotropin therapy was combined with and preceded by GnRH agonist (buserelin; Hoechst, Amsterdam, The Netherlands) treatment (3 × 400 mg/day, intranasally). GnRH agonist treatment was started on the first day of a spontaneous or progestagen

withdrawal bleeding and was continued for a period of three weeks, followed by concomitant treatment with randomly chosen gonadotropins (Humegon^R, [Organon Int, Oss, The Netherlands] n=4; or Metrodin^R, [Serono, Amsterdam, The Netherlands] n=3). Starting on the first day of gonadotropin administration, daily blood withdrawal (just preceding gonadotropin injections) and transvaginal sonography took place. Sonography was performed between 12:00 and 16:00 P.M. by the same observer (DCS), according to previously validated methods (Pache et al., 1990). A 5-MHz transvaginal transducer (Model 1550; Philips Medical Systems, Eindhoven, The Netherlands) was used. Mean size of ovarian follicles (> 2 mm diameter) was computed measuring three dimensions (longitudinal, antero-posterior, and tranverse).

The initial dose of gonadotropins was 150 or 225 IU/day im. The dose was decreased by ½ or 1 amp (=75 IU)/day if at least 1 follicle exceeded a diameter of 9 mm. Further decrease to 75 IU/day was continued until administration of human chorionic gonadotropin ([hCG] Pregnyl^R; Organon Int). When the mean diameter of the dominant follicle exceeded 18 mm, buserelin and gonadotropins were discontinued, and 10,000 IU of hCG was administered. No luteal support was provided.

Hormone assays: After withdrawal, blood was centrifuged within 30 min, and serum was stored at –20°C until assayed. FSH serum levels were determined using a commercially available immunoradiometric assay kit (Medgenix, Fleurus, Belgium) as described previously (Fauser et al., 1991). Data are expressed in terms of MRC 78/549 reference preparation, and intra- and interassay coefficients of variation were less than 3 and 8%, respectively. Serum E_2 levels were estimated by radioimmunoassay (Pache et al., 1992b). Intra- and interassay coeffients of variation were 5 and 8%, respectively.

Data analysis: Endocrine and sonographic data obtained on the last day before the spontaneous LH peak in controls were compared with data obtained on the day of hCG administration in PCOS patients (estimated interval between LH and ovulation 12 h, versus that between hCG and ovulation 36 h). 'Presumed selection' was defined, based on previous studies under normal conditions (Pache et al., 1990), as the day that one follicle could be visualized at a size exceeding a mean diameter of 10 mm. The minimum size of a dominant follicle measured during gonadotropin induction of ovulation in PCOS patients was decided to be 12 mm due to the potential interference of one or more large (8–12 mm) secondary follicles. Results are presented as mean ± SD, unless otherwise stated. Comparison of hormone patterns between the treatment group and controls, was performed using Mann-Whitney's test and Repeated Measurements Analysis of Variance. Sonographic observations were compared using Mann-Whitney's test or Wilcoxon's test. *P*-values given are two-sided, and 0.05 was considered the limit of statistical significance.

3.3.3 Results

On the day of presumed selection mean FSH levels in the control group were 4.4 ± 0.8 IU/L, whereas FSH concentrations in the PCOS group were 6.8 ± 2.3 IU/L (p=0.3) (Fig. 8). FSH levels at the day before the LH surge in controls were not significantly different from those in the PCOS group on the day of hCG administration (3.3 ± 1.8 IU/L in controls, versus 3.4 ± 2.2 IU/L in PCOS). Daily serum FSH levels in PCOS patients decreased more rapidly as compared to controls (–0.3 ± 0.2 IU/day in controls, versus –0.7 ± 0.4 IU/day in PCOS; P< 0.02). Mean FSH levels on the first day of gonadotropin administration were not significantly different from the day of hCG administration in the PCOS group (2.9 ± 1.6 IU/L, versus 3.4 ± 2.2 IU/L; p=0.06).

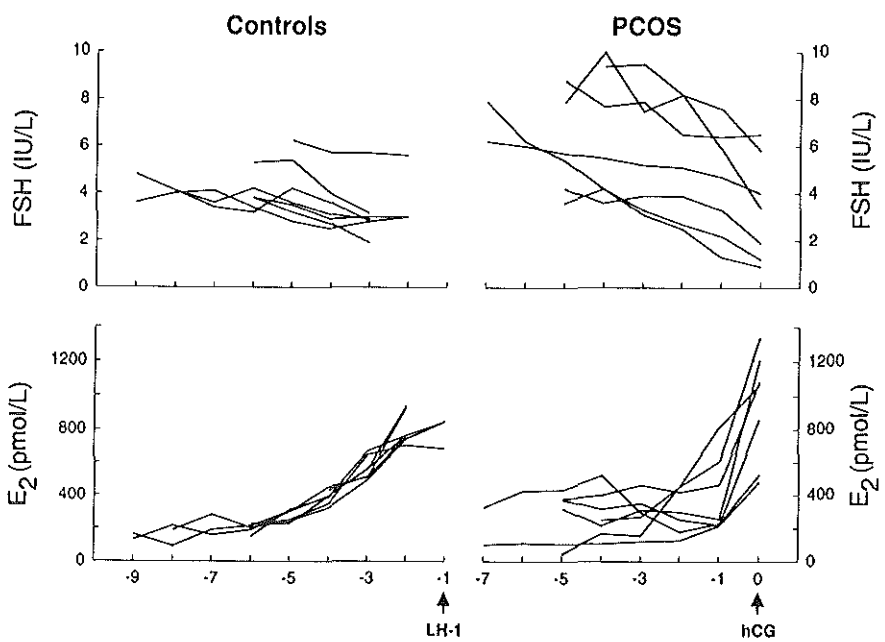

Figure 8 Daily changes in individual serum concentrations of FSH (IU/L) and E_2 (pmol/L) on the days prior to the LH surge in 7 controls (left panel), and days before hCG administration in 7 gonadotropin-treated PCOS patients (right panel).

Mean E_2 concentrations on the day of presumed selection in the treatment group were 255 ± 137 pmol/L compared to 211 ± 108 pmol/L in controls. No statistically significant differences were seen in daily increase (30% in controls and PCOS) and mean peak levels (825 ± 94 pmol/L in controls, versus 937 ± 231 pmol/L in PCOS) of E_2 serum levels comparing both groups. The interval between the day of selection and the day before the spontaneous LH surge in controls, and the day of hCG administration in PCOS patients was not significantly different (6.7 ± 1.9 days in controls versus 5.4 ± 1.1 in PCOS).

The mean diameter of the dominant follicle on the day before the LH peak in controls was 18.4 ± 1.0 mm, and 18.0 ± 0.3 mm in PCOS on the day of hCG administration (Fig. 9). Mean daily growth of the dominant follicle (1.7 ± 0.4 mm in controls, versus 1.9 ± 0.6 mm in PCOS) was not significantly different.

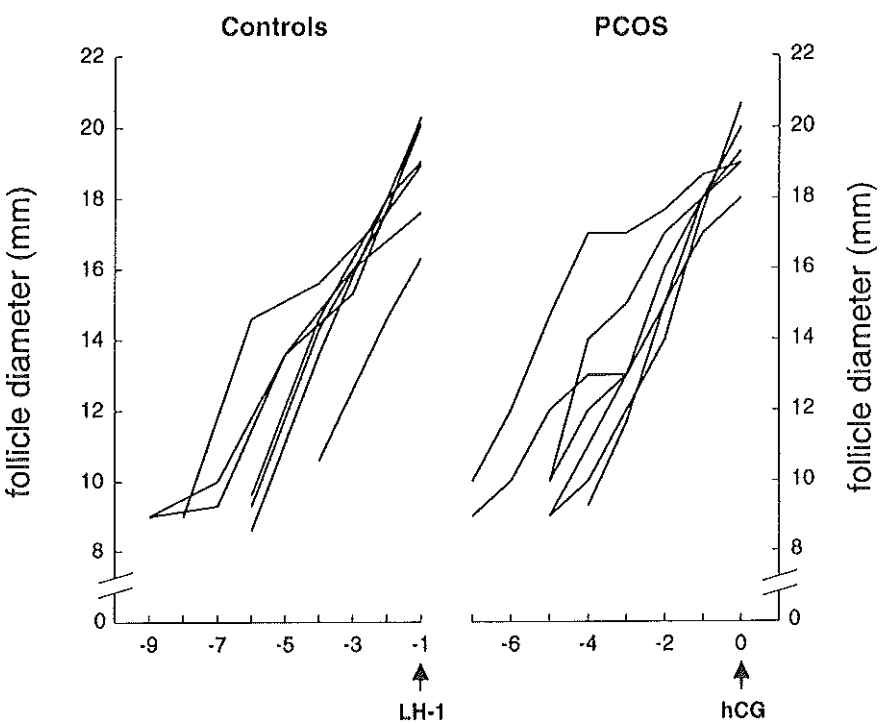

Figure 9 Size (mm) of individual dominant follicles on days prior to the LH surge in 7 controls (left panel), and days before hCG administration in 7 gonadotropin-treated PCOS women (right panel).

3.3.4 Discussion

Based on previous studies (Pache et al., 1990), it is assumed that selection of the dominant ovarian follicle is disturbed in women suffering from PCOS. Normal FSH serum levels in the patients (Fauser et al., 1991) are insufficient to induce adequate aromatase activity (Erickson et al., 1979; Pache et al., 1992b) (i.e. the FSH threshold for follicle stimulation is augmented). In these patients, follicles can be stimulated to ongoing development by a transient increase of FSH serum concentrations subsequent to exogenous administration of gonadotropins. It has been demonstrated (Mango et al., 1988) that diminished stimulation of nondominant follicles beyond the day of presumed selection, by decreasing the daily dose of exogenous gonadotropins, reduces number and size of functional medium sized follicles. Moreover, follicles that have reached a certain stage of maturation will continue their development in the presence of decremental FSH serum concentrations (Mango et al., 1988).

Comparison of previous studies concerning follicle development in gonadotropin-induced cycles was hampered by differences in patient criteria, applied preparations and dose regimens (Sallam et al., 1982; Schmidt et al., 1981). In addition, the definition of "monofollicular" growth in induced cycles revealed discrepancies, partially due to the trans-abdominal technique of sonography (Coleman et al., 1988). Simultaneous development of secondary medium-sized follicles and differences in timing of hCG injections can explain differences in growth slopes, estrogen production and preovulatory size of large follicles (Templeton et al.,1986). Using pulsatile intravenous administration of FSH, in a low-dose, incremental dose regimen, follicle growth was also suggested to be similar to normal (Polson et al., 1987). In the present study exhibiting decreasing serum FSH concentrations – due to the applied decremental dose regimen – it was demonstrated that growth and estrogen production by the dominant follicle is similar as compared to the normal menstrual cycle. This finding is in agreement with animal studies (Zeleznik & Kubik, 1986) suggesting that the dominant follicle is less dependent on FSH stimulation. This may be due to intra-ovarian auto- or paracrine up-regulation (Fauser & Hsueh, 1988). Although all gonadotropin-treated patients exhibiting monofollicular growth selected for this study fulfilled the same strict criteria for definition of PCOS, it cannot be ruled out that this study includes only a specific proportion of all PCOS patients. It may be concluded from this preliminary study, that if monofollicular development occurs in gonadotropin-treated PCOS patients using decremental dose regimens (and adjuvant GnRH agonist treatment), growth and steroidogenic activity of the dominant follicle is indistinguishable from normal. Once formation of a dominant follicle has been induced by a transient increase in serum FSH, further development is normal despite

decreasing serum FSH levels. Serum FSH levels during the late follicular phase in gonadotropin-treated PCOS patients showed a return to early follicular FSH serum concentration and were similar to normal preovulatory levels. This gives further support to the notion that in at least a proportion of PCOS patients early follicle development is normal and only selection of the dominant follicle is disturbed.

3.4 INITIAL ESTRADIOL RESPONSE PREDICTS OUTCOME OF EXOGENOUS GONADOTROPINS USING A STEP-DOWN DOSE REGIMEN FOR INDUCTION OF OVULATION IN PCOS PATIENTS

3.4.1 Introduction

Patients suffering from anovulation due to PCOS frequently exhibit insufficient response to clomiphene citrate therapy. Exogenous gonadotropins are subsequently administered, commonly in stepwise incremental doses ("step-up" regimens) (Thompson & Hansen, 1970). Daily dosages remain unchanged once response is judged to be adequate (Sagle et al., 1991) i.e. the FSH threshold (Brown, 1978) for ovarian stimulation has been reached. However, this approach does not take into account the notion that the dominant follicle needs less FSH for ongoing development (Zeleznik & Kubik, 1986; Abbasi et al., 1987) presumably due to intra-ovarian up-regulation (Hsueh et al., 1984). It may be hypothesized that in step-up regimens accumulation of FSH in the late follicular phase – due to the relative long half-life of FSH (Mizunuma et al., 1990) – could unintentionally disturb the selection process, leading to development of multiple follicles (Ben-Rafael et al., 1986; Fauser et al., 1993c). This in turn may underlay complications of treatment such as multiple pregnancy, and ovarian hyperstimulation (Blankstein et al., 1987). In this context it has been reported that even during low-dose step-up regimens a considerable proportion of multiple follicle growth can be observed (Herman et al., 1993). Recently, efforts to apply an alternative decreasing dose regimen revealed evidence that stimulation of secondary follicles is less pronounced during a step-down regimen (Schoot et al., 1992a) and that monofollicular growth can be achieved in a considerable proportion of patients (Schoot et al., 1993).

Ovarian response during conventional gonadotropin induction of ovulation is monitored by serum E_2 levels or pelvic ultrasound mainly focusing on the late follicular phase. Comparison of both monitoring techniques revealed no clear differences (Mango et al., 1988). During previous studies (Schoot et al., 1992a; Schoot et al., 1993) using the step-down approach (focusing on diminished interference with the selection

process) the decrease in gonadotropin doses administered was based on sonographic criteria only. This was related to observations under normal conditions where a dominant follicle could be visualized from a diameter of 9 mm upwards (Pache et al., 1990) with only thereafter a considerable rise in serum E_2 levels (Fauser et al., 1993a). A similar endocrine profile was found indeed in gonadotropin-treated PCOS patients exhibiting monofollicular growth (Schoot et al., 1993). However, the use of ultrasound alone in PCOS patients may be hampered by major inter-individual differences in E_2 biosynthesis following similar FSH stimulation (Caruso et al., 1993). The present study involves an endocrine analysis of ovarian response during gonadotropin-induced cycles using a step-down dose regimen (and adjuvant gonadotropin releasing hormone [GnRH] -agonist treatment) based on sonographic criteria only in infertile anovulatory clomiphene-resistant PCOS patients.

3.4.2 Materials and methods

Subjects: Twenty-eight infertile (duration; 4.5 ± 2.2 [SD] years) patients diagnosed as PCOS participated after informed consent was obtained. This study was approved by the Ethics Review Committee of the Erasmus University/Dijkzigt Hospital. PCOS was defined – as published previously (Fauser et al., 1991) – as women presenting with infertility and oligo/amenorrhea, scoring positive in at least three out of four of the following criteria: 1) Obesity (body mass index [BMI] > 26 kg/m^2), 2) Hirsutism (Ferriman & Gallwey score > 8), 3) Elevated androgen levels (free androgen index [FAI]; testosterone [T] X 100 / sex-hormone-binding globulin [SHBG]) ≥ 5, and/ or dehydroepiandrosterone sulphate (DHEAS) ≥ 10 ± mol/L), and 4) Polycystic appearance of ovaries by transvaginal sonography (Pache et al., 1992a). Luteinizing hormone (LH) concentrations in these patients were 7.9 ± 0.6 IU/L (see Table 5). They were all clomiphene-resistant, defined as anovulation (diagnosed by basal body temperature, vaginal ovarian ultrasound, or progesterone serum estimates) during at least 3 months of 150 mg/d clomiphene citrate for 5 subsequent days. In all these patients thyroid-stimulating hormone levels and prolactin concentrations were within normal limits (data not shown).

Study protocol: In all PCOS patients gonadotropin medication was combined with and preceded by GnRH agonist treatment to prevent premature luteinization (Dodson et al., 1987) and reduce chances of interference between pituitary gonadotropins and exogenous medication. Buserelin (Hoechst, Amsterdam, The Netherlands) was administered at a daily dose of 3 × 400 μg intranasally (in), starting on the first day of a progestagen withdrawal bleeding or spontaneous bleeding. After

Table 5 Clinical and endocrine (mean ± SEM) characteristics of 28 anovulatory, clomiphene-resistant PCOS patients participating in this study.

Age (years)	27.4 ± 1.1
BMI (kg/m²)	28.4 ± 0.8
Amenorrhea (n)	14
Polycystic ovaries (n)[a]	25
LH (IU/L)	7.9 ± 0.6
FSH (IU/L)	4.3 ± 0.4
E_2 (pmol/L)	216 ± 17
T (pmol/L)	2.8 ± 0.2
FAI (Tx100/SHBG)	10.6 ± 1.5
DHEAS (µmol/L)	6.8 ± 0.6

[a]:number of patients presenting with polycystic appearance of ovaries as estimated by transvaginal sonography (Pache et al., 1992a).

randomization, human menopausal gonadotropins (HMG) (Humegon®; Organon, Oss, The Netherlands) or purified urinary FSH (pFSH) (Metrodin®; Serono, Weesp, The Netherlands) was added 3 weeks later at an initial daily dose of 150 IU (= 2 ampules of 75 IU each) im. Both HMG and pFSH administered during this study were derived from one batch (#890421-018 and #90 G23, respectively) to exclude potential batch differences in bioavailable hormones (Cook et al., 1988). Starting on the first day of gonadotropin administration, daily blood-withdrawal (just preceding im injections, and 24 h following the previous injection) and transvaginal sonography was performed until hCG (Pregnyl®; Organon, Oss, The Netherlands) administration. If at least 1 follicle exceeded a diameter of 9 mm, daily doses of gonadotropins were reduced to 1½ ampules for a fixed period of 2 consecutive days, followed by a further reduction to 75 IU/day im. Daily doses of 75 IU were continued and remained unchanged until at least one follicle (and no more than 3) attained a diameter of 18 mm or more. On that day buserelin and HMG medication was discontinued, and 10,000 IU of hCG was administered im. If more than 3 follicles exhibited a diameter of > 15 mm, further stimulation was canceled. If growth of follicles was absent during 7 consecutive days medication was increased and these patients were considered to be drop-outs from the study. No luteal support was provided. Transvaginal ovarian sonography was performed daily between 12:00 and 16:00 P.M. by the same observer (DCS), according to previously validated methods (Pache et al., 1990). A 5-MHz transvaginal transducer (Model 1550; Philips Medical Systems, Eindhoven, The

Netherlands) was used. The diameter of small follicles was measured in 2 dimensions. If the mean of the longitudinal and antero-posterior diameter exceeded 7 mm, a diameter in the third lateral plane was added.

Hormone assays: After withdrawal, blood was centrifuged within 30 min, and serum was stored at –20°C until assayed. Immunoreactive FSH and LH serum levels were determined using a commercially available immunoradiometric assay (IRMA) kit (Medgenix, Fleurus, Belgium) as described previously (Fauser et al., 1991). Data are expressed in terms of MRC 78/549 (FSH) and MRC 68/40 (LH) reference preparation, and intra- and interassay coefficients of variation were less than 3 and 8% (FSH) and 5 and 15 % (LH), respectively. All steroid hormones were measured by radioimmunoassay (RIA) as previously described (Fauser et al., 1991). Intra- and interassay coeffients of variation were less than 4 and 5% for SHBG, less than 5 and 8% for E_2, less than 3 and 5% for T, and less than 4 and 6% for DHEAS, respectively.

Data analysis: Due to the absence of differences following extensive analysis in studied endocrine (Fig. 10) and sonographic (data not shown) parameters between both subgroups treated with HMG or pFSH, data of the two groups were pooled. Endocrine data obtained on the first day of gonadotropin administration (day 1) were compared with those obtained on day 3. Furthermore, data obtained on the day of first dose reduction were compared with those of day 1 of gonadotropin treatment as well as with data obtained at the moment of hCG administration. Sonographic observations and endocrine data were compared using Mann-Whitney's test or Wilcoxon's test. E_2 levels were logarithmically transformed to eliminate skew distributions. The probabilities of hyperresponse (E_2 exceeding 3000 nmol/L) (Schenker & Weinstein, 1978) and successful stimulation in relation to E_2 levels were assessed using logistic-regression. Results are presented as mean ± SEM or median and range (box and whisker plots (Brown & Swanson Beck, 1990), Fig. 11) unless stated otherwise. P-values given are two-sided, and 0.05 was considered the limit of statistical significance.

3.4.3 Results

Clinical and endocrine characteristics of 28 PCOS patients participating in this study are depicted in Table 5 (above). There was no difference in endocrine or sonographic parameters tested between 14 patients treated with HMG or pFSH (data not shown). Following 3 weeks of buserelin treatment, serum E_2 (115 [43–263] nmol/L) and LH (2.2 [0.3–7.7] IU/L) levels lowered significantly ($p < 0.001$) as compared to pretreatment levels (E_2 (228

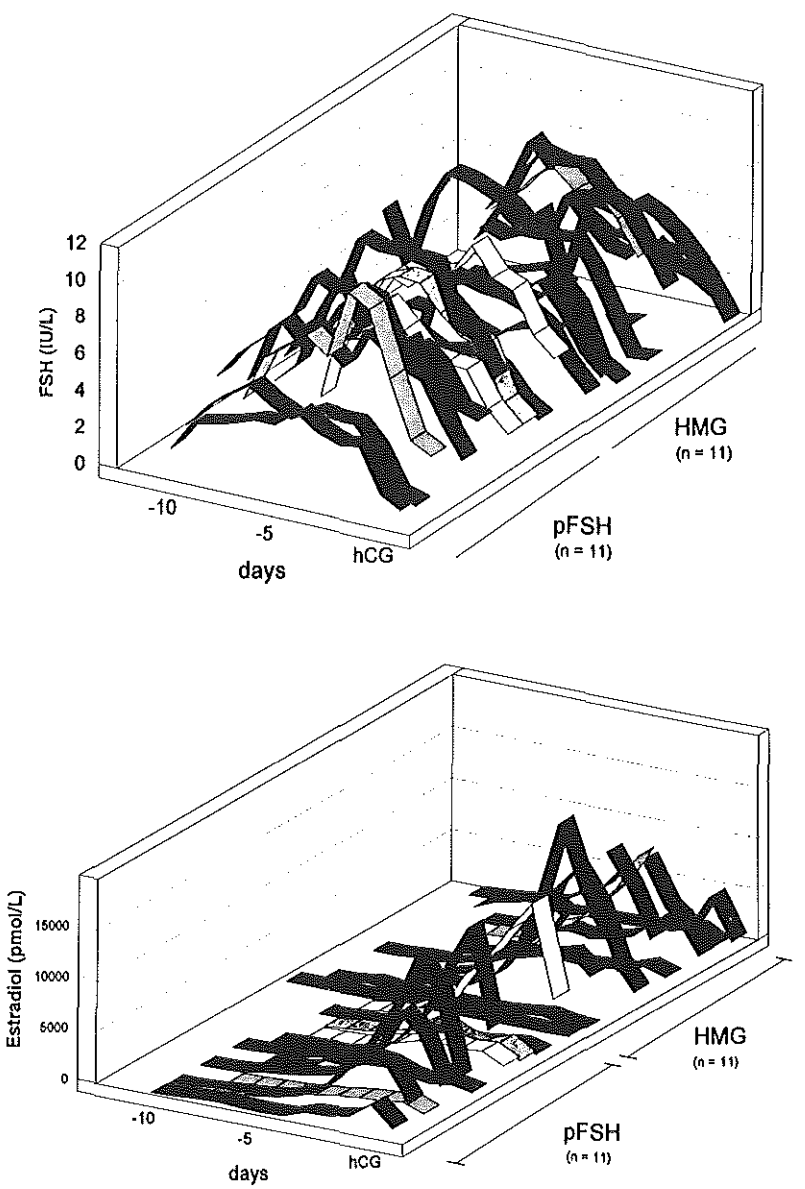

Figure 10 Individual daily follicular phase serum FSH and E_2 levels in PCOS patients treated with HMG (n=11) or FSH (n=11) in a decremental dose regimen with adjuvant GnRH agonist medication. hCG represents day of hCG injection to induce ovulation.

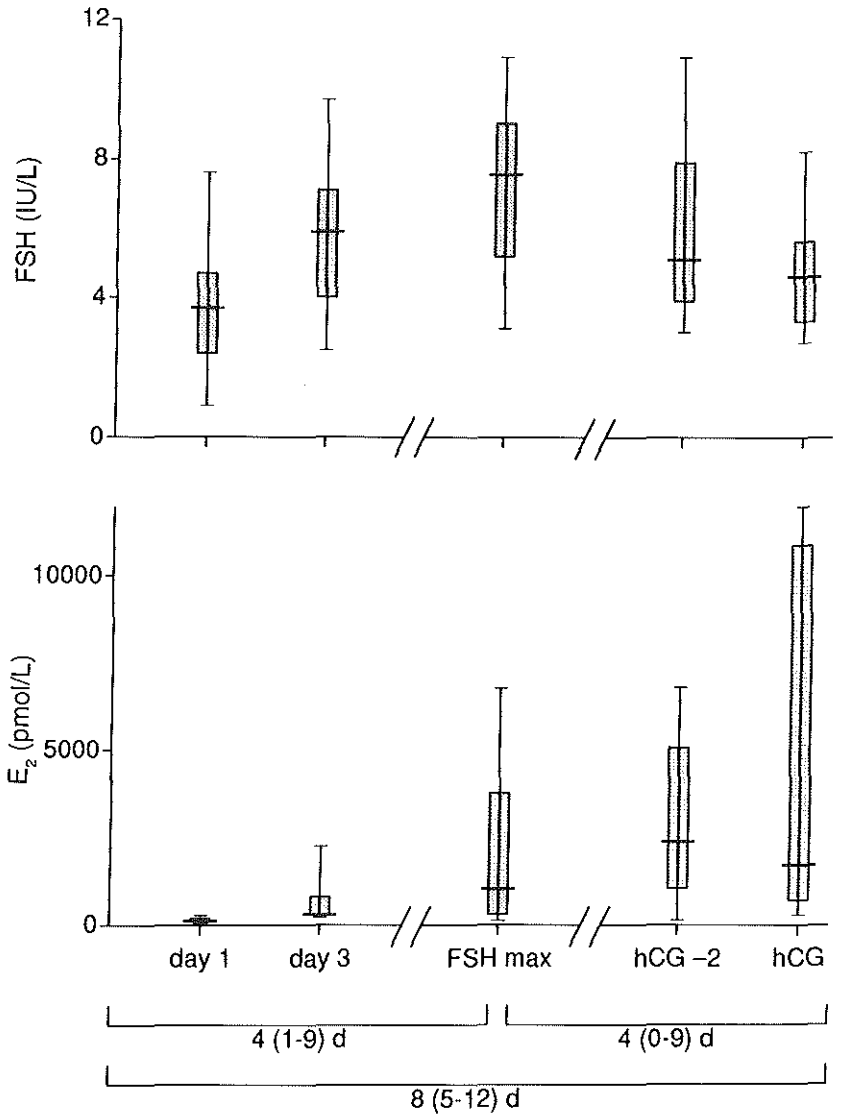

Figure 11 Box and whisker plots indicating serum levels of FSH (upper panel) and E_2 (lower panel) on day 1 and 3 of gonadotropin administration, the day of maximum FSH concentrations and 2 days prior to and on the day of hCG administration. Boxes encompass 50% of the values between the 25th and 75th percentiles. The whiskers give the range of the values. The horizontal line in the boxes gives the median value for the group. FSH concentrations day 3 vs day 1; p= 0.001. FSH concentrations day hCG vs day max; p=0.001.

[98–412] nmol/L; LH (8.1 [4.2–13.1] IU/L) in the total group studied. Immunoreactive FSH concentrations (3.6 [0.9–7.6] versus 4.2 [1.3–7.7] IU/L) showed no significant ($p=0.4$) changes following GnRH agonist treatment.

In the overall group, 22 patients (HMG=11; pFSH=11) received hCG, whereas 6 patients (=21%) were canceled from the study due to strict protocol criteria (absent follicle growth during 7 days of 2 amps of gonadotropins [n=5] or deterioration of follicle development 1 day after gonadotropin dose reduction [n=1]). After receiving hCG, ovulation was confirmed by sonography and midluteal progesterone 37.1 ± 4.2 pmol/L serum assays. Individual daily follicular phase serum levels for FSH and E_2 are shown in Fig. 10. Duration of the follicular phase ranged between 5 and 12 days (median 8 days), whereas the total number of ampules of gonadotropins administered was 13 [10–18]. Serum FSH concentrations increased significantly ($p=0.001$) on day 3 (5.9 [2.5–9.7] versus 3.6 [0.9–7.6] IU/L on day 1) (Fig. 11). Further increase of serum FSH levels (6.2 [3–10] IU/L) was observed towards the day of first dose reduction (day 4 [2–7] days). Maximum FSH levels (7.6 [3.9–10.9] IU/L) were reached after 4 [1–9] days of gonadotropin treatment, and due to reduction of exogenous gonadotropins administered, decreased to 4.7 (2.7–8.2) IU/L at the day of hCG administration. The interval between day of maximum FSH concentrations and day of hCG injection was 4 [0–9] days. Median daily decrease in serum FSH levels after maximum levels were reached was 10 [3–19] %. Serum FSH concentrations on the day of hCG administration were still significantly ($p=0.02$) higher as compared to initial levels.

Following GnRH-agonist pretreatment initial serum E_2 concentrations (115 [43–263] nmol/L) were significantly correlated ($r=0.62$; $p=0.03$) with the number of small ovarian follicles (both ovaries; 29 [20–42]). On day 3 the range of E_2 concentrations revealed 12-fold differences (1297 [247–3,178] nmol/L) whereas no correlation between E_2 and FSH levels was observed. On the day of first dose reduction serum E_2 concentrations did not correlate with serum FSH ($r=0.32$; $p=0.2$), and with the total number of follicles ($r=0.26$; $p=0.4$). E_2 concentrations on the day of first dose reduction correlated significantly ($r=0.72$; $p=0.001$) with E_2 on day of hCG administration (1,793 [279–18,414] nmol/L). Increments of E_2 levels before hCG administration did not correlate with the absolute decrease in FSH serum concentrations following the day of gonadotropin dose reduction ($r=0.05$; $p=0.4$), but did correlate ($r=-0.6$; $p < 0.01$) with the number of days of decreasing FSH levels. E_2 levels on day of hCG correlated ($r=0.67$; $p < 0.05$) with the number of large ovarian follicles (> 13 mm).

Increase of serum E_2 on day 3 compared to day 1 (E_2 increase= E_2 day 3/ E_2 day 1) was significantly correlated ($p < 0.001$) to the chance of receiving hCG afterwards. No significant initial increase in E_2 concentrations was observed in those women with canceled cycles. E_2 increase until day 3 showed a stronger correlation with the chance of obtaining hCG than E_2 increase until

day 2. The predictive value for dropping out of this study due to follicle maturation arrest following the starting dose of 150 IU daily was related to the percentage of E_2 increase on day 3 (Fig. 12A). Serum E_2 levels on the day of first dose reduction correlated ($r=0.65$; $p < 0.05$) with E_2 concentrations on the day of hCG administration. The chance of E_2 exceeding 3,000 nmol/L on the day of hCG administration (ovarian hyperresponse) (Schenker & Weinstein 1978) was significantly correlated ($p < 0.005$) with the concentration of E_2 on the day of dose reduction (Fig. 12B). Furthermore, non-responders demonstrated a significant ($p=0.02$) higher BMI compared to patients who received hCG (30.2 ± 0.9 versus 27.4 ± 0.7 kg/m^2).

3.4.4 Discussion

Clinical application of exogenous gonadotropins using a decreasing dose regimen may be hampered by large inter-individual variability in serum FSH levels following similar doses (Harlin et al., 1986) as well as differences in ovarian response to similar FSH stimulation (i.e. differences in FSH threshold). In practice, initially one has to administer an adequate quantity of gonadotropins to support ongoing growth and the formation of a dominant follicle. This is followed by a reduction in the dose in such a way that the selected follicle is sufficiently supported by FSH whereas further development of secondary follicles is not sustained (Fauser et al., 1993b). Although large differences in serum FSH and E_2 concentrations after GnRH-agonist pretreatment were observed in the present study, the FSH pattern revealed a significant initial 2.1-fold increase, followed by decreasing serum concentrations of 10% per day for 4 days towards the day of hCG administration. Median length of the follicular phase was 8 days using a total of only 13 ampules per cycle. This observation is in agreement with previous studies in the monkey model showing continued follicle growth despite decreased FSH stimulation of 12.5% daily (Zeleznik & Kubik, 1986). This study was also undertaken to investigate potential differences in ovarian steroid production and follicle growth, following administration of urinary gonadotropin preparations with low (pFSH) or high (HMG) LH content with combined adjuvant down-regulation of endogenous pituitary gonadotropin secretion in PCOS patients. Analogous to previous comparative reports (Homburg et al., 1990) using step-up regimens, no significant differences in E_2 levels or follicular growth were observed comparing both groups.

Former studies by our group (Fauser et al., 1993c) and others (Mizunuma et al., 1991) have suggested the step-down approach to be clinically safe and effective with acceptable pregnancy and complication rates. Effective application of a step-down regimen does require adequate tools to monitor ovarian function in order to: a) estimate effectivity of initial

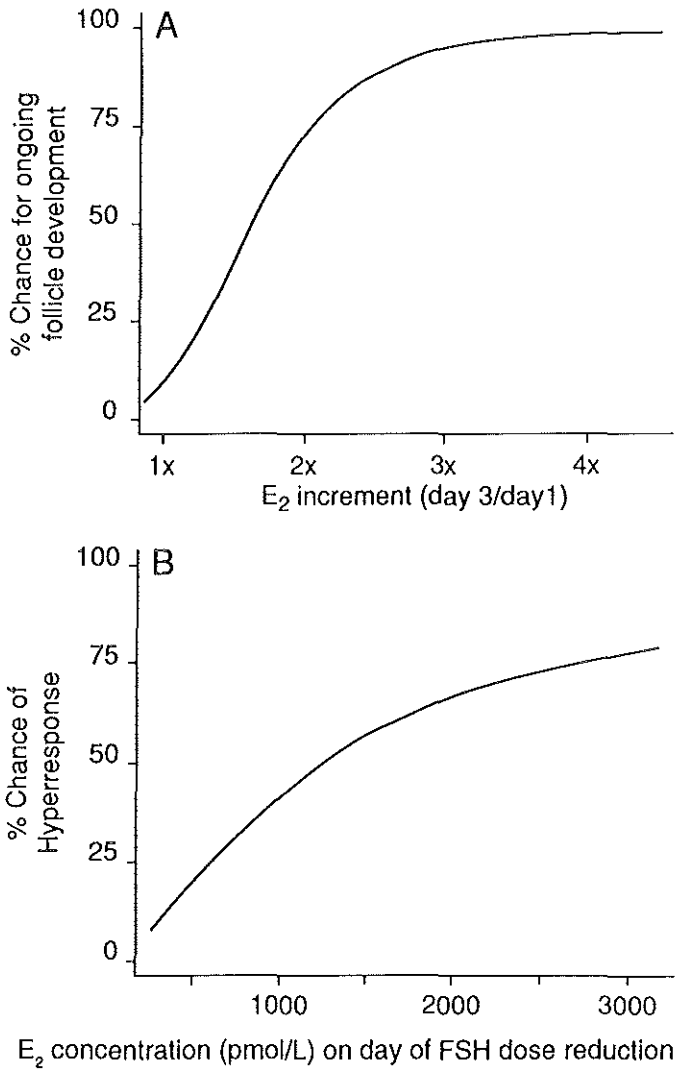

Figure 12 (A) Probability of developing ongoing ovarian follicle growth and ovulation related to E_2 increment ([E_2 day 3]/ E_2[day 1]) during the first 2 days of gonadotropin medication in 28 PCOS patients using GnRH agonist and a step-down regimen for gonadotropin induction of ovulation. (B) Probability of developing ovarian hyperresponse (E_2 concentrations ≥ 3,000 pmol/L on day of hCG administration) related to E_2 serum levels on the day of gonadotropin dose reduction (≥ 1 ovarian follicle ≥ 9mm) in 22 ovulatory PCOS patients using GnRH-agonist and a step-down regimen for gonadotropin induction of ovulation.

gonadotropin doses on an individual basis, b) decide when the first and subsequent decreases in dose should take place, and finally c) to time the administration of hCG. However, using gonadotropin step-up regimens, differences in response during the late follicular phase are not related to early follicular phase events but rather to the number of days of gonadotropin administration and the total amount of ampules administered (i.e. magnitude of FSH accumulation). Since in the present study the mode of reduction of gonadotropins was similar for all subjects, it was possible to investigate the influence of the initial dosage on treatment outcome. No correlation could be observed between increments of serum FSH and E_2 concentrations on day 3 of gonadotropin administration. Differences in E_2 levels at the moment of comparable sonographic ovarian appearance (1 follicle $\geq$ 9mm, which was chosen as the day of initial dose reduction) were intriguing. Ovarian E_2 production seemed to be unpredictable and extremely variable following a relative short (3–4 days) period of gonadotropin stimulation in combination with commonly accepted "sufficient" sonographic response (1 follicle $\geq$ 9mm) to gonadotropins. Furthermore, the wide range in E_2 levels on this first day of dose reduction demonstrates a poor correlation between sonographic and estrogenic ovarian response in the early follicular phase during stimulated cycles. This has been mentioned previously (O'Herlihy et al., 1982), but has not gained wide attention. It should be emphasized that the difference in the initial E_2 response is not related to observed differences in FSH serum concentrations. The major individual variability in E_2 response (indicating different FSH threshold) may reflect differences in underlying mechanisms of ovarian dysfunction in PCOS patients. As suggested previously a functional test to investigate individual ovarian sensitivity to clomiphene citrate (Navot et al., 1987) or gonadotropins (Caruso et al., 1993) may predict treatment outcome. It may therefore be suggested that measurement of serum FSH levels during gonadotropin induction of ovulation in PCOS patients is of limited clinical significance. Moreover, it is to be defined what can be gained from weekly incremental low-dose step-up regimens (Buvat et al., 1989) where the FSH threshold is reached after an extended period of time. It cannot be excluded, however, that ovaries are sensitized for FSH stimulation using this regimen. We speculate that in step-down regimens the FSH threshold may be surpassed as long as this occurs for just a limited period of time (short FSH window). In the present study, using follicle development as marker for the moment of dose reduction, the wide range in observed E_2 levels at the day of hCG suggests that in a considerable proportion of patients the dosage was still reduced too late.

In summary, in the present study ovarian response was evaluated by serum hormone estimates and transvaginal sonography in PCOS patients using a gonadotropin step-down regimen for induction of ovulation. Obtained

data suggest that: a) FSH serum levels show a significant decrease following dose reduction of administered gonadotropins, b) despite decreasing FSH serum concentrations follicle growth is sustained and ovulation does occur, c) initial estrogenic response of gonadotropin-stimulated PCOS patients may reflect differences in underlying mechanisms of ovarian dysfunction, d) using a step-down dose regimen the initial E_2 increment can be used as a predictor of low response or hyperresponse and doses administered can be adjusted accordingly.

4

Conclusions

This thesis has focused on two major issues: a) ovarian response during administration of recombinant human FSH (without LH activity) to hypogonadotropic volunteers, and b) ovarian effects of a step-down dose regimen for gonadotropin induction of ovulation in anovulatory, clomiphene-resistant PCOS patients. Fevold (using pituitary gonadotropin preparations) (Fevold, 1941) as well as Eshkol & Lunenfeld (using purified urinary extractions) (Eshkol & Lunenfeld, 1967) demonstrated in the rodent model differential effects of FSH and LH on ovarian steroidogenesis and folliculogenesis. Couzinet (Couzinet et al., 1988) confirmed the synergism of the gonadotropins in hypogonadotropic females using purified urinary FSH. Although bioactive LH remains present in low concentrations in purified urinary FSH preparations, results supported the two-cell two-gonadotropin hypothesis indicating that sufficient amounts of LH are needed to stimulate theca cell androgen production, which in turn provides the substrate to be converted to estrogens by FSH-induced granulosa cell enzyme activity.

The recently developed recombinant FSH preparations (without LH bioactivity) provided the challenging opportunity to investigate separate effects of FSH and LH in hypogonadotropic females. Data presented in the first part of this thesis indicate the significance of sufficient amounts of LH – next to FSH – for adequate E_2 production by ovarian follicles. Moreover, it suggests differential regulation of mitogenic activity and estrogen synthesis of the granulosa cell by FSH. FSH alone can induce growth of preovulatory follicles without stimulating E_2 synthesis. It therefore seems questionable whether estrogen biosynthesis is mandatory for follicular development in the human. Effects of low intrafollicular E_2 levels on oocyte quality, the capacity to ovulate and to transform into a corpus luteum are unknown at present. In addition, rhFSH stimulation in hypogonadotropic women resulted in a rise in immunoreactive INH similar to the increase in INH serum levels in the follicular phase of regularly cycling women, indicating that granulosa cell function per se is normal. It may be speculated that follicle growth and rising E_2 levels are merely associated events and that the growth of the follicle is driven by other as yet unidentified factors. Growth of the dominant follicle continued despite decreasing serum FSH levels and appeared to be similar to normal regular cycle. The observed dissociation between follicular growth and estrogen biosynthesis should elicit future research on the

significance of estrogens and other intra-follicular factors for growth of ovarian follicles.

In the second part of the present thesis, patients with infertility and anovulation due to PCOS were investigated. Based on previous studies by our group it has been postulated that early follicle growth is normal and selection of the dominant follicle is disturbed in PCOS patients (Fauser, 1994b). 'Normal' serum FSH levels are observed in these PCOS patients, suggesting that the necessary intercycle rise in FSH does not occur (van Dessel et al., 1995). Moreover, the minimal requirement of FSH for ongoing follicle stimulation (FSH-'threshold') may be increased in some patients due to intra-ovarian abnormalities. Sufficient amounts of exogenously administered gonadotropins can mimic the intercycle rise or overcome this elevated FSH-threshold, causing increased aromatase activity and stimulating other intra-ovarian mechanisms and subsequent follicle growth. However, gonadotropin administration to oligoamenorrheic patients frequently causes multiple follicle development and consequently complications such as multiple gestation and ovarian hyperstimulation. In normally menstruating women, estrogen feedback mechanisms reduce late follicular FSH levels in such a way that multiple dominant follicles rarely develop. The exception to this rule is families with dizygotic twins (Martin et al., 1991). It has been shown in the monkey that interference with these feedback actions (Zeleznik et al., 1985) indeed results in multiple follicle development. In addition to the FSH-threshold (minimal required level of serum FSH to start recruitment of follicles), the concept of the FSH-window has been introduced (the number of days that serum FSH concentrations remain above threshold levels) (Fauser et al., 1993b; Fauser et al., 1994c). In a mono-ovulatory cycle, this is the period necessary to allow just one follicle to gain dominance. Preservation of the selected follicle due to local up-regulation despite decreasing gonadotropin levels, ensures monofollicular development. Other follicles from the recruited cohort go into atresia, since FSH levels drop below their threshold. An extended opening of the 'FSH-window' may interfere with dominant follicle selection, eventually leading to dominance of multiple follicles and ongoing follicle recruitment.

Incremental dose regimens – even if the slow incremental protocol is applied (Hull, 1991; Herman et al., 1993) still cause iatrogenic complications. In an attempt to diminish gonadotropin-related complications, it was suggested to increase dosages of gonadotropins in a prudent way, slowly surpassing the individual FSH-threshold (Brown, 1978). As yet unknown factors influencing the individual FSH-threshold as well as differences in individual pharmacodynamics appeared to be of major importance. Complications may also be due to prolonged maintenance of FSH levels above threshold values (hence a wide FSH-window) in step-up regimens. This continued stimulation may cause ongoing growth of multiple follicles.

The clinical studies discussed in the third chapter of the present thesis represent attempts to systematically monitor ovarian response by serum hormone estimates and transvaginal sonography to:

1) Test the hypothesis that lowering serum FSH in the late follicular phase would result in reduced multiple follicle development and still lead to ongoing growth and ovulation of the dominant follicle.
2) Find the appropriate starting dose and decremental steps for clinical use of step-down gonadotropin induction of ovulation.
3) Compare patients with 'step-down' gonadotropins exhibiting mono-follicle development with the unstimulated normal menstrual cycle.
4) Provide endocrine background information regarding the step-down dose regimen focusing on FSH serum levels.
5) Study the predictive value of early ovarian response regarding treatment outcome.

Implementation of the step-down concept in gonadotropin induction of ovulation can theoretically influence the number of medium-sized follicles, and consequently the possible incidence of complications. However, several questions remain to be answered including: 1) How do we reach the threshold dose? (i.e. can we find a parameter to predict major differences in individual sensitivity to FSH, and adjust the starting dose accordingly), and 2) When should gonadotropin doses be reduced? It should be emphasized that single-center, non comparative data suggest that the step-down approach can be used in clinical practice and does result in a favourable outcome (van Santbrink et al., 1995).

In our initial study evaluating the step-down regimen, the chosen gonadotropin dose of 225 IU/d for all patients (as described in section 3.1), appeared to cause an extended period of elevated FSH levels. This resulted in absent or delayed effects on serum FSH concentrations and follicle growth following reduction of gonadotropin dosages. In the third study, large inter-individual variations in sonographic and steroidogenic ovarian response to a standardized starting dose of gonadotropins were observed. No correlations between steroidogenic and sonographic ovarian changes were noticed. However, the increment in serum E_2 levels following two days of gonadotropin (150 IU/day) administration appeared to predict high and low responders to a step-down dose regimen. The dose regimen of administered exogenous gonadotropins, – step-up (stepwise search for effective dose) or step-down (immediate implementation of effective dose) – may not affect the number of recruited follicles. If a high standard dose of gonadotropins is administered at the start, parameters of ovarian response may help to individualize further dose adjustment. Since no objective predictors of ovarian responsiveness to initial gonadotropin dose are available, a challenge test prior to initiation of therapy might be useful. We feel, based on studies in this thesis, together with our experience in clinical patient care (van Santbrink

et al., 1995), that the concept of decremental gonadotropin dose regimens for induction of ovulation is promising. Step-down dose regimens may lead to reduction of clinical complications during gonadotropin induction of ovulation. However, one should be cautious at present and it is mandatory to increase the number of PCOS patients treated in a step-down fashion. Moreover, multi-center, comparative studies are needed in order to obtain conclusions that are meaningful for clinical practice.

5

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6

Summary

Chapter 1

This chapter starts with a general introduction covering highlights in the history of glycoprotein hormones, especially FSH. Improvements in the process of purification of gonadotropic hormones are discussed. The newly developed human recombinant follicle-stimulating hormone (rhFSH) offers therapeutical and investigational potential.

Interaction of gonadotropic hormones on ovarian target tissue level is discussed. FSH appears to be mandatory for recruitment of ovarian follicles, whereas estrogen production of the growing dominant follicle plays an important role in the process of selection. Development of multiple ovarian follicles during gonadotropin induction of ovulation in infertile PCOS patients is hypothesized to be the result of increased or sustained FSH levels following the moment of presumed selection. This hypothesis of temporary FSH stimulation to minimize ongoing growth of secondary follicles is called the 'FSH window' concept. Analogous to the normal menstrual cycle, it is believed that development of secondary ovarian follicles is less pronounced in a dose regimen where FSH decreases following selection of the dominant ovarian follicle ('step-down' dose regimen for gonadotropin induction of ovulation).

An historical overview of 30 years of gonadotropin induction of ovulation represents developments in treatment; i.e. gonadotropin preparations, dose regimens, monitoring facilities and adjuvant medication. Although success rates of gonadotropin induction of ovulation have improved, complications are still of great concern to clinicians.

Chapter 2

Section 2.1 Some background information with regard to human recombinant FSH is provided. The lack of intrinsic LH activity of this compound provides the opportunity to investigate separately the roles of FSH and LH with regard to folliculogenesis and steroid production.

Section 2.2 To evaluate the importance of LH for normal estrogen production and subsequent development of ovarian follicles, a woman with isolated

gonadotropin deficiency was monitored during rhFSH administration with respect to ovarian follicular growth and steroid production. During the first week (75 IU/day rhFSH im), a significant rise in serum FSH (4.9 IU/L) was observed in the absence of changes in serum E_2 concentrations (36–76 pmol/L). During the following five days, 150 IU/day rhFSH was administered resulting in a further increase of serum FSH levels (maximum 8.5 IU/L). Development of multiple follicles – maximum diameter 22 mm as observed by transvaginal sonography – emerged together with a minor rise in E_2 levels (from 76 to 236 pmol/L) and with a minimal increase in endometrial thickness (below 6 mm). Six days following the last injection of rhFSH, aspiration of 3 follicles (13, 15 and 18 mm) was performed and low intrafollicular AD (< 675 nmol/L) and E_2 (< 9400 pmol/L) concentrations, as compared to normal follicles, were found.

These first data on rhFSH administration in the human suggest that: a) FSH alone can induce growth of preovulatory follicles, b) follicle growth does occur in the presence of subnormal E_2 levels, c) LH is needed for adequate AD biosynthesis as substrate for aromatase activity. This indicates that growth and steroidogenic granulosa cell activity may be differentially regulated.

Section 2.3 Seven females suffering from hypogonadism due to previous hypophysectomy, isolated gonadotropin deficiency or Kallman syndrome volunteered to participate in a study to assess ovarian response following multiple-dose administration of rhFSH (Org 32489). Mean baseline serum FSH and LH levels were 0.25 (<0.05–1.15) IU/L and 0.06 (<0.05–0.37) IU/L, respectively. Subjects received daily im injections of rhFSH for 3 weeks (week 1: 75 IU/d, week 2: 150 IU/d, week 3: 225 IU/d). Blood sampling and sonographic investigations were performed on alternate days. Steady-state FSH concentrations were reached approximately 3 to 5 days after alterations of doses administered. Maximum FSH levels were between 7.1 and 11.8 IU/L, whereas serum LH concentrations remained unchanged. Due to the abscense of follicle development and lack of a rise in immunoreactive INH (presumably due to early ovarian failure) in 2 subjects, analysis of ovarian response was restricted to 5 volunteers. Serum androstenedione (AD) levels showed no significant changes during rhFSH administration. Although serum immunoreactive INH concentrations reached normal, late follicular levels (659 [388–993] U/L), serum E_2 revealed only a minor increase (77 [18–210] pmol/L). Moreover, growth of (multiple) ovarian follicles was observed up to preovulatory sizes (>15 mm) in these patients.

It may be concluded from the present study that: 1) rhFSH exhibits no intrinsic LH activity, 2) rhFSH stimulation in hypogonadotropic women resulted in an immunoreactive INH rise which was similar as compared to normal, whereas in contrast only a minor increase in E_2 concentrations was observed (suggesting normal granulosa cell function, and low availability of

androgens as a substrate for aromatization), and 3) despite the minimal estrogen increase, ovarian follicles developed normally to the preovulatory stage.

Chapter 3

Section 3.1 During treatment of anovulation with exogenous gonadotropins, a high incidence of complications (mainly multiple pregnancies and ovarian hyperstimulation) is observed. Since ovarian response may vary from patient to patient and from one cycle to the other, careful monitoring is mandatory. Frequent determinations of serum E_2 levels may be of help in this respect. However, high estrogen levels can be caused by a single dominant follicle or by a number of smaller ones.

The importance of the 'FSH- threshold' and 'FSH- window' concepts with regard to gonadotropin induction of ovulation is dicussed. In normal regularly cycling women decreasing serum FSH levels are observed following selection of the dominant follicle. This concept was tested in anovulatory PCOS patients, by inducing follicle growth giving exogenous gonadotropins in a decreasing dose regimen. This would potentially stimulate growth of follicles during a restricted time (to prevent multiple follicle development) with reduced interference in the selection process. Accurate monitoring of follicle development is possible since the recent introduction of transvaginal pelvic sonography. Insight in ovarian changes comparing different gonadotropin dose regimens may eventually lead to better understanding of regulation of ovarian function. In addition, this may lead to more effective prevention of multiple follicle development with subsequent reductions in complication rates.

Section 3.2 Data presented in this study indicate that ovulation can be induced in PCOS patients using GnRH analogs combined with HMG in a decreasing dose regimen. Based on the observed decline in numbers of functional, medium-sized follicles comparing a fixed versus a decreasing dose regimen, it may be speculated that the risk of ovarian hyperstimulation during gonadotropin induction of ovulation in PCOS patients may be reduced under circumstances where stimulation by FSH is reduced in the late follicular phase.

Section 3.3 The aim of this study was to investigate the late follicular phase of 7 gonadotropin-treated PCOS patients exhibiting monofollicular growth, and compare developmental characteristics with the dominant follicle in 7 control women with regular menstrual cycles. Daily serum FSH levels in PCOS patients decreased more rapidly as compared to controls (-0.3 ± 0.2 IU/day in controls, versus -0.7 ± 0.4 IU/day in PCOS; $P < 0.02$). No

statistically significant differences were seen in daily increase (30% in controls and PCOS) and mean peak levels (825 ± 94 pmol/L in controls, versus 937 ± 231 pmol/L in PCOS) of E_2 serum levels comparing both groups. Mean daily growth of the dominant follicle (1.7 ± 0.4 mm in controls, versus 1.9 ± 0.6 mm in PCOS) was not significantly different.

It is concluded that in PCOS patients using a decreasing dose regimen gonadotropin plus adjuvant GnRH-agonists, levels of serum FSH are decremental and growth of a single ovarian follicle is demonstrated. In addition, growth of the dominant follicle and estrogen production in PCOS patients are not significantly different from follicle growth under normal conditions.

Section 3.4 Daily ovarian E_2 production and follicle growth (by transvaginal ultrasound) were investigated during gonadotropin administration of HMG or pFSH for induction of ovulation in 28 PCOS patients suffering from clomiphene-resistant anovulation. In contrast to regular increasing dose regimens, gonadotropins were administered in a 'step-down' fashion combined with GnRH-a in an attempt to reduce the incidence of multiple follicle development and subsequent complications. No differences were observed in ovarian response comparing HMG versus pFSH administration. Growth of ovarian follicles was sustained and ovulation achieved in the majority of patients despite a significant decrease in serum FSH levels of 10% per day. Major variability in early E_2 increase (not related to differences in FSH serum concentrations) without changes in ovarian follicle number and size suggests differences in ovarian sensitivity to FSH stimulation. Early E_2 increments predict chances of ovarian hyperresponse or hyporesponse (absent follicle maturation) during gonadotropin induction in the applied step-down fashion.

These findings suggest that gonadotropin induction of ovulation according to a decremental dose regimen may be useful for clinical practice and underline the significance of monitoring early E_2 response to further improve treatment outcome.

7

Samenvatting

Hoofdstuk 1

Dit hoofdstuk begint met een algemene inleiding met historisch overzicht over de zuivering van gonadotrope hormonen, m.n. FSH. Het recent ontwikkelde humaan recombinant follikel stimulerend hormoon (rhFSH) biedt naast een aanvulling van therapeutische mogelijkheden een unieke kans om fysiologisch onderzoek te verrichten.

Verschillende effecten van gonadotrope hormonen op ovarieel weefsel worden besproken. FSH lijkt onmisbaar in het proces van recrutering ('recruitment') van ovariële follikels, terwijl tijdens de selectie van de dominante follikel oestrogenen een belangrijke rol spelen. Tijdens conventionele gonadotrofine toediening voor inductie van ovulatie in het kader van infertiliteits behandeling bij PCO patienten stijgen of stabiliseren serum FSH spiegels na het moment van selektie van de dominante follikel. Het ontstaan van meerdere ovariele follikels wordt beschouwd als een gevolg van aanhoudende FSH stimulatie. Naar analogie aan de normale menstruele cyclus lijkt ontwikkeling van secundaire ovariële follikels minder sterk indien FSH in de laatste dagen van de follikulaire fase dalende is.

Een historisch overzicht van dertig jaar ovulatie inductie met behulp van gonadotrofines wordt gegeven (o.a. gonadotrofine preparaten, doserings-schema's, monitoring en adjuvante medicatie). De kans op succes van behandeling neemt toe, de complicaties (meerling zwangerschap en ovarieel hyperstimulatie syndroom) blijven echter in de praktijk veel zorgen geven.

Hoofstuk 2

paragraaf 2.1 Enige achtergrond informatie met betrekking tot humaan recombinant FSH (rhFSH) wordt gegeven. Het gebrek aan intrinsieke LH activiteit van rhFSH biedt de mogelijkheid om de afzonderlijke rol van FSH en LH voor follikelgroei en steroidogenese te onderzoeken.

paragraaf 2.2 Om de waarde van LH voor oestrogeen produktie en ontwikkeling van ovariële follikels te onderzoeken werden ovariële veranderingen (follikels en steroidogenese) tijdens toediening van rhFSH bij een vrouw met geïsoleerde gonadotrofine uitval vastgelegd. Tijdens de

eerste week (75 IU rhFSH/dag im) bleek een significante stijging van serum FSH (4.9 IU/L), zonder veranderingen in serum oestradiol (E_2) concentraties (36–76 pmol/L). De serum FSH spiegel nam toe (maximum 8.5 IU/L) gedurende de volgende vijf dagen (150 IU rhFSH/dag). Multiple follikels – maximum diameter 22 mm (transvaginale echoscopie) – werden zichtbaar zonder een bijpassende stijging in E_2 concentratie (76–236 pmol/L) en zonder duidelijke toename van de totale endometrium dikte (minder dan 6 mm). Zes dagen na de laatste injectie met rhFSH werden 3 follikels (13, 15 and 18 mm) leeggezogen. Lage intrafollikulaire AD (< 675 nmol/L) en E_2 (< 9,400 pmol/L) concentraties – vergeleken met normale follikels – werden gevonden. Deze eerste data betreffende rhFSH toediening bij de mens geven aan dat: a) FSH ontwikkeling van preovulatoire follikels kan induceren, b) follikelgroei in de ovaria kan optreden in aanwezigheid van lage E_2 spiegels, c) LH nodig is voor adequate biosynthese van AD zodat het als substraat kan dienen voor aromatase activiteit. De hier genoemde bevindingen geven aan dat verschillende activiteiten van de granulosacel (groei en steroidproductie) verschillend gereguleerd worden.

Paragraaf 2.3 Zeven vrijwilligsters met hypogonadotroop hypogonadisme als gevolg van hypofysectomie, geïsoleerde hypofysaire gonadotrofine uitval of syndroom van Kallman participeerden in een studie waarin ovariële response werd vastgesteld na toediening van multiple dosis rhFSH (Org 32489). Uitgangswaarden voor FSH en LH in het serum waren respectievelijk 0.25 (< 0.05–1.15) IU/L en 0.06 (< 0.05–0.37) IU/L. De vrijwilligsters kregen dagelijks im injecties met rhFSH gedurende 3 weken (week 1: 75 IU/d, week 2: 150 IU/d, week 3: 225 IU/d). Bloed afname en echoscopische onderzoekingen werden drie maal per week uitgevoerd. Steady state concentraties van FSH werden bereikt ongeveer 3 tot 5 dagen nadat de dagelijks toegediende dosis was opgehoogd. Hoogste individuele serum FSH spiegels bij rhFSH dosering bleken tussen 7.1 en 11.8 IU/L. Serum LH concentraties bleven onveranderd laag. De analyse van ovarium respons werd beperkt tot die 5 deelnemende vrouwen waarbij follikelgroei werd aangetoond. In 2 vrouwen werd geen follikelgroei gezien en bleek geen verandering in immunoreaktief INH opgetreden; waarschijnlijk tengevolge van premature ovarium uitval. Serum AD levels lieten wienig verandering zien tijdens rhFSH toediening. Alhoewel serum immunoreactief INH concentraties bleken overeen te komen met laat folliculaire spiegels (659 [388–993] U/L), toonde serum E_2 weinig toename (77 [18–210] pmol/L). In tegenstelling hiermee groeiden meerdere ovariële follikels door tot preovulatoire afmetingen (>15 mm).

Uit deze studie mag worden geconcludeert dat: 1) rhFSH geen LH activiteit bezit, 2) stimulatie met rhFSH in hypogonadotrope vrouwen resulteert in toename van immunoreactive INH concentraties (vergelijkbaar met de normale cyclus), terwijl slechts een geringe toename in serum E_2 concentraties

optrad (suggererend dat er bij normale functie van de granulosacellen een geringe beschikbaarheid van androgenen is en, 3) ondanks zeer geringe toename van E_2 ovariële follikels in staat zijn door te groeien tot preovulatoire grootte.

Hoofdstuk 3

Paragraaf 3.1 Tijdens behandeling van anovulatie en kinderwens met gonadotrofines wordt een hoge incidentie van complicaties (meerlingzwangerschappen en ovariële hyperstimulatie) vastgesteld. Vanwege grote verschillen in respons tussen patienten en behandelingscycli onderling is nauwgezette controle noodzakelijk. Herhaaldelijke bepalingen van serum E_2 kunnen in dit opzicht behulpzaam zijn. Hoge serum E_2 spiegels kunnen echter door enkele grote maar ook door veel middelgrote ovariële follikels worden geproduceerd.

Het concept van 'FSH- drempel' en 'FSH- venster' met betrekking tot ovulatie inductie met gonadotropines wordt besproken. Naar analogie aan de normale menstruele cyclus waarbij in de follikulaire fase een daling van het serum FSH wordt vastgesteld, werd besloten in anovulatoire PCO patienten dit concept te testen door toediening van gonadotrofines in afnemende hoeveelheid. Verondersteld werd dat hierdoor de periode van stimulatie van follikel groei zou afnemen met als gevolg preventie van groei van multiple follikels door verminderde beinvloeding van het proces van selectie van de dominante follikel.

Recente ontwikkeling van transvaginale echoscopie maakt accurate monitoring van follikelgroei in het ovarium mogelijk. Vergelijking van ovarium respons tussen verschillende doseringsschema's voor gonadotrofines is hierdoor mogelijk. Tevens wordt meer inzicht geboden in de functie van het ovarium tijdens inductie van ovulatie met gonadotrofines. Wellicht wordt een meer effectieve preventie van overmatige groei van follikels hierdoor mogelijk hetgeen kan helpen bij de vermindering van de complicaties van behandeling.

Paragraaf 3.2 Uit de gepresenteerde gegevens van deze studie blijkt dat ovulatie inductie mogelijk is bij patienten met PCO na gebruik van een dalend doseringsschema voor gonadotrofines ('step-down'schema) in combinatie met GnRH-agonisten. Deze 'step-down' behandeling wordt vergeleken met een ander gonadotrofine behandelingsschema (onveranderde hoeveelheid; 'fixed-dose') in een vergelijkbare groep PCO patienten. Een afname van de aantallen functionele middelgrote follikels tijdens de 'step-down' dosering wordt vastgesteld. Er wordt gespeculeerd dat risico's samenhangend met groei van multipele follikels (en complicaties van behandeling) mogelijk minder vaak voorkomen na een 'step-down' schema.

Paragraaf 3.3 Vergelijkend onderzoek van biometrische en hormonale parameters tijdens de laat follikulaire fase wordt beschreven in 7 met gonadotrofines behandelde PCO patienten met monofollikulaire groei en van 7 regelmatig menstruerende vrouwen (controles). Serum FSH spiegels in PCO patienten daalden sneller dan in de controlegroep (–0.3 ± 0.2 IU/dag in controlegroep, versus –0.7 ± 0.4 IU/dag in PCO groep; P< 0.02). Geen statistisch significante verschillen werden vastgesteld m.b.t. dagelijkse E_2 toename (30% in beide groepen) en gemiddelde maximum E_2 spiegels (825 ± 94 pmol/l: controlegroep; 937 ± 231 pmol/l in PCO-groep). Ook de gemiddelde dagelijkse groei van de dominante follikel was niet verschillend (1.7 ± 0.4 mm: controlegroep; 1.9 ± 0.6 mm PCO-groep).

Uit de gepresenteerde gegevens van deze studie blijkt wederom dat ovulatie inductie mogelijk is bij PCO-patienten, na gebruik van een dalend doseringsschema voor gonadotrofines ('step-down' schema) in combinatie met GnRH-a. Tevens blijkt uit de gegevens dat groei en E_2 productie van de dominante follikel in PCO-patienten tijdens het beschreven ovulatie inductie schema niet significant verschilt van follikelgroei onder normale omstandigheden.

Paragraaf 3.4 Dagelijks onderzoek (serum E_2 spiegels en transvaginale echoscopie) werd verricht tijdens gonadotrofine toediening (HMG of pFSH) voor ovulatie inductie in 28 clomifeen resistente anovulatoire PCO-patienten. In tegenstelling tot de gangbare dosering voor gonadotrofines (toenemende doses ='step-up') werden gonadotrofines toegediend volgens het 'step-down' model. Geen verschillen werden gevonden tussen de groepen met pFSH (n=18) en HMG (n=18). Groei van follikels werd vastgesteld en ovulatie trad op in een meerderheid van de patienten ondanks significant dalende serum FSH spiegels (10% per dag). Grote variabiliteit in vroeg-follikulair optredende E_2 stijging (niet gecorreleerd met serum FSH concentraties) zonder zichtbare veranderingen in aantal en diameter van ovariële follikels suggereert verschillen in gevoeligheid voor FSH stimulatie. Vroege E_2 stijging (tot dag 2) voorspelt de kans op ovariële respons (hyperrespons/ hyporespons) tijdens gonadotrofine toediening in het beschreven 'step-down' doseringsschema.

Deze observaties bevestigen de mogelijke klinische toepasbaarheid van het step-down schema voor gonadotrofine ovulatie inductie en onderstrepen het nut van meting van vroege E_2 respons ter verbetering van toepasbaarheid van het genoemde doseringsschema.

8

Publications

List of publications included in the present thesis (numbers in bold refer to chapter sections)

2.2 Schoot D.C., Coelingh Bennink H.J.T., Mannaerts B.M.J.L., Lamberts S.W.J., Bouchard P. and Fauser B.C.J.M. (1992) Human recombinant follicle-stimulating hormone induces growth of preovulatory follicles without concomitant increase in androgen and estrogen biosynthesis in a woman with isolated gonadotropin deficiency. Journal of Clinical Endocrinology and Metabolism, **74**, 1471–1473.

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3.2 Schoot D.C., Pache T.D., Hop W.C., de Jong F.H. and Fauser B.C.J.M. (1992) Growth patterns of ovarian follicles during induction of ovulation with decreasing doses of human menopausal gonadotropin following presumed selection in polycystic ovary syndrome. Fertility and Sterility, **57**, 1117–1120.

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Curriculum vitae auctoris and acknowledgements

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