Ovarian follicle dynamics in the rat: regulation and flexibility

Ovariële follikel dynamiek in de rat: regulatie en flexibiliteit

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Ter afsluiting

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List of abbreviations

ActRII(A,B)	Activin receptor type II(A,B)
ActRI	Activin receptor type I
BW	Body weight
CL	Corpus luteum
D1	Dioestrus-1
D2	Dioestrus-2
D3	Dioestrus-3
EGF	Epidermal growth factor
FSH	Follicle-stimulating hormone
GnRH	Gonadotrophin releasing hormone (=LHRH)
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone (=GnRH)
OE	Oestrus
Oestradiol	Oestradiol-17β
pff	Porcine follicular fluid
PMSG	Pregnant mare serum gonadotrophins
PO	Pro-oestrus
rhFSH	Recombinant human FSH
TGF	Transforming growth factor
ULO	Unilateral ovariectomy

Chapter 1

Ovarian follicle development

Introduction

Reproduction is the most important factor for the maintenance of a species. The key event in this process is fertilization: combination of haploid cells from male and female parents, spermatozoon and oocyte, respectively. Before spermatozoa and oocytes are formed and ready for fertilization many processes have taken place, which are very different for spermatozoa and oocytes. One of the most striking differences is the continuous production of newly formed spermatozoa in the fertile male, while the formation of oocytes is already complete before birth. The oocyte is surrounded by supporting cells. In the complex of the oocyte with the surrounding cells, called the follicle, close collaboration exists between the oocyte and the surrounding cells. The main part of this thesis describes the dynamics of follicle development and the hormonal factors influencing this development.

Development of the female germ cells

Oocytes are derived from germ cells that migrate from the yolk sac to the genital ridge and fill the indifferent gonads. In both male and female mice this process has taken place at about 11 days postfertilization (E11)(Hirshfield, 1991). The germ cells in the female mouse will then start to divide very rapidly until a number of 85.000 is reached at E18.5 (Hirshfield, 1991). In the rat 75.000 germ cells are found at E18.5 (Beaumont & Mandl, 1962). Histological recognition of the ovary and the oocytes is only possible after the end of the mitotic activity of the oogonia, when oocytes and the somatic cells are organized in discrete follicles (Hirshfield, 1991). In the rat ovaries can be recognized from E16 (Torrey, 1945). Germ cells differentiate into oocytes when they stop dividing and begin to undergo meiosis (Hirshfield, 1991). Meiosis progresses through the leptotene, zygotene and pachytene stages but stops in the diplotene stage of the first meiotic division (Bachvarova, 1985). By day E17 all germ cells have entered meiosis in the mouse (Evans, Robb, Tuckett, *et al.*, 1982).

Two days after birth the ovary of the rat contains about 27.000 oocytes (Beaumont & Mandl, 1962), each surrounded by a few flattened pregranulosa cells. All these oocytes are arrested in the prophase of the first meiotic division. The same holds true for the human, only the number of oocytes is 2.000.000 at birth (Baker, 1963). From this time on the number of oocytes decreases gradually until the end of reproductive life. The loss of oocytes per day after birth is highest in the prepubertal period, i.e. the period before the first ovulation. During reproductive life the number of growing follicles is correlated with the number of non-growing primordial follicles (Krarup, Pedersen & Faber, 1969). Around the time of first ovulation 6.000 (22% of the number present at birth) follicles were found in the rat (Meijs-Roelofs, van Cappellen, van Leeuwen, *et al.*, 1990) and 400.000 (20%) in the human (Baker, 1963). In women, who shed one oocyte per 4 week cycle, only 400 (0.1%) of these 400.000 follicles will eventually ovulate. Most other follicles will vanish by a process called atresia. In contrast to the human, the rat ovulates about 12 oocytes in every 4 or 5 day cycle (Greenwald, 1961; Welschen, 1972a). When the ovarian cycle stops in the rat at the age of about one year, about 800 follicles (13% of the number at first ovulation) will have ovulated and most of the other follicles will have disappeared by atresia.

During the period of ovarian cyclicity the ovary ovulates a species specific, constant number of follicles at constant time intervals (Lipschütz, 1928). This constancy is most striking in the rat and hamster, because a relatively large number of follicles are ovulated in every cycle (Greenwald, 1961; Welschen, 1972a). It should be realised that the genetic variance in inbred laboratory rat strains is probably much lower than in humans. This constancy is even more striking when one considers the fact that there are two ovaries that together produce the species dependent number of ovulating follicles. In humans only one ovary ovulates per cycle, while in the rat both ovaries will ovulate. Communication between the two ovaries is established by hormonal signals between the pituitary and the developing follicles in the ovaries. A tight feedback mechanism between these two components regulates the ovarian cycle, as will be described in the section about cyclic follicle development. The effectiveness of this mechanism can be shown by removing one ovary, which will result in normal ovarian cyclicity in the remaining ovary with unchanging total numbers of ovulations per cycle (Greenwald, 1961; Peppler & Greenwald, 1970; Hirshfield, 1982; Meijs-Roelofs, Osman & Kramer, 1982). This compensation is induced by a follicle-stimulating hormone (FSH) increase 6 to 8 hours after unilateral ovariectomy (Welschen, 1970; Welschen & Dullaart, 1974; Butcher, 1979; Osman, Meijs-Roelofs & Kramer, 1982). In the same way FSH containing preparations can be used to induce superovulations in intact immature or adult animals (Edwards, 1960; Wilson & Zarrow, 1962; Lostroh & Johnson, 1966; Greenwald, 1974; Nuti, McShan & Meyer, 1974; Chiras & Greenwald, 1978; Miller & Armstrong, 1981; Armstrong & Opavsky, 1988) or an inhibin-neutralizing antiserum can be used to increase endogenous FSH causing increased ovulation numbers in rats (Sander, Kramer, van Leeuwen, *et al.*, 1991a; Sander, Meijs-Roelofs, van Leeuwen, *et al.*, 1991b).

Follicular development

From primordial to Graafian follicle

At birth all oocytes are surrounded by a few flat pregranulosa cells. This stage of development is called a primordial follicle (Figure 1.1b). The whole follicle is surrounded by a non-cellular layer, the basement membrane which consists primarily of type IV collagen, laminin and fibronectin (Hirshfield, 1991). Primordial follicles are present from birth until almost the end of reproductive life, depending on the species studied. In the rat primordial follicles are still found at the end of reproductive life (Mandl & Shelton, 1959), while in humans and mice the primordial follicles have disappeared by this time (Gosden, Laing, Felicio, *et al.*, 1983, mice; Richardson, Senikas & Nelson, 1987, humans). However, the differences found also reflect a difference of the definition of the end of reproductive life. The reason why some follicles start their development directly after birth, while others remain quiescent for many years, as in the human, is still unknown.

The first sign of follicular development is the transformation of the flattened pregranulosa cells which surround the oocyte into round granulosa cells. At this time also the oocyte will resume its growth (Arendsen de Wolff-Exalto & Groen-Klevant, 1980). An oocyte surrounded by one layer of rounded granulosa cells is called a primary follicle (Figure 1.1b). The mitotic activity necessary for granulosa cell division can be monitored by injecting rats with tritiated thymidine. In this way it was shown that there is almost no mitotic activity in the primordial follicles (Hirshfield, 1989). Only after infusing rats for 7 days with tritiated

thymidine some incorporation was found in almost all primordial follicles, which indicates that almost all of these follicles are developing but at an extremely slow speed.

After the formation of a primary follicle, a second layer of round granulosa cells will be added. The follicle is now entering the secondary follicle stage (Figure 1.1c). During this period the follicles will start their so-called "slow growth phase"; in these follicles at least some dividing granulosa cells can be found (Pedersen, 1969). In contrast to the slow, ongoing growth of the number of granulosa cells, the oocyte will have grown to almost its final volume at the end of the formation of the third layer of granulosa cells (Arendsen de Wolff-Exalto & Groen-Klevant, 1980). At the same time the follicle will be surrounded by a layer of so-called theca cells at the outside of the basement membrane (Hirshfield, 1991). These cells appear to originate from the ovarian cords during embryonic development (Merchant, 1975). Although follicles will develop until the end of the secondary phase in the absence of gonadotrophins, there can be little doubt about the importance of the effect of gonadotrophins, especially FSH, secreted by the pituitary, on follicle development (for review see Greenwald & Roy, 1994). At the end of the secondary phase of development liquid filled spaces will be formed in between the granulosa cells. The liquid is not excreted by the granulosa cells, but is derived from serum from blood vessels surrounding the follicle (human: Shalgi, Kraicer, Rimon, *et al.*, 1973; pig: Chang, Jones, Ellefson, *et al.*, 1976). This liquid appears to arise from filtration of thecal blood through a molecular sieve which retains 50% of molecules with a molecular weight of 250.000 and is impermeable to proteins with a molecular weight above 850.000 (human: Shalgi, *et al.*, 1973).

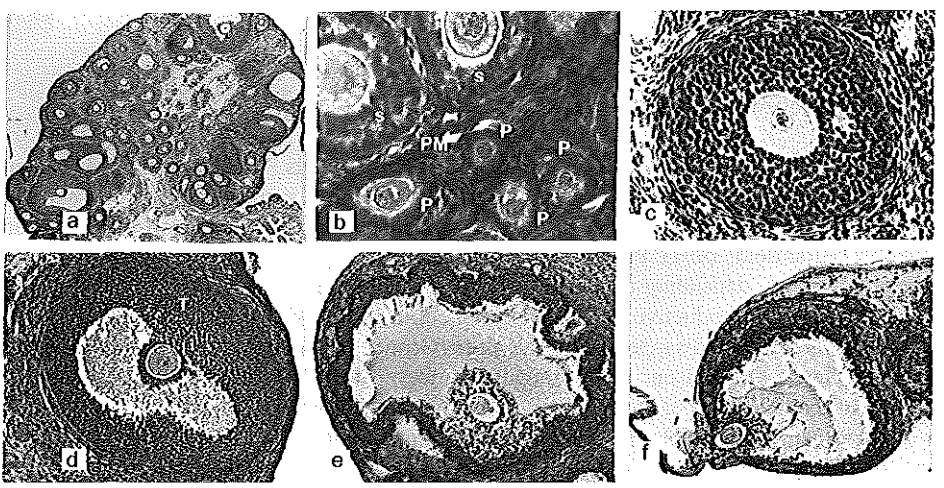

Figure 1.1 Different stages of follicle development. a) Ovary with different stages of developing follicles. b) Primordial follicle (PM, 1 layer of flat cells surrounding the small oocyte), primary follicle (P, 1 layer of cuboidal granulosa cells surround the growing oocyte) and secondary follicles (S, more than 1 layer of cuboidal granulosa cells surround the fully grown oocyte). c) Secondary follicle. d) Tertiary follicle (T, antrum between the granulosa cells). e) Preovulatory follicle after the preovulatory LH surge, a few hours before ovulation; note resumption of meiosis, first polar body and dispersion of the cumulus cells. f) Ovulation.

When the fluid-filled spaces associate with each other the follicular antrum is formed and the follicle now enters the "fast growth phase" of the tertiary or antral stage of development (Figure 1.1d) (Pedersen, 1969). This stage of development can only be reached when there is a sufficiently high concentration of FSH in the peripheral circulation; without FSH no antral follicles can be formed (Hirshfield, 1991). In this stage the follicle is composed of the oocyte, the granulosa cell layer surrounding the antral cavity, the non-cellular basal membrane, the theca interna cells and the theca externa cells. The follicle develops towards a preovulatory or Graafian follicle (Figure 1.1e) and will ovulate (Figure 1.1f) in the night after the luteinizing hormone (LH) surge. All the different stages of developing follicles, except the Graafian follicle, are continuously present in both ovaries during reproductive life (Figure 1.1a). The question whether follicles will continuously grow into the pool of antral follicles (Peters & Levy, 1966; Pedersen, 1970; Welschen, 1972b; Hirshfield & De Paolo, 1981; Hirshfield, 1988; Fauser & Van Heusden, 1997) or are recruited from a pool of preantral follicles at oestrus (Greenwald, 1973; Greenwald, 1974; Osman, 1985) is still not answered.

Atresia

The connection between the oocyte and the surrounding cells seems to be vital for the development of both the oocyte and the surrounding cells. Destruction of the oocytes in a prenatal ovary by busulfan treatment results in an ovary without developing follicles (Kasuga & Takahashi, 1986). Culturing oocytes without surrounding (pre)granulosa cells, results in regression of these oocytes (Buccione, Schroeder & Eppig, 1990). If, however, the oocytes are surrounded by granulosa cells, the oocytes can be cultured for some days (Li, Phillips & Mather, 1995).

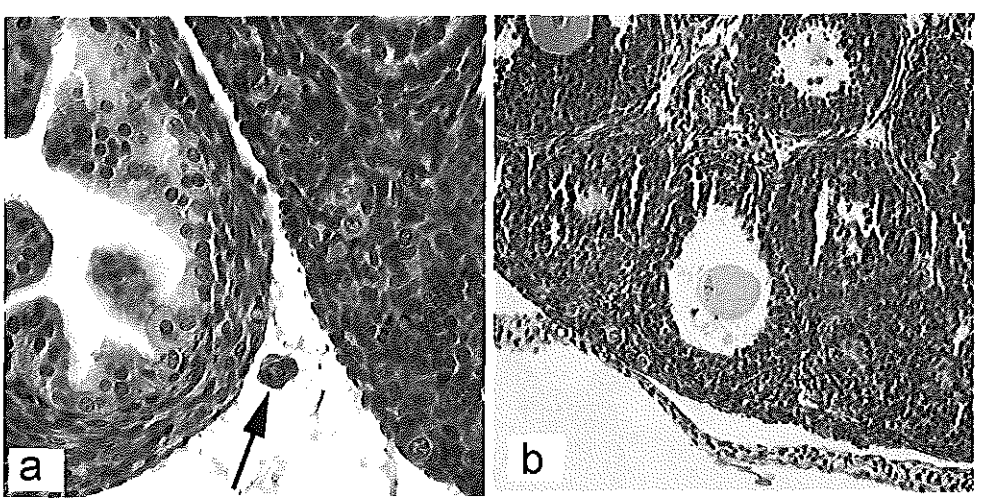

Figure 1.2 Atresia in preantral follicle in the prepubertal mouse. a) Extrusion of a primordial follicle (arrow). b) Degenerating oocyte surrounded by healthy granulosa cells.

The vital connection between the surrounding cells and the oocyte can also be seen *in vivo*. When the surrounding follicle cells regress the oocyte will also regress. This system of follicle regression is seen very often during reproductive life. It occurs mainly in antral follicles and is called atresia. A comparison between the number of developing follicles and the number of ovulations makes it clear that most follicles never reach ovulation and most of these follicle will become atretic in the antral stage of development. Three other types of regression may be seen especially during the prepubertal period. (1) In mice oocytes are lost during the first six days of life by migration of the oocytes through the ovarian epithelium (Figure 1.2a). These oocytes will be lost by elimination in the peritoneal cavity (Peters, 1969; Byskov & Rasmussen, 1973; Hiura & Fujita, 1977). (2) Also degeneration of primordial follicles is observed regularly in mice during the first 30 days of life (Edwards, Fowler, Gore-Langston, *et al.*, 1977). (3) Another type of follicular regression mainly seen in secondary follicles also occurs during the prepubertal period (Figure 1.2b). The first sign of regression normally seen during this period is the disappearance of the germinal vesicle of the oocyte followed by cleavage of the oocyte. By this time also the granulosa cells surrounding the oocyte will start their regression (Greenwald & Roy, 1994). This type of follicle regression is rarely seen in the adult rat.

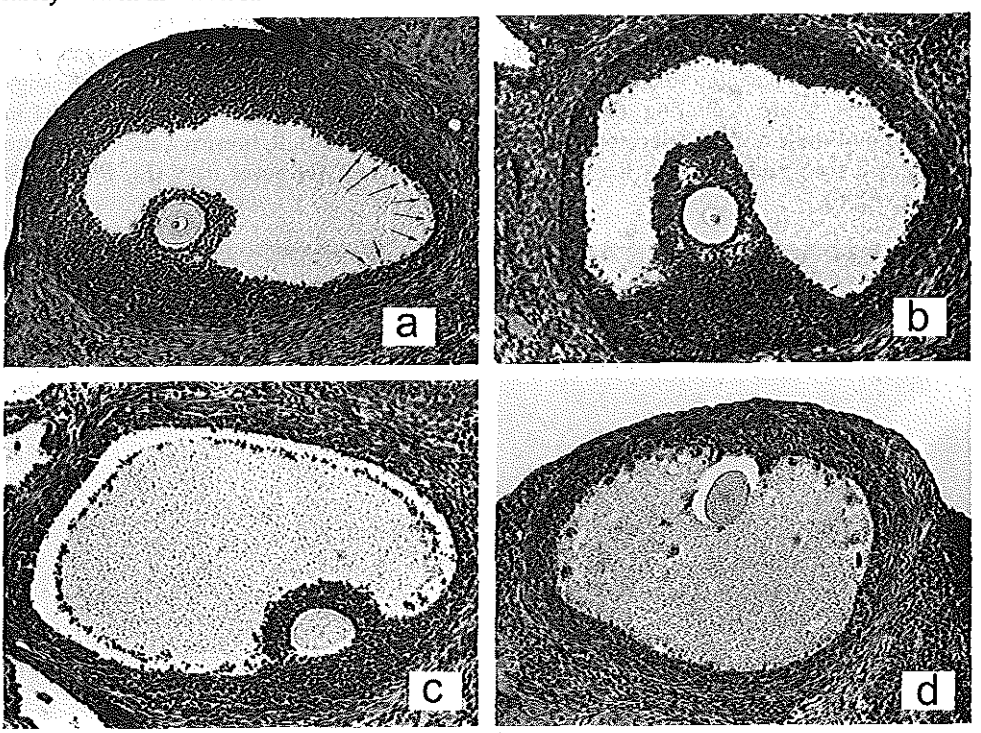

Figure 1.3 Different stages of follicular atresia. a) Early atretic follicle stage 1^a, local pycnosis in the granulosa cell layer (arrows), oocyte intact. b) Early atretic follicle stage 1^b, pycnosis all around the granulosa cell layer, oocyte intact. c) Late atretic follicle stage 2^a, pycnosis all around the granulosa cell layer, germinal vesicle breakdown in the oocyte nucleus. d) Late atretic follicle stage 2^b, germinal vesicle breakdown in the oocyte nucleus, naked oocyte.

During reproductive life most follicles will develop towards the antral stage of development and during this stage most follicles will become atretic. This type of atresia starts with pycnosis in the granulosa cells. Because pycnosis is always visible in some granulosa cells of every follicle it is difficult to define a starting point for this type of atresia. Criteria most often used are 5 pycnotic nuclei, 5 percent pycnotic cells (Byskov, 1974), or localized pycnosis at different spots (Osman, 1985) in the largest cross section of the follicle (Figure 1.3a). Within 24h after the first signs of atresia, pycnotic cells will be seen throughout the granulosa cell layer (Osman, 1985, Figure 1.3b). Thereafter regression is observed in the oocyte by the disappearance of the germinal vesicle and shrinkage of the granulosa cell layer (Figure 1.3c). The theca cells seem not to be affected in this stage of atresia (Mori, Fujita, Nihnobu, *et al.*, 1982). During the last stage of atresia the oocyte starts dividing and all granulosa cells will get lost by pycnosis (Figure 1.3d). The follicle shrinks and the remnant will be either destroyed or taken up by the interstitial stroma cells (Greenwald & Roy, 1994). The total process of atresia will take up about 3 (Osman, 1985) to 5 (Byskov, 1974) days in small animals like mice and rat.

Recently more and more evidence is accumulating that pycnosis can be regarded as apoptosis (Gerschenson & Rotello, 1992). Apoptosis is a way of programmed cell death. This would fit with the idea of tightly controlled follicular atresia as a tool to regulate the ovarian cycle in time and numbers. Lacker *et al* (1981; 1987a; 1987b) developed a model based on the feedback mechanism between the ovarian hormones and the gonadotrophins secreted by the pituitary to explain the regulation of the ovarian cycle. In this model the number of interacting follicles influences the timing and the number of ovulations. Best results in terms of timing of ovulation and the number of ovulations were found when many more follicles started their development than needed for ovulation. This means that most follicles are sacrificed to get a tightly regulated ovarian cycle.

Hormonal factors

Interovarian hormonal factors

Two types of hormones are involved in the hypothalamic-pituitary-ovarian feedback loop: protein and steroid hormones (Figure 1.4). Steroid hormones are produced in the ovary by the multi-step enzymatic conversion of cholesterol (Figure 1.5). Steroidogenesis is regulated by influencing the amount of substrate and the expression levels of the different enzymes.

The hypothalamus secretes gonadotrophin releasing hormone (GnRH) into the portal blood vessels of the pituitary. This hormone activates the secretion of LH and FSH from the pituitary. LH and FSH are both dimeric proteins composed of the same α-subunit but with different β-subunits. Both hormones exert their effects by activating specific membrane receptors in their target cells. The FSH receptor is located in the membrane of granulosa cells of primary and larger follicles (Oxberry & Greenwald, 1982; Mulheron, Quattropani & Nolin, 1989). The receptor consists of a hormone binding domain outside the cell, a trans-membrane domain which transverses the membrane 7 times and a signalling domain inside the cell (Savarese & Fraser, 1992). There is no significant change in FSH binding of the largest follicles during the ovarian cycle related to the amount of DNA (Uilenbroek & Richards, 1979). There seems to be some increase in the amount of FSH receptor mRNA on pro-oestrus

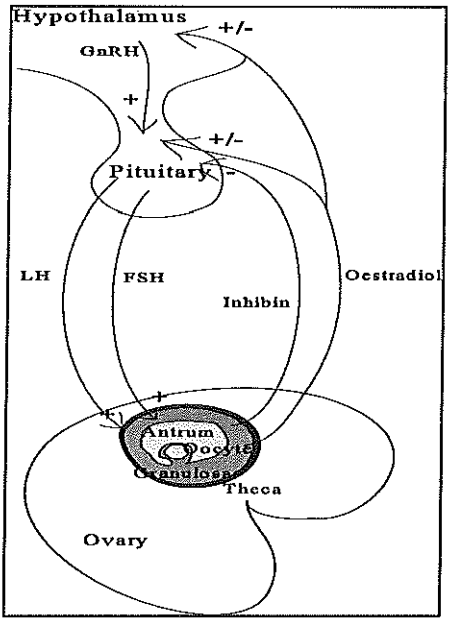

Figure 1.4 Hypothalamo-pituitary-ovarian feedback loop.

after the LH surge (Camp, Rahal & Mayo, 1991). Known actions of FSH are induction of mitosis in granulosa cells (Hirshfield, 1986), activation of the aromatase activity , induction of LH receptor formation (Moley & Schreiber, 1995) and stimulation of inhibin production (Lee, McMaster, Quigg, *et al.*, 1982).

LH receptors are found in the theca cells of antral follicles and in the mural granulosa cells of Graafian follicles (Camp, *et al.*, 1991). LH activates steroidogenesis by activation of the side chain cleavage enzyme (P540scc, Figure 1.5) which converts cholesterol into pregnenolone. In this way LH activates the formation of androgens in the theca cells, because theca cells contain the enzymes which convert pregnenolone to androgens. These androgens diffuse to the granulosa cells where they are converted to oestrogens, mainly oestradiol. The secreted oestradiol will, through its receptors in the pituitary, give rise to an increased sensitivity of the pituitary towards GnRH (Figure 1.4). However, the large amounts of oestradiol secreted during the last stage of the ovarian cycle will have a specific negative feedback effect on the GnRH, LH and FSH secretion. Inhibin which is also secreted by the granulosa cells will have a negative feedback action on the FSH secretion of the pituitary. This action is presumably performed by blocking the action of locally produced activin which stimulates the FSH secretion of the pituitary. Until now no specific receptor for inhibin has been found. Inhibin probably has its effect by inhibition of the activin binding to activin receptor type II (Martens, de Winter, Timmerman, *et al.*, 1997).

The secretion of gonadotrophins can also be influenced by treatment with GnRH agonists or antagonists. Treatment with a GnRH antagonist leads to a decreased secretion of LH and FSH from the pituitary which in turn will lead to a decreased ovarian stimulation. This treatment partly resembles the effect of hypophysectomy without abolishing the secretion of other factors from the pituitary. The secretion of LH and FSH, however, will not be completely suppressed. Treatment with a GnRH agonist can provoke different responses depending on the type of treatment. Treatment with a single dose of GnRH agonist will lead to an increased LH and FSH secretion which in turn will lead to more or less follicle growth stimulation depending on the stage of follicular development at the moment the treatment takes place (Naor, Zilberstein, Zakut, *et al.*, 1983). Chronic treatment with a GnRH agonist will lead to desensitisation of the GnRH receptor in the pituitary which will lead to the same effects as treatment with a GnRH antagonist. However GnRH (agonist) may also have a direct effect on the ovary itself trough local GnRH receptors (Knecht, Ranta, Feng, *et al.*, 1985). GnRH agonist treatment decreases the LH receptor formation in developing follicles which in turn decreases the progesterone production of these follicles (Jones & Hsueh, 1980; Naor, *et al.*,

1983), and suppresses the FSH receptor levels in granulosa cells (Ranta, Knecht, Baukal, *et al.*, 1984).

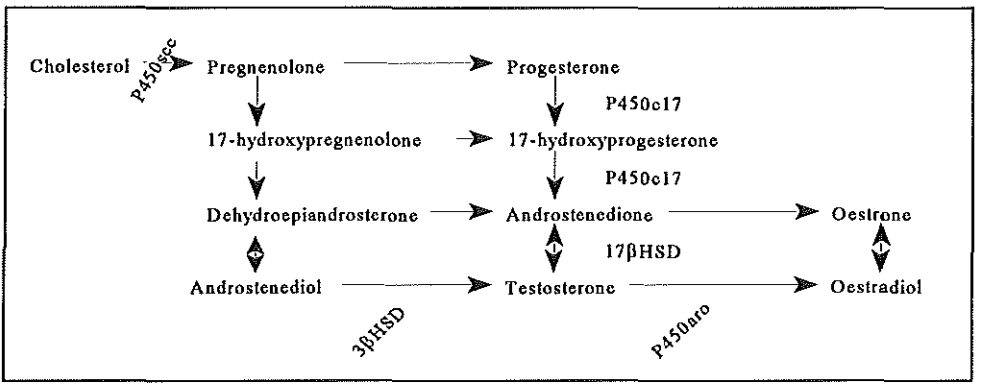

Figure 1.5. Ovarian steroid synthesis. P450ssc - cholesterol side chain cleavage enzyme. P450c17 - 17-hydroxylase. 17βHSD - 17β-hydroxysteroiddehydrogenase. 3βHSD - 3β-hydroxysteroiddehydrogenase. P450aro - aromatase.

Intraovarian hormonal factors

Follicle development is not only regulated by the hypothalamic-pituitary-ovarian axis, but also by intra-ovarian factors. Because intra-ovarian factors are locally produced and regulated, their local and peripheral concentrations may be very different. This makes it difficult to study them. The role of most of these factors is still not very clear.

Oestradiol

This steroid hormone is not only important as a feedback component but it also has effects on the follicle directly. Oestradiol is produced by the granulosa cells (Marut, Huang & Hodgen, 1983) by aromatisation of androgens secreted by the theca cells. This androgen secretion is stimulated by LH while the aromatisation of androgens to oestradiol in the granulosa cells is stimulated by FSH (Fevold, 1941; Falck, 1959). One of the important actions of oestradiol seems to be the prevention of atresia. When immature female rats are hypophysectomized, atresia in the antral follicles can be prevented for some time by injections with the synthetic oestrogen diethylstilbestrol (Schwall & Erickson, 1981). It was also found that oestradiol induces mitoses in granulosa cells (Richards, 1980). The granulosa cells do not only produce oestradiol, but they also contain oestradiol receptors (Hillier, Saunders, White, *et al.*, 1989), which makes autocrine and paracrine action possible. The importance of the oestradiol action was demonstrated with an oestradiol receptor knock-out mouse in which the female offspring was infertile with haemorrhagic cystic ovaries (Lubahn, Moyer, Golding, *et al.*, 1993). A constitutively active oestradiol receptor resulted in the formation of ovarian tumours (Fuqua, Fitzgerald, Chamness, *et al.*, 1991). Oestradiol can only be formed when the P450aromatase enzyme is present and active. Defects in this enzyme lead in human females to ovaries with a

higher than normal number of small antral follicles from 6-8 mm (Morishima, Grumbach, Simpson, *et al.*, 1995). No follicles larger than this size were found. This is the folliclar size at which follicles become FSH dependent and the size at which follicle selection takes place (Pache, Wladimiroff, de Jong, *et al.*, 1990). However, using data obtained after FSH treatment of patients with genetic defects which inhibited the oestradiol formation, it was shown that follicle development could take place in the absence of oestradiol. This led to the conclusion that oestradiol is not vital for human follicle development (Fauser & Van Heusden, 1997).

Inhibin and activin

Inhibin is a dimeric protein composed of an α- and a $ß_A$- or $ß_B$-subunit, linked by a disulphide bond. Alternatively, two ß-subunits can be linked to form activin. Inhibin was first described as a substance of gonadal origin that specifically decreases FSH release without altering the LH release. Both inhibin and activin were found in follicular fluid (Inhibin: de Jong & Sharpe, 1976, Activin: Ling, Ying, Ueno, *et al.*, 1986a; Vale, Rivier, Vaughan, *et al.*, 1986). Locally produced activin is known to stimulate the FSH secretion of the pituitary (Ling, *et al.*, 1986a; Vale, *et al.*, 1986). Activin is also produced in the ovary (Ling, Ying, Ueno, *et al.*, 1986b). Besides the pituitary actions of inhibin, more and more evidence for autocrine/paracrine actions of this hormone and of activin are found. Inhibin was found to reduce the FSH induced aromatase activity and progesterone production in rat granulosa cell culture (Ying, Becker, Ling, *et al.*, 1986). This result could not be reproduced by Hutchinson *et al* (1987) using bovine inhibin treated rat granulosa cells. Activin was found to stimulate the progesterone and inhibin production, and the FSH stimulated aromatase activity in rat granulosa cells (Xiao, Findlay & Robertson, 1990). This is in contrast with the findings *in vivo* that human recombinant inhibin positively influenced follicle development, while human recombinant activin treated ovaries were negatively influenced in there follicle development after injection directly into the ovary (Woodruff, Lyon, Hansen, *et al.*, 1990). Activin A can stimulate granulosa cell DNA replication *in vitro* (Li, *et al.*, 1995). The situation is complicated further by the presence of follistatin, a protein which is known to bind and deactivate activin, in follicular fluid (Klein, Robertson, Shukovski, *et al.*, 1991; Kogawa, Nakamura & Sugino, 1991). The inhibin α-subunit is found in all follicles from primary to Graafian (Cuevas, Ying, Ling, *et al.*, 1987; Merchenthaler, Culler, Petrusz, *et al.*, 1987; Tisdall, Hudson, Smith, *et al.*, 1994). The inhibin β-subunits are mainly found in the granulosa cells of large healthy antral follicles. At oestrus the β-subunits were also found in the smaller antral follicles (Meunier, Cajander, Roberts, *et al.*, 1988). An increase in the expression of inhibin α- and $ß_A$-subunit-mRNA was found in newly recruited follicles in the rat after the periovulatory FSH surge (Woodruff, D'Agostino, Schwartz, *et al.*, 1988; D'Agostino, Woodruff, Mayo, *et al.*, 1989).

Activin action in induced by binding of activin to an activin membrane receptor type II (ActRII, A or B). The activin receptors belong to the family of transforming growth factor-ß (TGFß) receptors. These ActRII mRNAs were found in oocytes, in the corpus luteum and in granulosa cells of rat follicles at dioestrus-1(Cameron, Nishimura, Mathews, *et al.*, 1994). The ActRII-activin complex will bind to an ActRI membrane receptor and subsequently induce phosphorylation of the ActRI receptor (Attisano, Wrana, Montalvo, *et al.*, 1996). All inhibin effects found are inhibitions of activin action (Mathews, 1994). However, not all activin actions are antagonized by inhibin (Mathews, 1994). This may be explained on the basis of

high number of ActRIIA receptors in the granulosa cells and the lower binding of inhibin to the ActRIIA receptor compared to activin (Martens, *et al.*, 1997). Further investigations on the role of inhibin and activin were carried out by making inhibin-α and inhibin-β subunit knock-out mice. The inhibin-α knock-out mouse develop gonadal stromal tumours after normal gonadal development. In case of the female mouse the tumour develops before normal ovarian cyclicity takes place which makes it impossible to identify the specific role of inhibin during normal follicular development (Matzuk, Finegold, Su, *et al.*, 1992). However, it is possible to stimulate follicle growth in prepubertal knock-out mice before tumour development, and in this way ovulated oocytes can be fertilized (Matzuk, Kumar, Shou, *et al.*, 1996). From this observation it can be concluded that follicle development and ovulation can take place without inhibin action. This model, however, does not reveal the auto- or paracrine role of inhibin during normal cyclic follicle development. Activin knock-out mice do have a disturbed embryonal development which makes the study of the effects on postnatal ovarian development very difficult (Matzuk, Kumar, Vassalli, *et al.*, 1995).

The role of inhibin in the endocrine feedback regulation of FSH production is quite clear, but the roles of activin and inhibin and their importance as local regulators still need further study.

Other intraovarian factors

FSH is generally accepted as a key factor in follicle selection. It rescues follicles from atresia, but it is unknown how FSH selects certain follicles for ovulation. Probably other (local) factors are involved in follicle selection. It was hypothesized that the physiological basis of follicle selection is the differential expression of factors that modulate the action of gonadotrophin on follicular cells at key points during the process of follicle development (Campbell, Scaramuzzi & Webb, 1995). Some of the possible factors are summarized in table 8.1. The problem with most these local factors is the lack of evidence that they are also working *in vivo* (Fauser & Van Heusden, 1997). The most atractive factors for follicle selection are the factors that have both a peripheral and a local activity. This could explain the coordination between both ovaries.

Follicle development in the rat

Prepubertal follicle development

The prepubertal period is the period from birth to the first ovulation. It was shown by Peters that there is active follicle development during this period both in girls (1976) and mice (1969). Also in other species pronounced follicle development has been observed during this period. In rat and mouse it was found that follicle growth during the prepubertal period is somewhat faster than during cyclic life (mouse: Pedersen, 1969; rat: Hage, Groen-Klevant & Welschen, 1978). Although some follicles do start their development in the prepubertal period most primordial follicles will remain in their resting stage of development. What could be the reason for follicles to start their growth shortly after birth ? One theory is that preantral follicle

Table 8.1. Factors influencing follicle selection

Factor	Species	Reference
Activins, inhibins, and follistatin	Human	Giudice, Chandrasekher & Cataldo, 1993
IGFBP-2 and -3	Human	San Roman & Magoffin, 1993
IGFBP-3	Human	Magoffin, San Roman & Muderspach, 1995
Naturally occurring aromatase inhibitors	Porcine, human, rat	DiZerega, Tonetta & Westhof, 1987
TGF-β	Rat	Knecht, Feng & Catt, 1987
Follistatin	Rat	Nakatani, Shimasaki, Depaolo, *et al.*, 1991
Anti-Mullerian hormone	Rat	Hirobe, He, Gustafson, *et al.*, 1994
Vascular endothelial growth factor	Primate	Ravindranath, Little-Ihrig, Phillips, *et al.*, 1992
Oestradiol	Ovine	Fortune, Sirois, Turzillo, *et al.*, 1991
Pulsatile LH	Ovine	McNeilly, Picton, Campbell, *et al.*, 1991
Vitamin A	Bovine	Schweigert & Zucker, 1988
IGFBP-2 and -4	Ovine	Perks & Wathes, 1996
Oestrogen	Porcine	Veldhuis, 1985
Bcl-xlong	Chicken	Johnson, Bridgham & Wagner, 1996

development is an autonomous process not regulated by extra-ovarian factors. This theory is based on the fact that in hypophysectomized rats there is still follicle development until the antral stage (Lane & Greep, 1935; Paesi, 1949). Another theory postulates that the high gonadotrophin levels, especially FSH during this period, induce prepubertal follicle development (Eshkol, Lunenfeld & Peters, 1970; Eshkol & Lunenfeld, 1972; Baker & Neal, 1973; Schwartz, 1974). During the first three weeks of life the FSH levels in female rats are much higher than during cyclic life with the exception of the ovulatory FSH peak (Goldman, Grazia, Kamberi, *et al.*, 1971; Kragt & Dahlgren, 1972; Ojeda & Ramirez, 1972; Meijs-Roelofs, Uilenbroek, Osman, *et al.*, 1973). These high gonadotrophin levels are very important for normal ovarian development (Schwartz, 1974). Reduction of the high gonadotrophin levels by treatment with testosterone propionate, anti-serum against FSH or 5α-dihydrotestosterone propionate resulted in a decreased number of preantral and small antral follicles 15 days after the last injection (Uilenbroek, Arendsen de Wolff-Exalto &

Blankenstein, 1976a; Uilenbroek, Arendsen de Wolff-Exalto & Welschen, 1976b; Uilenbroek & Arendsen de Wolff-Exalto, 1979). Due to their limited growth rate the first antral follicles in rat and mouse are seen around day 20 of life (Pedersen, 1969). When the first follicles reach the antral stage of development they start to produce inhibin (Sander, Meijs-Roelofs, Kramer, *et al.*, 1985), which is reflected in a decrease of FSH until the day of first pro-oestrus (Sander, Meijs-Roelofs, van Leeuwen, *et al.*, 1986). During the period between day 20 and a few days before first ovulation the follicles do not grow larger than a volume of 500 * 10^5 μm^3 (diameter $\leq$ 450 μm) before they become atretic and the amounts of inhibin produced are relatively small (Sander, *et al.*, 1986). During the last 4 days before first ovulation the largest antral follicles (vol. $\geq$ 350 * 10^5 μm^3, diameter $\geq$ 400 μm) do contain increasing amounts of inhibin, which correlate negatively with the decreasing peripheral FSH concentrations (Sander, *et al.*, 1986). The pulsatile LH pattern which can be seen from 10 days before first ovulation onwards (Meijs-Roelofs, Kramer & Sander, 1983), may be the reason why only during this last period follicles develop to volumes $\geq$ 500 * 10^5 μm^3.

Cyclic follicle development

After the first ovulation has taken place, the ovarian cycle starts. In our rat strain (Wistar R-Amsterdam) the very first cycle takes 7-8 days (Osman & Meijs-Roelofs, 1976). This first cycle is followed by a period of 4-6 months (Sander, 1988) during which 11 follicles will ovulate in every 5-day cycle (Welschen, 1970). In this thesis the days of the 5-day cycle will be referred to as oestrus (OE), dioestrus-1, -2, -3 (D1, D2, D3) and pro-oestrus (PO). This highly organized system is regulated by hormones secreted by the hypothalamus, the pituitary gland and the developing follicles.

Oestrus

In our strain of rats ovulation will take place between 02.00h and 04.00h in the early morning of oestrus (Osman, 1975). Characteristic for oestrus morning (about 10.00h) is a high FSH serum concentration (Figure 1.6) and the presence of a large number of small antral follicles in the ovaries (Welschen & Rutte, 1971). Also the incorporation of [^{3}H]thymidine in preantral follicles increases (Roy & Greenwald, 1986; Wang, Roy & Greenwald, 1991). It was postulated that the high FSH levels are responsible for recruitment of these small antral follicles (Welschen & Rutte, 1971; Welschen, 1972b; Hirshfield & De Paolo, 1981). Later it was demonstrated that suppression of the FSH levels at oestrus by injection of porcine follicular fluid (pff, a crude inhibin preparation) indeed decreases the number of antral follicles found at dioestrus-1 (Hirshfield & De Paolo, 1981). The number of small antral follicles (100-350 * 10^5 μm^3, diameter 265-400 μm) found at oestrus morning (about 30/ovary, Osman, 1985) is five times the number which will eventually ovulate. At this stage of development it is not possible to induce ovulation of these follicles by means of hCG (Welschen & Rutte, 1971). The inhibin-like activity found in the ovary at oestrus is low compared to levels found during the second part of the cycle (Sander, *et al.*, 1986). Also, the oestradiol serum levels are low at oestrus. Immuno-reactive inhibin in serum was found to increase slightly during oestrus (Watanabe, Taya & Sasamoto, 1990). The progesterone

secretion from the newly formed corpora lutea (CLs) at oestrus increases until dioestrus-2 whereafter the progesterone secretion decreases.

Dioestrus

The number of healthy antral follicles at oestrus will gradually decrease to about 14 medium to large antral follicles (vol. > 200 * 10^5 μm^3, diameter >350μm) per 2 ovaries at dioestrus-3 (Osman, 1985). This decrease is the result of atresia of a number of the antral follicles. At dioestrus-1 the number of early atretic follicles is much higher than during oestrus (Osman, 1985). The largest follicles found at 10.00h on dioestrus-1 have a volume between 350 and 500 * 10^5 μm^3 (diameter 350-400 μm). Ovulation can be induced in these follicles by treatment with 15 IU of hCG (Welschen & Rutte, 1971). During the dioestrus period the inhibin-like activity in the ovary increases gradually (Sander, *et al.*, 1986). At the end of the dioestrus period the oestradiol serum concentration will start rising. The increasing oestradiol concentrations will make the cells in the pituitary more sensitive to GnRH from the hypothalamus (Döcke, Rohde, Stürzebecher, *et al.*, 1990). Both LH and FSH are low during this period due to the feedback action of inhibin and oestradiol on the pituitary. The immuno-reactive inhibin-α serum concentration, which does not necessarily reflect the bioactive inhibin levels, does not change very much during this period (Watanabe, *et al.*, 1990).

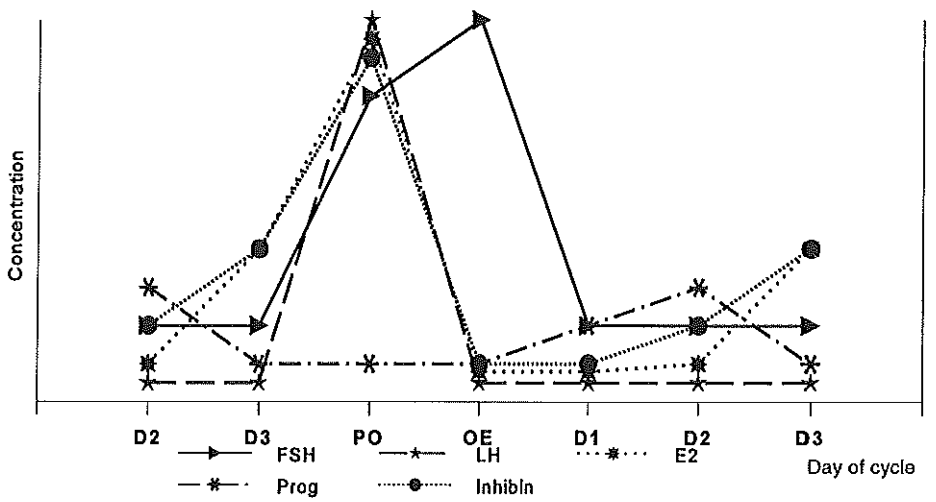

Figure 1.6 Hormonal patterns during the 5-day cycle of the rat

Pro-oestrus

On the morning of pro-oestrus big Graafian follicles will be found in the ovaries. These are the follicles that will ovulate the next morning. By now the number of ovulating follicles is fixed. The inhibin-like activity in the ovary is very high (Sander, *et al.*, 1986) and also the serum oestradiol concentration reaches a maximum level. The high oestradiol concentrations

in serum will increase the hypothalamic pulsatile secretion of GnRH, which leads to an increased secretion of LH and FSH from 13.00-17.00h in the afternoon of pro-oestrus. The LH surge stimulates the oocyte in the Graafian follicle to resume meiosis. The granulosa cells of the Graafian follicles stop producing oestradiol and switch to the production of progesterone. In contrast to the LH surge, which declines rapidly after 15.00h on pro-oestrus the FSH concentration remains high until the next day, oestrus 13.00h (Hirshfield & De Paolo, 1981). This second part of the FSH surge is often called the secondary FSH peak and is probably caused by the drop in the serum inhibin concentration which occurs shortly after the LH peak. In the rat there is not much distinction between the first and second part of the FSH surge.

The end of reproductive life

The period of regular cyclicity in our rats will last for about 4-6 months (Sander, 1988). This period is followed by a period in which the percentage of normal 5-day cycles will decrease. Due to different definitions for the end of reproductive life, different conclusions are drawn about its cause. Depletion of follicles as a main reason was found by some authors (Sopelak & Butcher, 1982, rats; Gosden, *et al.*, 1983, mice; Richardson, *et al.*, 1987; Gosden & Faddy, 1994, humans), changes in hormonal control by others (Jones & Krohn, 1961, mice). The change in hormonal control was also mentioned by the authors of the first theory. In women it seems clear that before follicle depletion occurs changes will be found in the ovarian-pituitary feedback system (Richardson, *et al.*, 1987; Gosden & Faddy, 1994). Because women will normally live long after the end of reproductive life, the ovary will end up completely devoid of follicles.

Aim and scope of this thesis

The aim of this thesis is to describe the effect of gonadotrophins, especially FSH, on the development of preantral and antral follicles in the rat. The peripheral concentrations of gonadotrophins are very high during the first two weeks of life in this species. Suppression of these high levels yields a good model system to study the effect of the high endogenous gonadotrophin concentrations on growth and development of preantral and small antral follicles. During cyclic life the effect of manipulation of the levels of FSH on antral follicle development was studied to determine when and from which class of follicles the follicles which will ovulate are selected. In order to reach this goal, a method was developed to describe dynamic events of antral follicle growth during the ovarian cycle of the rat. These studies were performed by measuring the number of ovarian follicles in the different size classes and the levels of different hormones involved in the ovarian-hypothalamo-pituitary feedback system.

In the next chapters studies will be presented investigating the role of the prepubertal FSH surge in the regulation of preantral follicle development and of first ovulation (chapter 2), the stability of the ovarian-hypothalamo-pituitary feedback loop before first ovulation (chapter 3), the dynamics of antral follicle growth during the cycle (chapter 4), the contents of inhibin-like activity of follicles during the ovarian cycle (chapter 5) and the influence of exogenous FSH on the ovarian-pituitary feedback loop (chapters 6 and 7). Chapter 8 will contain a general discussion of the studies performed. Finally the appendix presents physiological data fitted to the follicle inflow model presented in chapter 4.

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Chapter 2

Ovarian follicle dynamics in immature rats treated with a luteinizing hormone-releasing hormone antagonist (Org.30276)

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Ovarian Follicle Dynamics in Immature Rats Treated with a Luteinizing Hormone-Releasing Hormone Antagonist (Org. 30276)

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ABSTRACT

The high concentrations of gonadotropins present in immature female rats by the end of the second week of life were suppressed by treatment with an antagonist against luteinizing hormone-releasing hormone (LHRH-A; Org. 30276) on Days 6, 9, 12, and 15 of life. Differential ovarian follicle counts were made on Days 15, 22, 28, and on the day of first estrus of all growing follicles and follicles≥100 $\times 10^5$ μm^3 (mostly antral). In LHRH-A-treated rats, a retardation of follicle growth was noted on Day 15, followed by a gradual loss of growing follicles that amounted to 20% on Day 22 and 40% on Day 28; at first estrus, the total population of growing follicles was only 50% of that present in control rats. Antral follicles, first present at 22 days of age, were lower in number at 28 days of age and at first estrus in LHRH-A-treated rats; this was true for both healthy and atretic follicles. Ovarian weights were significantly reduced in LHRH-A-treated rats at 15 and 28 days of age and on the day of first estrus. However, the numbers of corpora lutea following the first, and normally timed, ovulation were the same in both groups.

It was concluded that for early recruitment of follicles to reach a full-sized pool of growing follicles at the age of puberty, high concentrations of gonadotropins early in life have a significant role.

INTRODUCTION

It has been well established that gonadotropins are indispensable for normal ovarian development already during the first weeks of life (see Schwartz, 1974). A role for follicle-stimulating hormone (FSH) in enhancing follicle movement into the proliferating pool and in achieving follicle stages with more than a few layers of granulosa cells has been demonstrated (Eshkol et al., 1970; Eshkol and Lunenfeld, 1972; Baker and Neal, 1973; Schwartz, 1974). Luteinizing hormone (LH) has been found to play a role in the development of ovarian vascularization and of interstitial tissue, as well as in the development of follicular theca and antrum formation (Eshkol and Lunenfeld, 1972; Schwartz, 1974).

On the other hand, the question as to the significance of the extremely high FSH concentrations present in immature rats by the end of the second week of life (Goldman et al., 1971; Kragt and Dahlgren, 1972; Ojeda and Ramirez, 1972; Meijs-Roelofs et al., 1973) has not been answered satisfactorily. Suppression of these high FSH levels by treatment with testosterone proprionate (TP) (Uilenbroek et al., 1976a,b), with 5-dihydrotestosterone proprionate (Uilenbroek and Arendsen de Wolff-Exalto, 1979), or antiserum against FSH (Uilenbroek et al., 1976b) results in decreased numbers of medium and large follicles prepubertally, but little or no change is detected in the timing of first ovulation and in subsequent cyclicity (Uilenbroek and Arendsen

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de Wolff-Exalto, 1979). However, early vaginal opening and the presence at that time of advanced growth of large follicles are found in TP-treated rats (Uilenbroek et al., 1976a); in these (androgenized) rats, no ovulation takes place. Anderson et al. (1976) have found that using antiserum to gonadotropins is inadequate; after such treatment, even elevated FSH levels have sometimes been found. No changes in follicle population have been detected on Day 15 (Anderson et al., 1976).

With the availability of LHRH antagonists, a new approach for suppression of gonadotropin concentrations has become possible. In the present study, we used an LHRH antagonist (LHRH-A) that appeared to effectively suppress the high gonadotropin levels normally present early in life. After LHRH-A treatment, the ovarian follicle population was studied at various prepubertal ages, up to the age at first estrus.

MATERIALS AND METHODS

Immature female rats of our inbred Wistar substrain (R-Amsterdam) were used. They were kept in a controlled temperature of 22–25°C with a light period from 0500–1900 h daily. Standard dry pellets and tap water were available ad libitum. Rats were weaned at 22 days of age and thereafter checked daily for vaginal opening. Starting on the day of vaginal opening, daily vaginal smears were made until the day of first estrus. On this day, a fully cornified smear was found and ovulation was confirmed by the presence of tubal ova.

Treatment

On Days 6, 9, 12, and 15 of age (day of birth counted as Day 0), at 1100 h, rats received an s.c. injection of either 500 μg/100 g body wt of LHRH-A, Org. 30276 (Ac-D-pClPhe-DpClPhe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH_2.CH_3.COOH, Organon, Oss, The Netherlands) or of saline. The peptide was dissolved in saline at a concentration of 1 mg LHRH-A/ml. Rats, 7–10 per group, were killed by decapitation between 1100 and 1300 h on Days 6, 7, 9, 12, 15, 18, 22, 28, 34, and on the day of first estrus. Rats killed on Days 6, 9, 12, and 15 did not receive the injection due that day. At the time rats were killed, trunk blood was collected and allowed to clot in a refrigerator before centrifugation. The serum was separated and stored at –20°C until assay of LH and FSH. Body, ovarian, and uterine weights were recorded; ovaries were fixed in Bouin's fluid for histological examination and follicle counts.

Histology and Follicle Counts

Serial sections (10 μm) of the ovaries, embedded in paraffin wax, were stained with hematoxylin and eosin. Follicle counts were made in both ovaries using two methods:

1) With one method, we measured all follicles in which the oocyte was surrounded at some places by at least two cell layers (roughly comparable to type 3b-4 of Pedersen and Peters, 1968). This means that medium and large follicles (Pedersen and Peters, 1968) were counted. Each fifth serial section was searched for follicles that showed the nucleus of the oocyte, which were then measured.
2) The method of Boling et al. (1941), as modified by Welschen (1973), was used for further analysis of the population of large follicles. Measurements were made of all follicles with a volume≥100 × 10^5 μm^3 (mean diameter≥about 275 μm; generally comparable to Types 6, 7, and 8 of Pedersen and Peters, 1968). These follicles, which mostly have an antrum and which show significant changes in volume and number during the adult cycle (Welschen, 1972), were classified into five volume classes (1: 100–199; 2: 200–349; 3: 350–499; 4: 500–999; 5: ≥1000 × 10^5 μm^3). Healthy and atretic follicles were counted separately; criteria for atresia were changes in granulosa cells and changes or loss of the oocyte nucleus (Meijs-Roelofs et al., 1982). This method involved examining each histological section; two perpendicular diameters were measured in the section in which the nucleolus of the oocyte was seen.

By measuring two perpendicular diameters with a Summagraphic MM 1201 Data Tablet, connected with an Olivetti M24 Microcomputer, and substituting the third diameter by the mean of the other diameters, the volume of the follicles was calculated. A computer program was developed to manage the measurements, to perform some statistical analysis, and to draw block diagrams. Additionally, in two age groups (15 days and at the day of first estrus) we also measured all type 3b follicles, i.e. including those with only one layer of granulosa cells surrounding the oocyte (the smallest

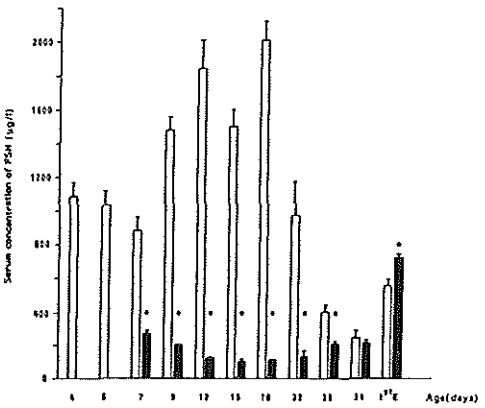


FIG. 1. Serum follicle-stimulating hormone (FSH) concentrations (mean ± SEM) in control *(open bars)* and luteinizing hormone-releasing hormone antagonist (LHRH- A)-treated rats *(solid bars)* at various ages. LHRH-A was administered at 6, 9, 12 and 15 days of age. Each *bar* represents the mean ± SEM of 6–12 rats. *$p<0.01$, LHRH-A-treated vs. saline-treated rats.

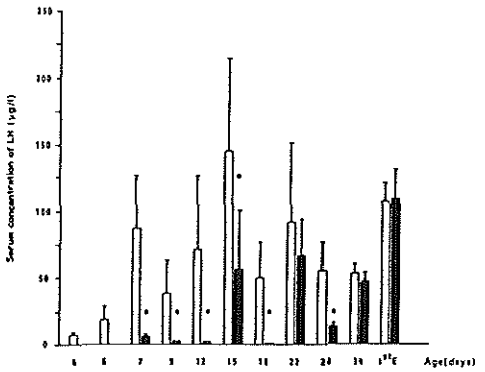


FIG. 2. Serum luteinizing hormone (LH) concentrations (mean ± SEM) in control *(open bars)* and in luteinizing hormone-releasing hormone antagonist (LHRH-A)-treated rats *(solid bars)* at various ages. LHRH-A was administered at 6, 9, 12, and 15 days of age. Each *bar* represents the mean ± SEM of 6–12 rats. *$p<0.01$, LHRH-A-treated vs. saline-treated rats.

growing follicles [Pedersen, 1969; Hage et al., 1978]).

Hormone Measurements

The concentrations of LH and FSH were estimated by radioimmunoassay, with anti-ovine LH or FSH as antiserum and rat LH or FSH as tracer (Welschen et al., 1975). Serum concentrations of LH or FSH are expressed as μg reference preparation/l (NIAMDD-rat LH or FSH RP-1). Samples were measured in duplicate and the intra- and interassay variability was 3 and 18% for LH and 9 and 16% for FSH.

Statistical Analysis

Statistical analysis was performed by the Mann-Whitney-U-test. A difference was considered significant if the double-tail probability was <0.05.

RESULTS

The LHRH-A treatment resulted in a striking and significant suppression of FSH levels to a level of about 200 μg/l, lasting from 7 until at least 28 days of age. Thus the extremely high levels present in control rats had been completely abolished (Fig. 1). Also LH concentrations in LHRH-A-treated rats between 7 and 28 days of age, were, with the exception of levels on Day 22, significantly suppressed compared with concentrations in control rats (Fig. 2). Vaginal opening (of a pinhole type) occurred about 5 days earlier in LHRH-A-treated rats than in saline-treated rats. However, neither age nor body weight on the day of first estrus differed between groups (38.3 ± 0.7 (n = 10) vs. 38.2 ± 0.5 days (n = 9) and 94.3 ± 2.2 vs. 94.8 ± 2.6 g). Also, the number of corpora lutea present in the ovaries at first estrus (9.8 ± 0.4 vs. 10.4 ± 0.3) were similar.

Ovarian Morphology

At 15 days of age, the ovaries of LHRH-A-treated rats were visibly smaller than those in saline-treated rats (Fig. 3-1), as was also apparent from their weight ($p<0.01$, Table 1). Follicles in the ovaries of LHRH-A-treated rats differed from those in saline-treated rats in that they often showed irregular oocytes, whereas granulosa cells were smaller and were crowded together (Fig. 3-3). The space between the oocyte and the surrounding granulosa cells was often larger in LHRH-A-treated rats (Fig. 3-2). Moreover, the interstitial tissue seemed smaller in volume and cells were less devel-

TABLE 1. Total number of follicles with ≥2 granulosa cell layers (mean ± SEM) present at various ages in the two ovaries of luteinizing hormone-releasing hormone antagonist (LHRH-A)-treated rats and saline-treated control rats.

Treatment	Age (days)	Body wt (g; mean ± SEM)	No. of rats	Total no. of follicles measured	Weight of 2 ovaries (mg)
Saline	15	25.2 ± 1.4	10	188 ± 7	3.5 ± 0.2
LHRH-A		26.0 ± 1.2	10	181 ± 6	1.9 ± 0.03*
Saline	22	38.8 ± 2.3	7	266 ± 9	9.5 ± 0.8
LHRH-A		39.5 ± 2.0	9	211 ± 10*	6.9 ± 0.8
Saline	28	52.8 ± 2.3	7	289 ± 18	16.6 ± 0.5
LHRH-A		54.2 ± 2.0	10	168 ± 10*	11.0 ± 0.3*
Saline	first estrus	94.8 ± 2.6	9	175 ± 6	36.5 ± 1.0
LHRH-A		94.3 ± 2.2	9	84 ± 4*	23.6 ± 0.5*

*$p<0.01$, LHRH-A-treated vs. saline-treated, Mann-Whitney U-test.

oped in LHRH-A-treated rats: cells were less disc-like than in the ovaries of saline-treated rats (Figs. 3-1 and 3-3).

In 22-day-old ovaries, no differences were seen in the follicles and interstitial tissue of LHRH-A-treated and saline-treated rats. Furthermore, there was no significant difference in absolute weight between the ovaries of LHRH-A-treated rats and those of saline-treated rats; however, expressed as relative ovarian weight (mg/100 g body wt), there was a difference ($p<0.01$) between LHRH-A-treated (17.0 ± 1.4) and saline-treated rats (24.5 ± 1.2). At 28 days of age, the histological picture was similar in LHRH-A-treated and saline-treated rats, but both absolute and relative ovarian weights were significantly lower ($p<0.01$) in the LHRH-A-treated group. Also, on the day of first estrus, absolute and relative ovarian weights were about 30% lower in LHRH-A-treated rats than in saline-treated rats ($p<0.01$, Table 1).

Counts of Medium and Large Follicles

Numbers of medium and large follicles present in saline- and in LHRH-A-treated rats on Days 15, 22, 28, and on the day of first estrus are shown in Figure 4. Follicles were arbitrarily subdivided into 12 volume classes.

On Day 15, the number of follicles present in the classes with a volume of 2–4 × 10^5 μm^3 and 4–6 × 10^5 μm^3 was significantly higher ($p<0.05$ and $p<0.01$, respectively) in LHRH-A-treated rats than in saline-treated controls. In the larger follicle classes, those between 10 and 40 × 10^5 μm^3, the number of follicles was significantly reduced in LHRH-A-treated rats compared with control rats. The smallest growing follicles (all type 3b follicles, Pedersen and Peters, 1968, 20–60 granulosa cells) were counted at Day 15 of age (not shown in Fig. 4), and a small difference ($p<0.05$) in the numbers was seen between LHRH-A-treated rats (112 ± 4) and saline-treated rats (131 ± 5).

Figure 4 shows that on Day 22 significant differences between groups were only seen in the volume class of 10–15 × 10^5 μm^3; the total number of follicles was significantly lower ($p<0.01$) in LHRH-A-treated rats than in saline-treated controls.

On Day 28, the number of follicles in the LHRH-A-treated rats was significantly lower in most of the volume classes between 2 and 80 × 10^5 μm^3; the most pronounced reduction was found in follicles between 6 and 40 × 10^5 μm^3 in volume.

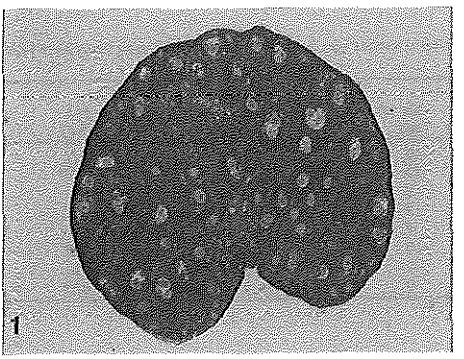
On the day of first estrus, the numbers of follicles from classes 6–8 × 10^5 μm^3 and greater were always significantly reduced in LHRH-A-treated rats compared with those in saline-treated rats. The number of type 3b follicles in LHRH-A-treated rats (62 ± 3) was not different from that in saline-treated controls (71 ± 4).

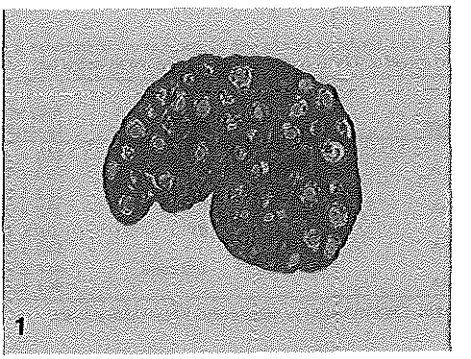
The total number of growing follicles present in the two ovaries was progressively and significantly ($p<0.01$) reduced in LHRH-A-treated rats, compared with control rats, on Days 22 and 28 and on the day of

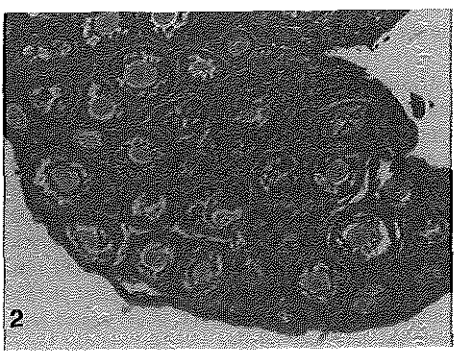
FIG. 3. Ovaries of 15-day-old control *(left)* and luteinizing hormone-releasing hormone antagonist-treated *(right)* rats. *1)* Note differences in ovarian size between control and LHRH-A-treated rats and the scarcely developed interstitial tissue in LHRH-A-treated rats (×40). *2)* Note irregular oocytes and spaces between oocytes and granulosa cells in LHRH-A-treated rats (×100). *3)* Note flat interstitial cells in LHRH-A-treated rats as well as irregular spaces between granulosa cells (×400).

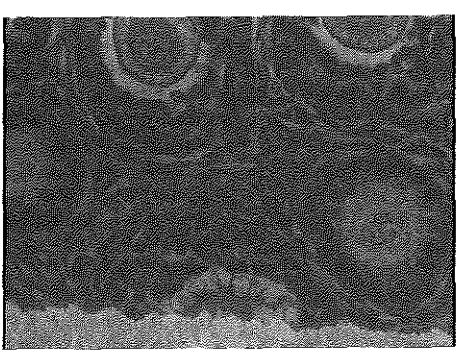
CONTROL

LHRH-A









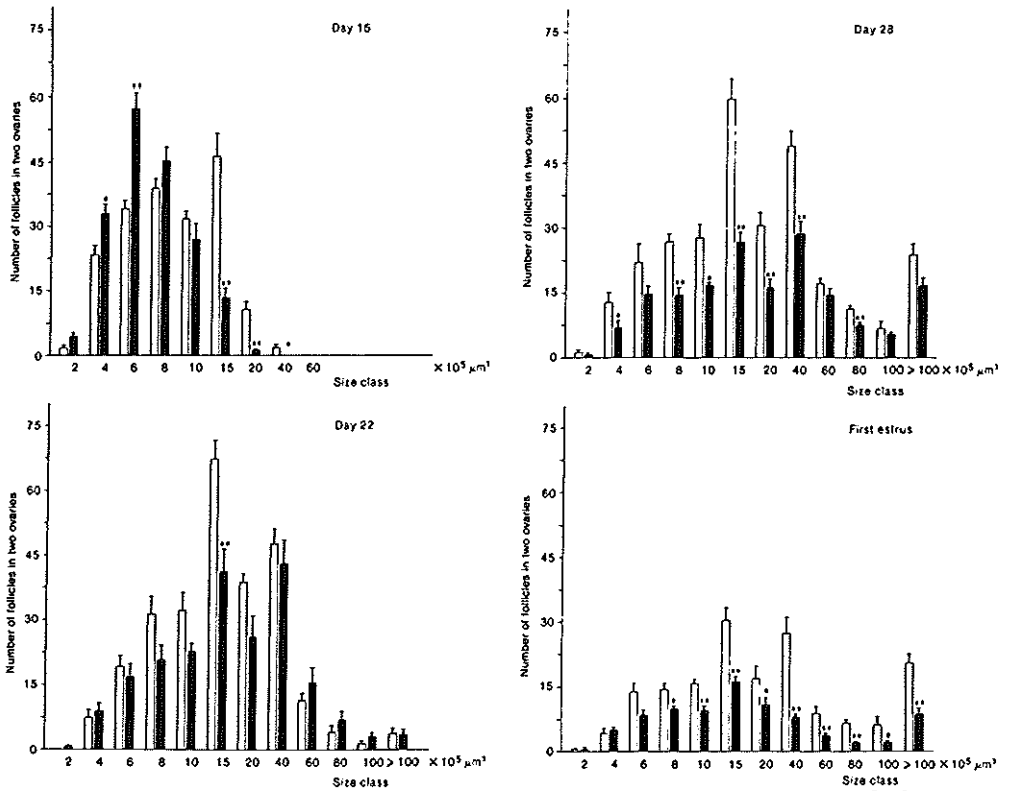


FIG. 4. Numbers of follicles of various size classes counted in two ovaries (e.g. *columns* marked δ contain follicles with a volume of 4–6 × 10^5 μm^3) at *15, 22,* and *28* days of age and on the *day* of *first estrus* in luteinizing hormone-releasing hormone antagonist (LHRH-A)-treated *(black bars)* and in saline-treated *(open bars)* rats. Each *bar* represents the mean ± SEM of 7–10 rats. *$p<0.05$ and **$p<0.01$, LHRH-A-treated vs. saline-treated rats, Mann-Whitney U-test.

first estrus (Table 1). On the day of first estrus, the total population of follicles measured had been reduced in LHRH-A-treated rats to about 50% of that in saline-treated rats (84 ± 4 vs. 175 ± 6).

Changes with age were seen in the total number of follicles, there was a significant increase in total number of follicles between 15 and 22 days of age, both in LHRH-A-treated ($p<0.05$) and in saline-treated rats ($p<0.01$). Maximal numbers of follicles were reached at 22–28 days of age in saline-treated rats and at 22 days of age in LHRH-A-treated rats; the reduction in number of follicles between 22 and 28 days in LHRH-A-treated rats was significant ($p<0.05$). In both saline-treated and in LHRH-A-treated rats, the total number of follicles had significantly decreased ($p<0.01$) on the day of first estrus compared with Day 28.

Counts of Follicles with a Volume≥100 × 10^5 μm^3

The numbers of follicles≥100 × 10^5 μm^3 (mostly antral follicles) subdivided into five volume classes and into the categories "healthy" or "atretic" are shown in Figure 5.

Follicles of these sizes, still absent from the ovaries on Day 15, seemed larger in number in the ovaries of

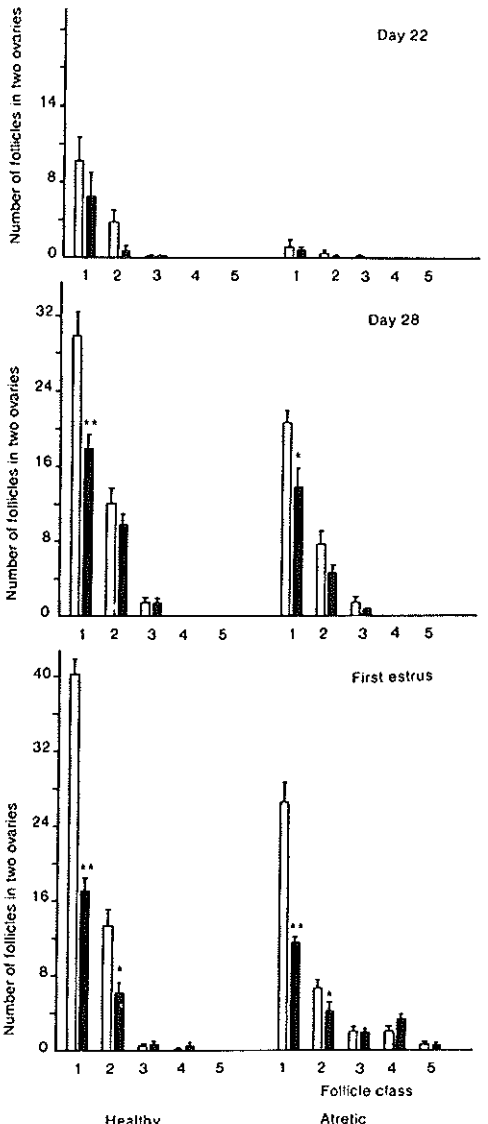


FIG. 5. Numbers of healthy and atretic follicles≥100 × 10^5 µm³ in two ovaries in the five size classes (class 1: 100–199; 2: 200–349; 3: 350–499; 4: 500–999; 5: ≥1000 × 10^5 µm³) at 22 and 28 days of age and on the day of first estrus in luteinizing hormone-releasing hormone antagonist (LHRH-A)-treated *(black bars)* and in saline-treated *(open bars)* rats. Each *bar* represents the mean ± SEM of 7–10 rats. *p<0.05 and **p<0.01, LHRH-A-treated vs. saline-treated rats, Mann-Whitney U-test.

22-day-old saline-treated rats than in LHRH-A-treated rats, but differences were not significant (Fig. 5, Table 2).

On Day 28, the number of healthy and atretic follicles in class 1 (100–199 × 10^5 µm³) was significantly lower (p<0.01 and p<0.05, respectively) in LHRH-A-treated rats than in saline-treated rats. The total numbers of healthy follicles and of atretic follicles, or the sum of both, were always significantly lower (p<0.01) in LHRH-A-treated rats than in saline-treated rats (Table 2), the difference in number being about 35%.

On the day of first estrus a similar picture was seen: the number of class 1 (p<0.01) and class 2 (p<0.05) follicles was significantly lower for both healthy and atretic follicles in LHRH-A-treated rats (Fig. 5). The total numbers of healthy or atretic follicles ≥100 × 10^5 µm³ in LHRH-A-treated rats were significantly lower (p<0.01) and amounted only about 50% of those in saline-treated rats (Table 2).

DISCUSSION

The LHRH-A-treatment effectively suppressed both FSH and LH concentrations over the period between 7 and 28 days of age, a result that is in agreement with earlier studies (van den Dungen, unpublished results). The treatment did not include the first week of life, the period during which gonadotropins have been reported to be essential for the development of ovarian FSH receptors (Smith and Ojeda, 1986). In a pilot experiment, rats received LHRH-A-treatment at 1, 4, and 7 days of age, and vaginal opening and first estrus occurred at an age and body weight not different from those in controls. At first estrus, there was a significant (p<0.01) difference in ovarian weight between LHRH-A-treated and control rats (31.9 ± 0.5, n = 8 vs. 37.0 ± 1.0 g, n = 7; unpublished results). Thus the decrease in ovarian weight was less severe than we found after LHRH-A-treatment from Day 6 on: about 30%. From the present work, a clear role emerges for the high concentrations of gonadotropic hormones present in the female rat by the end of the second week of life. After suppression of the peak concentrations of gonadotropins, rats were capable of ovulating normal numbers of ova at normally timed first ovulation, which is in agreement with previous studies (Uilenbroek and Arendsen de Wolff-Exalto, 1979). After LHRH-A treatment, a basic level of about 200 µg FSH/l always remained. This level presumably resulted from the au-

TABLE 2. Total numbers (mean ± SEM) of healthy and atretic follicles ≥100 × 10^5 μm^3 in the 2 ovaries of luteinizing hormone-releasing hormone antagonist (LHRH-A)-treated or saline-treated rats at various ages.

Treatment	Age (days)	No. of rats	No. of follicles		
			Healthy	Atretic	Total
Saline	22	7	14 ± 4	1.7 ± 1.2	16 ± 5
LHRH-A		9	7 ± 3	0.9 ± 0.5	8 ± 3
Saline	28	9	43 ± 3	30 ± 2	73 ± 4
LHRH-A		10	29 ± 1.8*	19 ± 2*	48 ± 3*
Saline	first estrus	9	54 ± 2	38 ± 3	92 ± 5
LHRH-A		9	24 ± 2*	21 ± 1.1*	46 ± 3*

*$p<0.01$, LHRH-A-treated vs. saline-treated, Mann-Whitney U-test.

tonomous, non-LHRH-stimulated FSH secretion (Schulling et al., 1984) and seemed to safeguard a basic follicular development leading to first ovulation. A similar low FSH level is normally reached only during late prepuberty (Meijs-Roelofs et al., 1982). However, the present data also showed that, after LHRH-A treatment, at the time of first estrus, the total population of growing follicles in the ovary was reduced to a mere 50% of the follicle population present in untreated rats. This seemed to be the result of a gradual process. After LHRH-A treatment, on Day 15, fewer follicles had grown into the larger volume classes (10–40 × 10^5 μm^3) than in control rats. Since the total numbers of follicles present were not different between groups, it seems that at this age follicle growth was merely delayed; however, irregularities in oocytes and in granulosa cell arrangement were seen, indicating a qualitative difference in the growing follicles that might lead to increased loss of follicles by atresia. No attempt was made to detect (early) atresia in the measurements of total follicle population in this study. Atresia in small and medium follicles is a relatively rapid process and involves changes in the oocyte nucleus at an early stage (Byskov, 1978). Therefore, since we used presence of the oocyte nucleus as a criterion for counting a follicle, it seemed sensible not to measure and count follicles in which a clear oocyte nucleus was absent.

The development of interstitial tissue seemed to lag in the LHRH-A treated rats, which may have added to the effect of diminished follicle growth by causing a reduction in ovarian weight. At 22 days of age, 7 days after the last LHRH-A injection, the morphological picture for both interstitial and follicular populations was largely restored, although serum FSH levels were still suppressed. Moreover, the total number of growing follicles had been reduced by about 20%. Due to the relatively large variations, only relative ovarian weights differed significantly between groups at this age. An even larger reduction, of about 40%, in total number of growing follicles and significantly reduced ovarian weights was seen at 28 days of age. Also, at the day of first estrus, a significant decrease in ovarian weight was seen in LHRH-A-treated rats, notwithstanding the equal numbers of corpora lutea present in the ovaries of LHRH-A-treated and control rats. This weight reduction probably was caused by the loss of about 50% of the growing follicles in LHRH-A-treated rats; lower follicle numbers were now found not only in nearly all preantral follicles (6–100 × 10^5 μm^3) but also in the total numbers of follicles ≥100 × 10^5 μm^3 (mostly antral). The more detailed study of antral follicles confirms this: no significant differences in numbers of antral follicles were seen on Day 22, but on Day 28, significantly less of the smallest antral follicles (class 1) were found in LHRH-A-treated rats. On the day of first estrus, significantly fewer class 1 and class 2 follicles were present. In the LHRH-A-treated rats, with their thoroughly reduced follicle population (to about 50%), a much lower number of follicles apparently was recruited to grow and reach the antral stage; at the same time, a lower number of these antral follicles became atretic, resulting in equal numbers of follicles reaching the ovulatory stage in LHRH-A-treated rats and in control rats.

The question arises whether a female rat reaching the stage of sexual maturity with an experimentally induced, significant reduction in follicle population will show deviations from the norm in terms of follicle recruitment, cycle length, numbers of ovulation, and length of the total reproductive period. Butcher (1985) reported that unilateral ovariectomy, and thus reduction of the number of growing follicles, during adulthood resulted in disturbance of subsequent estrous cycles and early acyclicity. It would be of interest to see whether

this also holds for rats with a prepubertally (experimentally) reduced population of growing follicles. An alternative possibility is suggested by the theory that follicle recruitment and maturation take place on the basis of follicle selection as described by Lacker et al. (1987). This would imply that a female rat, starting reproductive life with a much smaller follicle population size than normal, might still be capable of a cyclic period of normal length and of normal ovulation numbers, but with a greater spread in cycle length, by involving smaller numbers of follicles in the cyclic maturational processes, together with reduced follicular atresia. Indeed, the situation found at first estrus in our LHRH-A-treated rats may support this possibility. Normal numbers of ova ovulated, but lower numbers of both healthy and atretic small antral follicles were present in the ovaries.

The effects of LHRH-A treatment on ovarian morphology, i.e. irregularities in oocytes and differences in size and arrangement of granulosa cells as well as a less developed interstitial tissue, are in good agreement with those described earlier (Eshkol and Lunenfeld, 1972; Anderson et al., 1976), following treatment with an antiserum against gonadotropins. In the present study, we found that these morphological changes quickly disappeared: 1 wk after LHRH-A treatment was stopped, they were no longer clearly visible (Day 22). However, neither the size of the follicle population nor ovarian weight was restored.

It should be noted that the total numbers of follicles in the ovaries reported in the present study do not represent the absolute numbers of follicles present. Since follicles were measured in each fifth serial section of the ovary and sections were 10 μm thick, and since the nucleus was about 15 μm in diameter, a number of follicles were missed. (Multiplication of the numbers found with a factor of 2.5–5.0 would probably yield the absolute numbers.)

In conclusion, the present study reveals that in the female rat abolishing the early extremely high levels of gonadotropins–especially FSH–results in a 50% reduction of the population of growing follicles at the start of cyclic life. Whether this reduction is due mainly to loss of follicles by increased follicular atresia (as seen on Day 15), by a decrease in numbers of follicles entering the growing pool, or both is currently under study, as are consequences of the thorough reduction of numbers of growing follicles at puberty for later cyclic performance.

We conclude that the high levels of gonadotropins circulating early in life are of physiological significance for development of the population of growing follicles present in the ovary at pubertal age.

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Chapter 3

Prepubertal reduction of the ovarian follicle population by combined LH-releasing hormone antagonist treatment and unilateral ovariectomy influences follicle characteristics but not the ovulation rate at first oestrus

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Prepubertal reduction of the ovarian follicle population by combined LH-releasing hormone antagonist treatment and unilateral ovariectomy influences follicle characteristics but not the ovulation rate at first oestrus

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ABSTRACT

The effect on first ovulation of the massive reduction of the total pool of ovarian follicles during the infantile and late juvenile period was studied in rats. Treatment with an LH-releasing hormone antagonist (LHRH-A) during infancy (5 mg/kg body weight on days 6, 9, 12 and 15 of life) was combined with unilateral ovariectomy performed on either day 15 (early ULO) or 2–5 days before the expected day of first ovulation (late ULO). Rats were killed on the day of first or second oestrus, when blood was collected and the (remaining) ovaries were prepared for differential counting of follicles and corpora lutea. In addition, blood was sampled 8 h after ULO and the ovaries studied histologically in the group of rats which were unilaterally ovariectomized 2–5 days before first ovulation.

The time of first ovulation was not influenced by treatment with LHRH-A, early or late ULO, or a combination of LHRH-A treatment and ULO. Ovulation rate after LHRH-A treatment was decreased, but was still within the normal range in intact rats and in early ULO rats compared with saline-treated controls.

Serum FSH concentrations 8 h after ULO performed 2–5 days before first ovulation were similar in saline- and LHRH-A-treated rats (845 ± 59 and 801 ± 99 (S.E.M.) μg/l respectively) and had increased compared with intact controls (216 ± 15 μg/l).

Treatment with LHRH-A resulted in a reduction of more than 50% in healthy and atretic follicles, and late ULO reduced the number of healthy follicles even further. In saline-treated rats late ULO decreased the rate of atresia, but in LHRH-A-treated rats atresia was not reduced further by (late or early) ULO.

It is concluded that even after massive reduction of the pool of ovarian follicles by early LHRH-A treatment combined with late or early ULO, the timing of the first ovulation was normal and ovulation rates, although somewhat lower in some LHRH-A-treated rats, were within the normal range.
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INTRODUCTION

Ovulation rate in mammals is generally fairly constant and species-specific (Lipschütz, 1928; Greenwald, 1961; Welschen, 1972). Even after the loss of a large part of the total pool of ovarian follicles, e.g. after unilateral ovariectomy, a constant ovulation rate is maintained in both short-term and long-term experiments (Greenwald, 1961; Peppler & Greenwald, 1970; Welschen, 1972; Hirshfield, 1982; Meijs-Roelofs, Osman & Kramer, 1982).

A mathematical model was recently developed which takes into account regulatory mechanisms involved in maintaining constant ovulation rates under normal and a number of experimental conditions (Lacker, Beers, Meuli & Akin, 1987*a*,*b*). This model assumes independence of ovulatory numbers from the size of the total pool of growing ovarian follicles. It largely limits itself to the dynamics of antral follicles during the ovarian cycle and does not take into account prepubertal maturation of the total follicle population. It seemed possible that ovaries that undergo serious developmental disturbances during infancy would function differently in adulthood. In an earlier study we found that the total number of growing follicles present at the time of first ovulation

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in rats can be reduced by suppression of the high gonadotrophin levels normally present early in life (van Cappellen, Meijs-Roelofs, Kramer & van den Dungen, 1989); an antagonist against luteinizing hormone-releasing hormone (LHRH-A; Org 30276) was used for this purpose. In these LHRH-A-treated rats the number of corpora lutea as well as the timing of first ovulation were normal. Moreover, in adulthood the number and distribution of the ovarian follicles over the various size classes were not different from those in control rats. Thus, the induced long-term diminution of prepubertal follicle growth was compensated for and did not result in a permanent effect on adult ovarian function (Meijs-Roelofs, van Cappellen, van Leeuwen & Kramer, 1990).

A method often used to reduce the population of follicles by about 50% is unilateral ovariectomy (ULO). In both adult and prepubertal rats, ULO induces an increase in serum follicle-stimulating hormone (FSH) concentration within 6 to 8 h, and this causes a compensatory growth of, especially antral, follicles in the remaining ovary (Welschen & Dullaart, 1974; Butcher, 1977; Osman, Meijs-Roelofs & Kramer, 1982).

In the present study, a rigorous reduction of the population of growing follicles was achieved by combining the early LHRH-A treatment during infancy of the previous study (van Cappellen *et al.* 1989) with (1) the acute effect of ULO performed during the late juvenile period (late ULO) or (2) the long-term effect of ULO during the infantile period (early ULO). We investigated whether, under these circumstances of severe two-way reduction of the pool of growing follicles, the remaining ovary would, within a few days, be capable of compensatory follicle growth and ovulation and would thus still follow the model described by Lacker *et al.* (1987*a*).

MATERIALS AND METHODS

Immature female rats of our inbred Wistar substrain (R-Amsterdam) were used and kept in a controlled temperature (22–25 °C) with a light period from 05.00 to 19.00 h. Standard dry pellets and tap water were available *ad libitum*. The rats were weaned at 22 days of age and thereafter checked regularly for vaginal opening. In this strain of rats, the day of vaginal opening generally coincides with the day of first ovulation (Meijs-Roelofs, Kramer & Sander, 1983) and occurs around day 39 of age. Starting on the day of vaginal opening, daily vaginal smears were taken until the day of first or second ovulation when a fully cornified smear was found. Ovulation was confirmed by the presence of tubal ova and of corpora lutea in the ovaries.

Treatment

At 11.00 h on days 6, 9, 12 and 15 of age (day of birth counted as day 0), rats received an s.c. injection of either 500 μg LHRH-A/100 g body weight (Org 30276; Ac-DpClphe-DpClphe-DTrp-ser-tyr-DArg-leu-arg-pro-Dala-NH_2; Organon, Oss, The Netherlands) or 0·9% (w/v) NaCl as described previously (van Cappellen *et al.* 1989). In the first group of rats (saline-treated, $n=7$; LHRH-A-treated, $n=10$) the right ovary was removed 2–5 days before the day of expected first ovulation, between 34 and 40 days of age (late ULO). Blood was collected 8 h after late ULO. Rats were killed at first or second oestrus when the remaining ovary was removed and blood collected. Rats in which ULO had been performed on the day before first ovulation were excluded from the study. In the second group of rats the right ovary was removed at 15 days of age (early ULO) and rats were killed at first oestrus (saline-treated, $n=8$; LHRH-A-treated, $n=8$). At necropsy, blood was sampled and body, ovarian and uterine weights were recorded. With the exception of the ovaries removed at 15 days of age, all ovaries were fixed in Bouin's fluid for histological examination and counting of follicles.

Histology and counting of follicles

Serial sections (10 μm) of the ovaries, embedded in paraffin wax, were made and stained with haematoxylin and eosin. Follicle counts were made using the method of Boling, Blandau, Soderwall & Young (1941) as modified by Welschen (1973). All follicles with a volume $\geq 25 \times 10^5$ μm^3 (diameter ≥ 170 μm, with four granulosa cell layers or more, comparable to types 5^a and larger according to Pedersen & Peters, 1968) were measured in the section containing the nucleolus. Two perpendicular diameters were measured with a summagraphic MM 1201 Data Tablet, connected to a microcomputer. The volume was calculated by substituting the third diameter as the mean of the other two diameters. Follicles were divided into two volume classes: follicles with a volume of between 25×10^5 μm^3 and 100×10^5 μm^3 (diameter between 170 μm and 265 μm, corresponding with types 5^a and 5^b described by Pedersen & Peters, 1968) are called preantral follicles and follicles with a volume $\geq 100 \times 10^5$ μm^3 (types 6–7 according to Pedersen & Peters, 1968) are called antral follicles. All follicles which showed at least local pycnosis in the granulosa cell layers and often further signs of atresia were counted as atretic (Osman, 1985).

Hormone measurements

The concentration of FSH in serum samples was measured by radioimmunoassay, using anti-ovine FSH as

antiserum and rat FSH as tracer (Welschen, Osman, Dullaart *et al.* 1975). Measurements were made in 25 µl serum/tube, with a sensitivity of 4 ng/tube. Serum concentration of FSH are expressed as µg NIADDK-rat FSH RP-1/l. Samples were measured in duplicate. Intra- and interassay variabilities of the assay were 6 and 11% respectively.

Statistical analysis

All data were analysed by the Systat computer program (Systat Inc.). If two groups were compared, a Student's *t*-test was performed; if more than two groups were compared a one-way analysis of variance followed by Tukey's multiple comparison test was performed (Miller, 1985; Einot & Gabriel, 1975). Differences were regarded as significant when the two-tailed P value was $\leq 0{\cdot}05$.

RESULTS

The weight of the ovary removed at ULO shortly before first ovulation was lower ($P \leq 0{\cdot}01$) in LHRH-A-treated than in saline-treated rats ($6{\cdot}0 \pm 0.2$, $n = 10$ and $11{\cdot}3 \pm 0{\cdot}3$ mg, $n = 7$ respectively (means ± S.E.M.)). Serum FSH concentration 8 h after ULO were not different between saline- and LHRH-A-treated rats (845 ± 59, $n = 4$ and 801 ± 99 µg/l, $n = 8$ respectively) and were significantly higher than in sham-operated control rats (216 ± 15 µg/l, $n = 9$). Rats that were unilaterally ovariectomized shortly before first ovulation, reached first ovulation $2{\cdot}4 \pm 0{\cdot}2$ ($n = 7$) days after ULO (age at ULO $35{\cdot}7 \pm 0{\cdot}5$ days) in the saline-treated group, and $3{\cdot}5 \pm 0{\cdot}4$ ($n = 10$) (age at ULO $35{\cdot}2 \pm 0{\cdot}4$ days) after LHRH-A treatment. LHRH-A-treated rats often showed a pinhole type, i.e. a flat vaginal membrane with a tiny perforation, of vaginal opening preceding the day of first ovulation.

No differences were found between the ages and body weights at first ovulation in the groups treated with saline or LHRH-A (Table 1), or between uterine weights in these groups (data not shown). Although the number of corpora lutea (CL) was generally higher than seven (with the exception of one rat which ovulated six oocytes), numbers of CL were lower in LHRH-A-treated intact rats and in LHRH-A-treated rats with early ULO compared with their saline-treated controls ($P \leq 0{\cdot}05$). At first ovulation, ovarian weight was significantly ($P \leq 0{\cdot}01$) lower in LHRH-A-treated compared with saline-treated control rats which was the same for intact rats as for rats with late or early ULO (Table 1). Ovarian weights at first ovulation in the saline-treated early (27·2 mg) and late ULO rats (25·0 mg) were significantly ($P \leq 0{\cdot}01$) higher than the weight of one ovary of the saline-treated intact rats ($34{\cdot}7/2 = 17{\cdot}4 \pm 0{\cdot}8$ mg; Table 1). The same was true for the ovarian weights in early (17·6 mg) and late ULO LHRH-A-treated groups (15·2 mg) compared with those in intact LHRH-A-treated rats ($22{\cdot}3/2 = 11{\cdot}2 \pm 0{\cdot}3$ mg; $P \leq 0{\cdot}01$). Serum FSH concentrations at 11.00 h on the day of first ovulation were significantly ($P \leq 0{\cdot}01$) higher in the LHRH-A-treated rats with late ULO compared with saline-treated rats in the same group (Fig. 1).

After late ULO, the length of the cycle following first ovulation was significantly ($P \leq 0{\cdot}01$) longer in LHRH-A-treated than in saline-treated rats (Table 1). Also bodyweight at second oestrus was higher in

TABLE 1. Body and ovarian weights and number of corpora lutea (CL) on the day of first and second ovulation after treatment with LH-releasing hormone antagonist (LHRH-A) or saline on days 6, 9, 12 and 15 and unilateral ovariectomy (ULO) performed 2–5 days before first ovulation (late ULO) or at 15 days of age (early ULO). Data are means ± S.E.M. For intact rats the weight of the two ovaries is given

			At first ovulation				
	No. of rats	Time of ULO	Age (day)	Body weight (g)	Ovarian weight (mg)	No. of CL	Length of first cycle (day)
Treatment							
Saline	8		36·8 ± 0·7	92 ± 3	34·7 ± 1·5	10·5 ± 0·4	—
LHRH-A	7		35·3 ± 0·5	83 ± 3	22·3 ± 0·7**	9·0 ± 0·4*	—
Saline	7	Late	38·1 ± 0·7	94 ± 3	25·0 ± 0·5	10·6 ± 0·7	—
LHRH-A	10	Late	38·7 ± 0·4	89 ± 2	15·2 ± 0·3**	9·3 ± 0·6	—
Saline	8	Early	38·0 ± 1·0	93 ± 3	27·2 ± 0·5	9·7 ± 0·4	—
LHRH-A	8	Early	37·3 ± 1·0	88 ± 1	17·6 ± 0·3**	8·6 ± 0·2*	—
			At second ovulation				
Saline	7		46·3 ± 0·9	122 ± 3	54·7 ± 2·1	10·6 ± 0·6	7·9 ± 1·1
LHRH-A	11		47·3 ± 1·1	116 ± 4	42·4 ± 2·5**	10·9 ± 0·3	8·8 ± 1·2
Saline	6	Late	45·0 ± 0·4	109 ± 3	35·9 ± 1·5	10·7 ± 0·3	5·7 ± 0·3
LHRH-A	10	Late	48·9 ± 1·6	132 ± 5**	38·7 ± 2·3	10·5 ± 0·4	10·2 ± 1·2*

*$P \leq 0{\cdot}05$, **$P \leq 0{\cdot}01$ compared with saline treatment in the same group (Student's *t*-test).

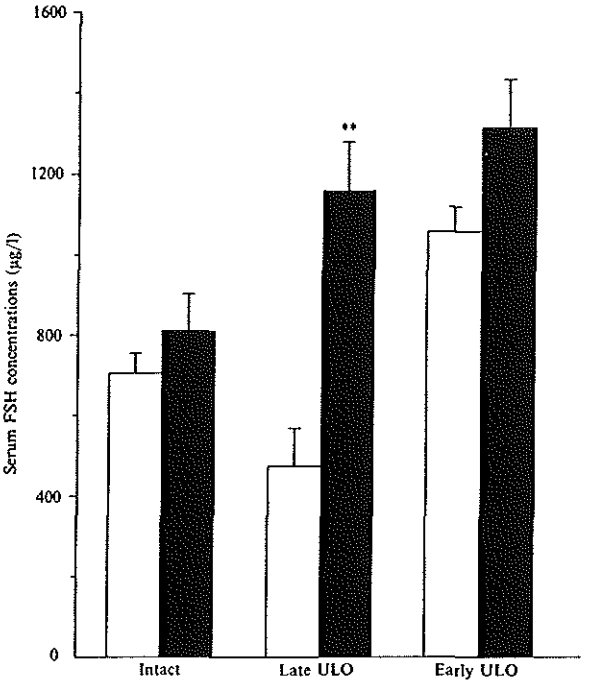

FIGURE 1. Mean ± S.E.M. serum FSH concentrations on the day of first ovulation (11.00 h) in rats that were in part subjected to unilateral ovariectomy (ULO) at different times (late and early ULO) and treated with saline (open bars) or LH-releasing hormone antagonist (LHRH-A; solid bars) on days 6, 9, 12 and 15 of age. $^{**}P \leq 0.01$ compared with the saline-treated control group (Student's *t*-test).

LHRH-A-treated rats compared with saline-treated rats after late ULO ($P \leq 0.01$). At second ovulation, ovarian weights in intact LHRH-A-treated rats were lower ($P \leq 0.01$) than in their saline-treated controls, but no difference was found between ovarian weights of LHRH-A- and saline-treated rats with late ULO (Table 1). The weight of one ovary at second ovulation in saline-treated intact rats (54·2/2 = 27·3 ± 1·0) was still lower than that in saline-treated rats with late ULO (35·9 mg; $P \leq 0.01$) as were

TABLE 2. Number of preantral and antral healthy and atretic follicles in one ovary 2–5 days before first oestrus after treatment with LH-releasing hormone antagonist (LHRH-A) or saline on days 6, 9, 12 and 15. Data are means ± S.E.M.

		Number of follicles			
		Healthy		Atretic	
Treatment	No. of rats	Preantral ($25–100 \times 10^5$ μm³)	Antral ($\geq 100 \times 10^5$ μm³)	Preantral ($25–100 \times 10^5$ μm³)	Antral ($\geq 100 \times 10^5$ μm³)
Saline	7	51·3 ± 4·2	24·7 ± 2·0	45·9 ± 3·1	26·3 ± 2·2
LHRH-A	10	19·2 ± 2·5**	12·3 ± 0·9**	17·9 ± 2·3**	11·2 ± 1·0**

**$P \leq 0.01$ compared with the saline-treated control group (Student's *t*-test).

TABLE 3. Number of preantral and antral healthy and atretic follicles at first oestrus after treatment with LH-releasing hormone antagonist (LHRH-A) or saline on days 6, 9, 12 and 15 and unilateral ovariectomy (ULO) performed 2–5 days before first ovulation (late ULO) or at 15 days of age (early ULO). Data are means ± S.E.M. For intact rats the number of follicles in two ovaries is given

			Number of follicles			
			Healthy		Atretic	
	No. of rats	Time of ULO	Preantral ($25–100 \times 10^5$ μm³)	Antral ($\geq 100 \times 10^5$ μm³)	Preantral ($25–100 \times 10^5$ μm³)	Antral ($\geq 100 \times 10^5$ μm³)
Treatment						
Saline*	7		$93{\cdot}6 \pm 9{\cdot}7^{a}$	$61{\cdot}1 \pm 4{\cdot}8^{a}$	$61{\cdot}0 \pm 4{\cdot}0^{a}$	$40{\cdot}4 \pm 3{\cdot}1^{a}$
LHRH-A*	6		$39{\cdot}7 \pm 1{\cdot}1^{bc}$	$30{\cdot}7 \pm 2{\cdot}8^{b}$	$33{\cdot}3 \pm 3{\cdot}5^{b}$	$13{\cdot}5 \pm 0{\cdot}9^{e}$
Saline	7	Late	$47{\cdot}6 \pm 2{\cdot}5^{b}$	$54{\cdot}9 \pm 2{\cdot}1^{a}$	$18{\cdot}0 \pm 2{\cdot}8^{c}$	$14{\cdot}4 \pm 1{\cdot}5^{bc}$
LHRH-A	10	Late	$17{\cdot}5 \pm 1{\cdot}5^{d}$	$14{\cdot}9 \pm 1{\cdot}8^{c}$	$12{\cdot}0 \pm 1{\cdot}7^{c}$	$11{\cdot}6 \pm 0{\cdot}6^{c}$
Saline	7	Early	$49{\cdot}1 \pm 5{\cdot}1^{b}$	$39{\cdot}9 \pm 3{\cdot}5^{b}$	$33{\cdot}7 \pm 5{\cdot}5^{b}$	$22{\cdot}7 \pm 3{\cdot}4^{b}$
LHRH-A	8	Early	$20{\cdot}4 \pm 1{\cdot}5^{cd}$	$16{\cdot}8 \pm 1{\cdot}5^{c}$	$16{\cdot}0 \pm 1{\cdot}4^{c}$	$16{\cdot}6 \pm 1{\cdot}3^{bc}$

Rows with different letters are significantly different ($P \leq 0{\cdot}05$) (Tukey's multiple comparison test).
*Number of follicles/two ovaries.

those of LHRH-A-treated intact rats ($42{\cdot}4/2 = 21{\cdot}2 \pm 1{\cdot}3$ mg) compared with LHRH-A-treated rats with late ULO (38·7 mg; $P \leq 0{\cdot}01$; Table 1). The number of CL was not different between the groups at second ovulation.

Follicle counts

In the ovaries removed a few days before first ovulation (late ULO), the number of healthy and atretic preantral and antral follicles of LHRH-A-treated rats was lower than those of saline-treated controls ($P \leq 0{\cdot}01$; Table 2) and generally amounted to less than 50%.

Late ULO in saline-treated rats resulted in a significant ($P \leq 0{\cdot}05$) diminution of the number of preantral healthy follicles in one ovary to about 50% of the number found in two ovaries in the saline-treated intact rats at first ovulation (Table 3). The number of preantral and antral atretic follicles in one ovary in the late ULO saline-treated rats was reduced ($P \leq 0{\cdot}05$) to about 35% of that in two ovaries in the intact saline-treated rats. The number of healthy antral follicles in two ovaries in the saline-treated intact rats was not different from the number of follicles in one ovary of the late ULO saline-treated rats.

Early ULO in the saline-treated rats resulted in a reduction ($P \leq 0{\cdot}05$) of the numbers of preantral and antral healthy and atretic follicles in one ovary to about 50%, compared with numbers in the two ovaries of saline-treated intact rats (Table 3).

LHRH-A treatment in intact rats caused a decrease ($P \leq 0{\cdot}05$) in the number of preantral and antral healthy and atretic follicles compared with saline-treated intact rats (Table 3).

Combining LHRH-A treatment with late ULO resulted in a reduction ($P \leq 0{\cdot}05$) of the number of preantral and antral healthy follicles and of the number of preantral atretic follicles in one ovary compared with those in the two ovaries in intact rats after LHRH-A treatment (Table 3). No difference was found between the number of atretic antral follicles in these two groups. After LHRH-A treatment and early ULO the numbers of antral healthy and preantral atretic follicles in one ovary were reduced ($P \leq 0{\cdot}05$) compared with that in the two ovaries in LHRH-A-treated intact rats. No differences were found between the LHRH-A-treated early and late ULO groups.

DISCUSSION

The present study addressed the question of whether prepubertal reduction of the follicles population to about 25% of the normal number would still allow for normal first ovulation in terms of timing and ovulation rate.

Two clearly different experimental situations were tested: (1) a double reduction in the number of follicles by LHRH-A treatment and ULO during the infantile period, long before first ovulation (early ULO) and (2) a first reduction in follicle number by LHRH-A treatment, resulting in a 50% reduced population shortly before first ovulation, and an acute second reduction 2–5 days before first ovulation (late ULO).

Both in the long-term (early ULO) and in the short-term (late ULO) situation the flexibility of the follicle population appeared to result in normally timed first ovulation with a normal or near normal (within the physiological range) ovulation rate.

Follicle dynamics, during the days preceding this ovulation, must be clearly different in the various experimental situations, because different numbers of follicles 2–5 days before first oestrus led to the same

or a slightly decreased number of ova shed a few days later. To examine these differences, the population of preantral follicles (volume 25–100 × 10^5 μm^3; diameter between 170 and 265 μm) with four or more granulosa cell layers was also studied. Thus, possible changes in inflow of preantral follicles into the antral class could be detected.

Ovaries taken out 2–5 days before first ovulation showed that LHRH-A treatment early in life not only reduced the population of healthy preantral and antral follicles, but also reduced the number of atretic follicles to a similar degree, i.e. to about 45%. Follicles becoming atretic take 2–5 days before they totally collapse (Byskov, 1979; Osman, 1985), and the number of atretic follicles therefore provides information about the rate of atresia during the last few days before surgery. It should be realized that in advanced stages of atresia, because of shrinkage, follicles will no longer be included in the counts (Byskov, 1979; Osman, 1985) or small antral follicles becoming atretic may be counted as preantral atretic stages because of reduced size. These errors were not compensated for in the countings.

In agreement with earlier studies (Welschen & Dullaart, 1974; Butcher, 1977), ULO shortly before first ovulation induced an increase in serum FSH concentration 8 h later, in both saline- and LHRH-A-treated rats. No difference was found in the FSH serum concentration between saline- and LHRH-A-treated rats 8 h after ULO, suggesting that there were no direct effects of the LHRH-A any more. In an earlier study, suppression of FSH and LH by LHRH-A was no longer found at day 34 (van Cappellen *et al.* 1989). As observed previously, in both adult and prepubertal rats, an increase in serum FSH concentration causes an increase in the number of healthy (antral) follicles (volume ≥ 100 × 10^5 μm^3; diameter ≥ 265 μm; Osman *et al.* 1982) which have entered the final accelerated growth phase in the remaining ovary (Pedersen, 1970; Welschen, 1973; Hirschfield, 1982) and which determine the number of ovulations at the end of the cycle.

Compensatory ovulation in the remaining ovary may then be achieved by (a combination of) two mechanisms: (1) reduction of the rate of atresia of antral follicles present (Greenwald, 1961; Welschen, Dullaart & de Jong, 1978; Hirschfield, 1982; Gosden, Telfer, Faddy & Brook, 1989) or (2) recruitment of an additional number of small antral follicles (Peppler & Greenwald, 1970; Osman *et al.* 1982; Meijs-Roelofs, Kramer, Osman & Sander, 1984).

ULO 2–5 days before first ovulation (late ULO) in saline-treated rats caused a reduction of atresia of preantral and antral follicles in the remaining ovary to 35% of the number of such follicles in the two ovaries of saline-treated intact rats, and the number of healthy antral follicles at first oestrus was not different from that in the two ovaries of intact rats. The reduction in the number of healthy preantral follicles did not exceed 50%, so no indication of increased inflow from preantral to antral follicle stages was found at first oestrus. It should be realized that the average time it takes a preantral follicle to grow from four granulosa cell layers (volume ≥ 25 × 10^5 μm^3, type 5[a], according to Pedersen & Peters, 1968) to an ovulatable size is about 8·5 days in untreated mice and rats (Pedersen, 1969; Hage, Groen-Klevant & Welschen, 1978).

In an earlier study it was found that the LHRH-A-treated rats had a normal population of primordial and small follicles with a volume < 6 × 10^5 μm^3, diameter < 105 μm (Meijs-Roelofs *et al.* 1990), whereas this population is obviously halved by early ULO. This main reduction of the population of the smallest follicles did not have a significant influence on the number of growing follicles with a volume ≥ 25 × 10^5 μm^3 present at first ovulation. This is clearly in contrast with earlier findings in cyclic mice, where a linear relationship between the number of non-growing and growing follicles was found (Krarup, Pedersen & Faber, 1969). A possible explanation for this discrepancy is the fact that, in the present study, exclusively prepubertal rats were studied, whereas Krarup *et al.* (1969) used mice of all ages.

Combining LHRH-A treatment with late or early ULO resulted in a further reduction in the number of healthy preantral and antral follicles, but atresia was only reduced in the preantral follicles of LHRH-A-treated rats after early ULO compared with the saline-treated controls after late or early ULO. This suggests that the number of atretic follicles cannot be reduced any further by this combined treatment. Probably the number of atretic follicles is not directly related to the number of healthy follicles when the number of healthy follicles becomes very low. In this situation, the percentage of preantral plus antral follicles that are atretic increases compared with late and early ULO saline-treated control rats. Notwithstanding the low numbers of healthy antral follicles recruited at first oestrus, a normal ovulation rate at second oestrus could still be achieved, as could be observed in LHRH-A-treated rats with late ULO. Probably LHRH-A-treated late ULO rats can, by reducing the inflow of follicles into the antral stage, use their follicles much more economically than intact rats.

The low number of growing follicles present shortly before ovulation, in the ovaries of late ULO LHRH-A-treated rats (Table 2), was sufficient to achieve a normal number of ovulations. This phenomenon could be predicted from the model of Lacker *et al.* (1987*a*), where a normal number of ovulating oocytes even after a severe reduction of the interacting population of follicles (i.e. antral follicles that secrete oestradiol) are generated. According to this model, changes

might be expected in the timing of ovulation, but these were not observed at first ovulation in the present study.

In contrast to a previous study (van Cappellen *et al.* 1989), a somewhat lower number of CL at first ovulation was found in some LHRH-A-treated rats, but the number of CL was still within the physiological range (7–11; Meijs-Roelofs, Kramer & Osman, 1985).

The low ovarian weights after LHRH-A treatment may be explained by the smaller number of follicles present in the ovaries. The increase in weight of the remaining ovary in ULO rats, observed after first and second ovulation, was probably due to the larger (doubled) number of CL in this ovary. After the second ovulation, a difference in ovarian weights between saline- and LHRH-A-treated rats subjected to (late) ULO was no longer seen. At that time two full sets of CL were present in the ovaries, which probably had a predominant influence on ovarian weight. The difference in bodyweight between saline-treated and LHRH-A-treated late ULO rats at second oestrus could be caused by the longer first cycle.

Differences in the FSH serum concentration between saline-, ULO- and LHRH-A-treated rats at first ovulation might explain differences in recruitment of antral follicles and could be an indication that the responses of the FSH feedback system are augmented by ULO and by the LHRH-A treatment in the infantile period. Alternatively, a difference in timing of the secondary FSH peak under the various experimental conditions could also explain our observation of differences in FSH concentration (see also Butcher, 1977). A direct effect of LHRH-A on FSH secretion is not very likely because this treatment ended more than 20 days earlier.

It may be concluded that a normally timed first ovulation with a normal or slightly reduced number of ova shed can be accomplished with a quarter of the normal number of growing follicles. The pool of primordial and small slowly growing follicles (volume $< 6 \times 10^5\ \mu m^3$; diameter $< 105\ \mu m$) did not influence the number of healthy antral follicles present at first oestrus.

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Chapter 4

Model of antral follicle dynamics during the 5-day cycle in rats based on measurement of antral follicle inflow

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Model of antral follicle dynamics during the 5-day cycle in rats based on measurement of antral follicle inflow

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Antral follicles were counted in ovaries from young adult Wistar rats, collected on the 5 days of the ovarian cycle. Follicles were classified as healthy, early atretic or late atretic and divided into five volume classes. From these data, a model was developed in which the inflow of healthy follicles into the various size classes was quantified. This model describes the follicle dynamics during a normal 5-day cycle. It was concluded that the stage of early atresia takes between 20 and 24 h. The inflow of follicles into the antral stage (volume $\geq 100 \times 10^5$ μm^3) was continuous but not constant. The highest inflow was found during pro-oestrus and oestrus, at about the time of the first and second FSH surge. The total inflow during each cycle was about 120 follicles of which only 10% ovulated. These ovulating follicles were recruited during the previous pro-oestrus and oestrus. Follicle selection took place in volume classes 1 and 2 (volume 100–350 $\times 10^5$ μm^3) during oestrus and dioestrus 1. At dioestrus 2, the follicles that will ovulate have been selected and can be recognized on the basis of their bigger size.

Introduction

Recent developments in *in situ* hybridization and immunohistochemical techniques make it possible to study the regulation of a large number of endocrine and paracrine factors in the ovary. It is important to have a model of follicle dynamics during the ovarian cycle so that the results obtained with these new techniques can be interpreted correctly. It is not known, for instance, whether growing follicles continuously enter the antral follicle stage throughout the cycle (Peters and Levy, 1966; Pedersen, 1970; Hirshfield and DePaola, 1981; Hirshfield, 1988) or whether there is a resting pool of preantral follicles from which follicles are recruited only during oestrus (Greenwald, 1973, 1974; Osman, 1985). This question is particularly important in relation to treatments aiming at increasing the number of ovulatory follicles. For a basic understanding of ovulatory quota reached at superovulation it is of interest to know the total number of follicles involved in cyclic follicle development. Moreover, knowing the developmental path of the follicles destined to ovulate may be of great help in interpreting results of histological and *in vitro* studies. In this study a model will be developed in which the inflow of antral follicles is quantified. This model should provide a tool to discriminate between the effect of increased/decreased follicle inflow and decreased/increased atresia in *in vivo* experiments. The model, although derived from static data, presents a dynamic picture of antral follicle development. In this study we also try to fit isolated statements about follicle dynamics, found in the literature, into one model.

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Materials and Methods

The histological material previously used and the follicle counts made earlier (Osman, 1985) served as a basis for the development of a model of inflow into the antral follicle stages.

Adult female rats of our inbred Wistar substrain (R-Amsterdam) were used and kept in a controlled temperature (22–25°C) with lights on from 05.00 to 19.00 h. Standard dry pellets and tap water were available *ad libitum*. Vaginal smears were taken daily at 10.00 h. The first day after oestrus when an influx of leucocytes became visible in the vaginal smears was designated as dioestrus 1. Only animals displaying at least two consecutive 5-day cycles were used.

Groups of 6–7 animals were killed on each day of the cycle, at 10.00 h and 17.00 h with an overdose of ether. The ovaries were dissected out, fixed in Bouin's fluid and embedded in paraffin wax. Serial sections of 10 µm were stained with haematoxylin and eosin.

The number of follicles was counted in one ovary per animal. All follicles with a volume $\geq 100 \times 10^5$ μm^3 (diameter ≥ 265 µm) were measured in the section containing the nucleolus of the oocyte to ensure that all follicles were measured only once. These follicles were all at the antral stage of development. Follicles were divided in 5 volume classes: class 1, 100–200 $\times 10^5$ μm^3 diameter 265–350 µm); class 2, 200–350 $\times 10^5$ μm^3 (diameter 350–400 µm); class 3, 350–500 $\times 10^5$ μm^3 (diameter 400–450 µm); class 4, 500–1000 $\times 10^5$ μm^3 (diameter 450–575 µm) and class 5 $\geq 1000 \times 10^5$ μm^3 (diameter ≥ 575 µm). All follicles were classified as healthy, early atretic or late atretic. A follicle was regarded as early atretic when there was at least localized pycnosis in the granulosa cell layer, but the nucleolus

Table 1. Number of healthy, (early atretic) and [late atretic] follicles in one ovary at 10 h in the morning of the various days of the ovarian cycle of rats

Volume class ($\times 10^5$ µm^3)	Oestrus	Dioestrus 1	Dioestrus 2	Dioestrus 3	Pro-oestrus
5	0.0	0.0	0.0	3.5 ± 0.9	5.6 ± 0.8
(≥ 1000)	(0.5 ± 0.3)	(0.0)	(0.0)	(0.3 ± 0.2)	(0.3 ± 0.2)
	[0.2 ± 0.2]	[0.0]	[0.0]	[0.0]	[0.0]
4	0.0	1.3 ± 0.6	5.7 ± 1.0	3.2 ± 0.8	0.3 ± 0.2
(500–1000)	(0.5 ± 0.3)	(0.2 ± 0.2)	(0.2 ± 0.2)	(1.3 ± 0.3)	(1.0 ± 0.4)
	[0.0]	[0.0]	[0.2 ± 0.2]	[0.0]	[0.3 ± 0.2]
3	0.3 ± 0.2	4.3 ± 0.7	3.2 ± 1.1	0.0	0.0
(350–500)	(0.0)	(0.7 ± 0.3)	(1.8 ± 0.8)	(1.5 ± 0.3)	(0.0)
	[0.3 ± 0.2]	[0.0]	[0.2 ± 0.2]	[0.3 ± 0.2]	[0.9 ± 0.3]
2	8.3 ± 0.7	14.7 ± 0.7	5.8 ± 1.4	0.3 ± 0.2	0.3 ± 0.2
(200–350)	(1.5 ± 1.0)	(5.0 ± 1.3)	(5.5 ± 0.7)	(5.2 ± 0.9)	(1.3 ± 0.4)
	[2.8 ± 0.9]	[0.2 ± 0.2]	[3.3 ± 1.1]	[3.3 ± 0.9]	[3.0 ± 1.0]
1	19.7 ± 1.5	11.3 ± 1.9	9.3 ± 1.3	5.8 ± 0.8	8.4 ± 1.6
(100–200)	(2.3 ± 0.9)	(6.3 ± 1.8)	(5.2 ± 0.6)	(7.8 ± 1.2)	(6.1 ± 2.1)
	[11.2 ± 1.0]	[2.7 ± 1.0]	[8.3 ± 1.6]	[12.0 ± 1.6]	[9.4 ± 1.8]
Number of rats	6	6	6	6	7

Values are means ± SEM.

was still visible. Late atresia was defined by the presence of pycnosis all over the granulosa cell wall and changes of the oocyte such as breakdown of the nuclear membrane with or without formation of a maturation spindle, and oocyte fragmentation.

Results

During the cycle a daily change was found in the number of antral follicles in the different volume classes of healthy follicles (Table 1). Class 1 follicles were present throughout the cycle and reached highest numbers at oestrus and lowest numbers at dioestrus 3. Large numbers of class 2 follicles were present at oestrus, dioestrus 1 and dioestrus 2. At dioestrus 3 and pro-oestrus there were few class 2 follicles. Class 3 follicles were nearly exclusively present at dioestrus 1 and 2 and class 4 follicles at dioestrus 1, 2 and 3. Class 5 follicles were seen only at the end of the cycle (dioestrus 3 and pro-oestrus).

Early atresia was infrequent in all volume classes (but not always lowest) at oestrus and tended to increase to maximum values during dioestrus (Table 1). Late atresia did not show much variation except for the low total number at dioestrus 1. Late atresia on all days is highest in volume class 1 (Table 1).

During the cycle, follicles are growing through the different follicle classes and finally ovulate or become atretic. By counting the number of follicles in histological sections the number of healthy follicles at 10.00 h and 17.00 h was known. To calculate the inflow of follicles from one class into the other, it is necessary to know how many follicles left a healthy follicle class by early atresia between two time points. For this it is necessary to know how long a follicle stays at the stage of early atresia as well as the number of early atretic follicles at a time point. From the follicle counts an indication of the duration of early atresia could be found. The rise in the number of early atretic follicles between oestrus and dioestrus 1 (from 4.8 ± 1.6 to 12.2 ± 1.7, Table 1) is followed 24 h later by a rise in the number of late atretic follicles (from 2.8 ± 0.9 to 12.0 ± 1.8, Table 1), indicating that early atresia takes about 24 h.

This value of 24 h was used to construct a first model in which the inflow of follicles was calculated. Only the numbers of follicles counted in the rats at 10.00 h were used.

On dioestrus 2, no class 5 follicles were seen. On dioestrus 3, 3.5 healthy and 0.3 early atretic follicles were counted: 3.5 + 0.3 = 3.8 follicles must therefore have entered volume class 5 between dioestrus 2 and dioestrus 3. At pro-oestrus, 5.6 healthy and 0.3 early atretic class 5 follicles were counted. The 0.3 early atretic follicles differed from the 0.3 early atretic follicles of dioestrus 3: 5.6 + 0.3 − 3.5 (3.5 follicles were already present the day before) = 2.4 follicles must therefore have entered class 5 over the past 24 h.

In the same way the number of follicles that entered class 4 from oestrus to dioestrus 1 and from dioestrus 1 to dioestrus 2 could be calculated (Fig. 1) using the formula:

Inflow = number of healthy follicles + number of early atretic follicles − number of healthy follicles present on previous day

Between dioestrus 2 and dioestrus 3 healthy follicles leave class 4 by atresia as well as by growing, that is developing into the bigger follicles of class 5. The formula has to be changed to:

Inflow = number of healthy follicles + number of early atretic follicles + inflow to bigger size class − number of healthy follicles present on previous day

In this way the inflow into class 4 between dioestrus 2 and dioestrus 3 could be calculated as 3.2 + 1.3 + 3.8 − 5.7 = 2.6. Repeating this calculation, inflow values for the five volume classes could be calculated throughout the cycle (Fig. 1).

The sum of all follicles entering class 1 during the 5 days of the cycle represents the number of follicles involved in antral

Table 2. Inflow of follicles from one follicle class to another during the 5-day cycle of the rat. Models were calculated separately for the rats killed at 10 and at 17 h

Follicle volume class	Factor[a]	Time[b] (h)	Inflow of follicles in 24 h of rats killed at 10 h						Inflow of follicles in 24 h of rats killed at 17 h					
			OE	D1	D2	D3	PO	Inflow total	OE	D1	D2	D3	PO	Inflow total
5	0.2	120		0.0	0.0	3.6	2.2	10.8		0.0	0.2	3.2	1.1	4.5
4	0.2	120	−0.2	1.3	4.4	1.4	−0.5	6.4	0.0	1.1	3.9	0.6	−0.4	5.2
3	0.2	120	0.1	5.4	3.7	−1.5	−0.5	7.2	1.2	5.2	2.1	−1.6	−0.6	6.3
2	0.2	120	8.4	12.8	−4.1	−6.0	−0.2	10.9	14.3	5.7	−5.7	−3.2	−0.9	10.2
1	0.2	120	20.2	5.7	−5.1	−7.9	3.6	16.5	26.7	−1.1	−7.5	−1.7	0.3	16.7
5	0.5	48		0.0	0.0	3.7	2.2	10.9		0.0	0.2	3.2	1.1	4.5
4	0.5	48	0.0	1.4	4.5	1.9	−0.2	7.6	0.1	1.1	3.9	0.9	−0.3	5.7
3	0.5	48	0.3	5.7	4.3	−0.5	−0.2	9.6	1.3	5.4	2.6	−1.0	−0.4	7.9
2	0.5	48	9.1	14.6	−1.8	−3.4	0.4	18.9	14.5	7.0	−4.2	−1.1	−0.4	15.8
1	0.5	48	21.6	9.4	−1.2	−3.0	6.1	32.9	27.2	1.7	−5.0	2.0	2.7	28.6
5	0.67	36		0.0	0.0	3.7	2.3	11.0		0.0	0.2	3.2	1.1	4.5
4	0.67	36	0.0	1.4	4.5	2.1	0.1	8.1	0.2	1.1	3.9	1.2	−0.2	6.2
3	0.67	36	0.3	5.8	4.6	−0.1	0.1	10.7	1.4	5.4	3.0	−0.4	−0.2	9.2
2	0.67	36	9.3	15.6	−0.6	−2.1	1.0	23.2	14.7	7.7	−3.1	0.4	0.0	19.7
1	0.67	36	22.2	11.4	0.9	−0.4	7.7	41.8	27.6	3.4	−3.2	4.5	4.4	36.7
5	1	24		0.0	0.0	3.8	2.4	11.2		0.0	0.2	3.2	1.2	4.6
4	1	24	0.2	1.5	4.6	2.6	0.5	9.4	0.4	1.1	3.9	1.6	0.0	7.0
3	1	24	0.5	6.2	5.3	0.9	0.5	13.4	1.6	5.6	3.7	0.4	0.1	11.4
2	1	24	10.0	17.6	1.9	0.6	1.8	31.9	15.0	9.3	−1.1	3.1	0.7	27.0
1	1	24	23.6	15.5	5.1	4.9	10.5	59.6	28.4	7.0	0.2	9.2	7.7	52.5
5	1.2	20		0.0	0.0	3.9	2.4	11.3		0.0	0.2	3.2	1.2	4.6
4	1.2	20	0.3	1.5	4.6	3.0	0.7	10.1	0.5	1.1	3.9	1.9	0.1	7.5
3	1.2	20	0.6	6.3	5.7	1.6	0.7	14.9	1.7	5.7	4.1	0.9	0.3	12.7
2	1.2	20	10.4	18.7	3.4	2.3	2.2	37.0	15.2	10.3	0.1	4.8	1.1	31.5
1	1.2	20	24.5	17.9	7.6	8.2	12.2	70.4	28.9	9.2	2.2	12.2	9.7	62.2
5	1.5	16		0.0	0.0	4.0	2.5	11.5		0.0	0.2	3.2	1.3	4.7
4	1.5	16	0.5	1.6	4.7	3.5	1.1	11.4	0.7	1.1	3.9	2.3	0.3	8.3
3	1.5	16	0.8	6.6	6.4	2.6	1.1	17.5	1.9	5.9	4.8	1.7	0.6	14.9
2	1.5	16	11.1	20.5	5.8	4.9	3.0	45.3	15.5	11.8	2.0	7.3	1.8	38.4
1	1.5	16	25.9	21.6	11.5	13.1	14.8	86.9	29.6	12.5	5.4	16.5	12.7	76.7

[a]Factor to multiply the number of early atretic follicles by to simulate an early atretic stage which takes more (factor < 1) or less (factor > 1) than 24 h.
[b]Simulated time of the duration of early atresia.
OE: oestrus; D1: dioestrus 1; D2: dioestrus 2; D3: dioestrus 3; PO: pro-oestrus.

follicle dynamics leading towards ovulation during that cycle. This sum amounts to 59.6 (15.5 + 5.1 + 4.9 + 10.5 + 23.6) follicles per ovary (Fig. 1). During one cycle in the rat strain used about 120 follicles, therefore, grow into the antral follicle classes $\geq 100 \times 10^5\ \mu m^3$ per two ovaries. Only about 10% of this number will ovulate.

There was a continuous inflow of follicles into class 1 (that of the smallest antral) follicles, but not at a constant rate. The inflow from pro-oestrus at 10.00 h to oestrus at 10.00 h is very high (23.6 follicles in 24 h) and this inflow remains high between oestrus at 10.00 h and dioestrus 1 at 10.00 h (15.5 follicles in 24 h). Thus during the periovulatory period, the time of the first and secondary FSH peak, inflow was highest.

With the model (Fig. 1) it was possible to track down the path of follicles that ovulate. Follicles that eventually ovulate (class 5 follicles) have entered class 5 during dioestrus 3 and pro-oestrus or, referring to the procedure of follicle counting, between dioestrus 2 at 10.00 h and pro-oestrus at 10.00 h. These follicles were class 4 follicles during dioestrus 2 and dioestrus 3, class 3 follicles during dioestrus 1 and dioestrus 2, class 2 follicles during oestrus and dioestrus 1 and class 1 follicles during oestrus and pro-oestrus of the preceding cycle. Thus we could trace the time-track of the follicles destined to ovulate, including the moment of their recruitment (i.e. inflow into the antral class 1) which was during pro-oestrus and oestrus and the moment they were selected (i.e. selection to continued growth up to ovulatory size instead of becoming atretic) which was during the days of oestrus and dioestrus 1.

The model was also developed from the follicle counts made in a group of rats killed at 17.00 h (Fig. 2). Follicle dynamics did not show much difference compared with those of the group of rats killed at 10.00 h (Fig. 1).

Once the type and number of follicles from which the ovulating follicles would emerge were known for every day of the cycle, it seemed of equal interest to characterize follicles which will become atretic. In an attempt to achieve this, follicles were

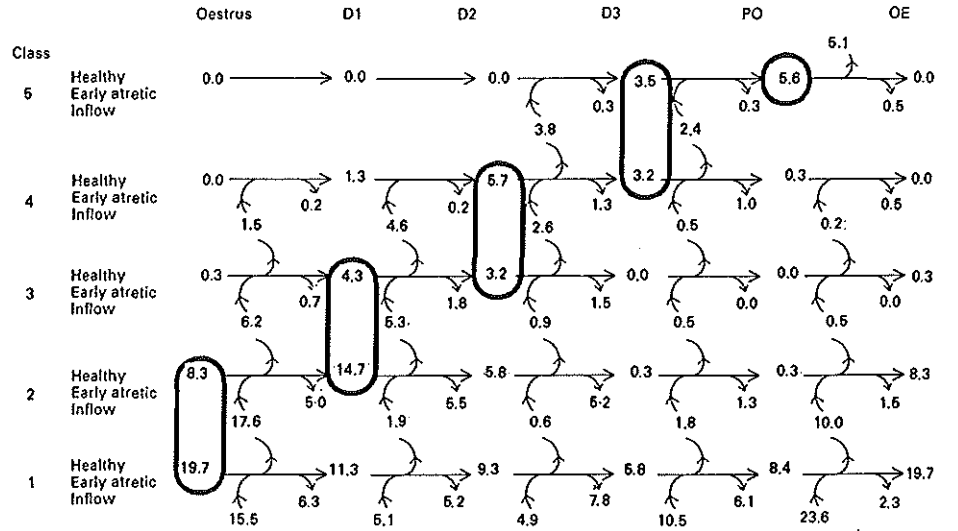

Fig. 1. Inflow of antral follicles in one ovary into the different volume classes during the cycle of rats. Upward arrow: number of follicles entering a class (inflow). Downward arrow: number of early atretic follicles. Numbers of healthy and atretic follicles were counted at 10.00 h in the morning. The inflow data are based on an early atretic period of 24 h. Numbers surrounded by an ovoid indicate the type of follicle containing those that will ovulate at pro-oestrus. OE: oestrus; D1: dioestrus 1, D2: dioestrus 2, D3: dioestrus 3, PO: pro-oestrus.

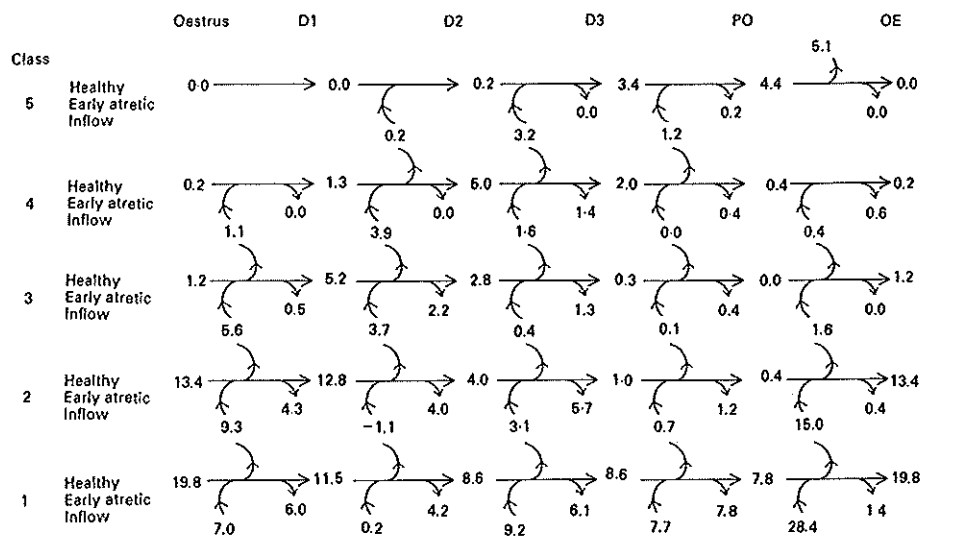

Fig. 2. Inflow of antral follicles in one ovary into the different volume classes during the cycle of rats. Upward arrow: number of follicles entering a class (inflow). Downward arrow: number of early atretic follicles. Numbers of healthy and atretic follicles were counted at 17.00 h. The stage of early atresia was assumed to take 24 h. OE: oestrus; D1: dioestrus 1; D2: dioestrus 2; D3: dioestrus 3; PO: pro-oestrus.

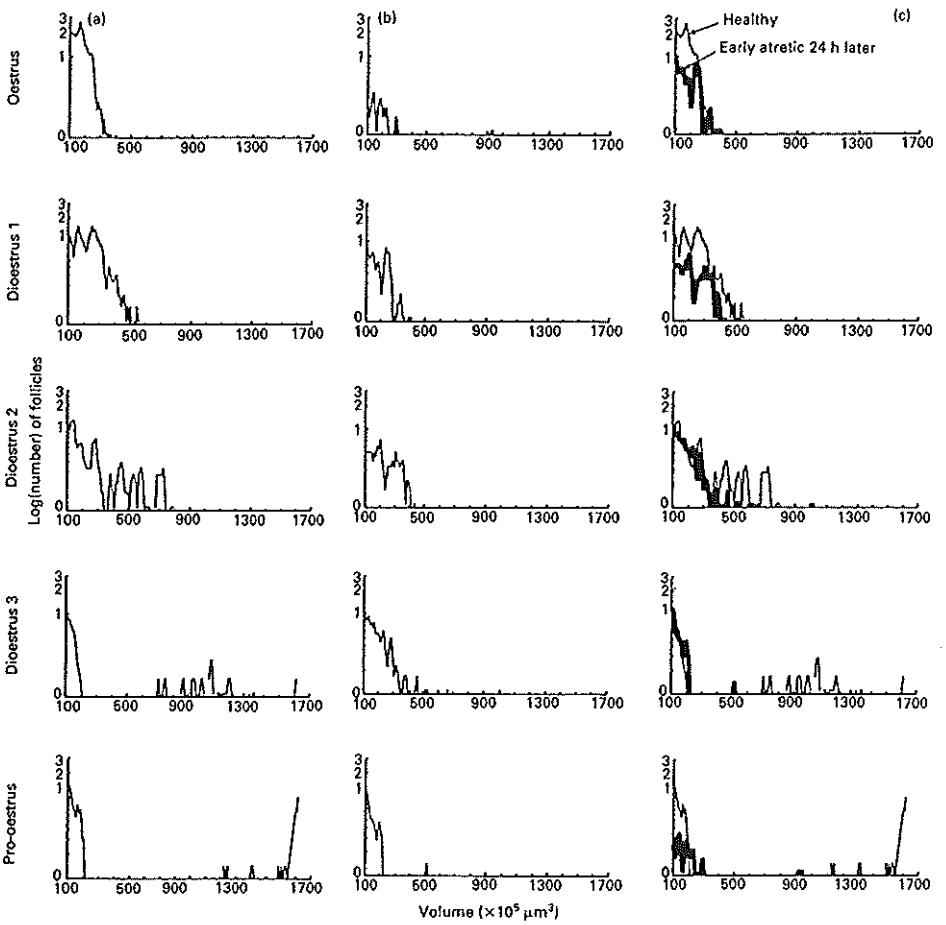

Fig. 3. Profiles of (a) healthy and (b) early atretic follicles in one ovary at 10.00 h during the various days of the cycle; (c) represents the number of healthy follicles (thin line) with the number of early atretic follicles (thick line) 24 h later projected on top of it. For instance the top right panel displays the number of healthy follicles at oestrus (thin line) and the number of early atretic follicles at dioestrus 1 (thick line). Follicles are classified in 151 classes with a width of 10×10^5 μm³. The values were smoothed with a moving average of three values. Class 151 contains all follicles with a volume $> 1600 \times 10^5$ μm³. The *y*-axis is logarithmic in all graphs.

divided into much smaller classes of equal width (10×10^5 μm³) ranging from 100 to 1600×10^5 μm³. The results of this classification, after smoothing with a moving average of three values, are shown (Fig. 3). The right five panels show for each day of the cycle the number of healthy follicles in combination with the number of early atretic follicles 24 h later (Fig. 3c). This was done to show which part of, for instance, the healthy follicle population of oestrus at 10.00 h will be early atretic at dioestrus 1 at 10.00 h. Thus the early atretic follicles of dioestrus 1 at 10.00 h are projected on top (thick line) of the healthy follicles of oestrus 10.00 h (Fig. 3a top panel).

From the group of healthy follicles present at oestrus, only a small part will become atretic within 24 h (Fig. 3c, note that the *y*-axis is logarithmic). On dioestrus 1 more of the follicles with a volume between 100 and 350×10^5 μm³ (class 1 and 2) will become atretic. At dioestrus 2, almost all follicles $< 350 \times 10^5$ μm³ will become atretic. This means that the larger follicles present at dioestrus 2 did grow into the larger size classes and were finally selected to ovulate. An interesting phenomenon was seen at dioestrus 3, when a clear gap between the smaller (up to 350×10^5 μm³) antral and the larger (above 700×10^5 μm³) follicles appears. This gap was still present at pro-

oestrus. The smallest antral follicles ($< 350 \times 10^5$ µm³, Fig. 3c) present at pro-oestrus will no longer become atretic and they will be joined by the high inflow into class 2 follicles during the next 2 days.

Discussion

The model of follicle inflow developed here was based on the assumption that the passage of follicles through the early stages of atresia takes about 24 h. Moreover, no correction was made for shrinkage of atretic follicles that might lead to disappearance of follicles into smaller volume classes. This latter point will be discussed first. Because in different studies criteria for atresia have been used, it is difficult to compare the reported or implied speed of shrinkage of atretic follicles. Byskov (1974) in an electron microscope study in mice described atresia as a process lasting 3–4 days, ending in follicles reaching the collapsed luteinized state. This state is comparable to the late atretic 2^b phase according to Osman (1985), which is reached in rats more than 48 h after the start of atresia. Considering the normal shape of early atretic follicles and the fact that there are early atretic follicles in all volume classes in which healthy follicles are found, it is likely that follicle shrinkage only takes place at a very low rate in early atretic follicles. Probably, late atretic follicles will shrink much faster as shown in this study in which almost no large (class 3, 4 and 5) late atretic follicles are seen, although large early atretic follicles were counted. Because the rate of shrinkage of early atretic follicles is not known (but is probably low, as discussed above) an alternative model was made in which a correction was made for follicle shrinkage. It was assumed that 30% of the early atretic follicles in a volume class had shrunk to a volume of the previous (smaller) volume size class. This was corrected for by increasing the number of early atretic class 5 follicles by 30%. The number of early atretic follicles of class 4, left after reduction of the 30% of class 5, was then increased by 30%. The same procedure was applied for the number of the early atretic volume classes 3, 2 and 1. This correction cannot be made for pro-oestrus and oestrus class 4 early atretic follicles because there are no class 3 early atretic follicles. In this case there was no increase in the number of early atretic class 4 follicles.

Use of the thus corrected model presented here resulted in only minor changes in follicle inflow. Thus the possible shrinkage of early atretic follicles does not seem to have much influence on the model. For the same reason, growth of early atretic follicles, which is not very likely because of the low mitotic index of early atretic follicles (Hirshfield, 1989), does not have much influence on the model. Reversal of early atresia does influence the model, but Hirshfield (1989) concluded that there is no return to the ovulatory pathway after follicles become atretic.

With regard to the assumption that early atresia takes 24h, the following points can be raised. The period of early and late atresia has been estimated as 3–4 days (Byskov 1974; Osman 1985; Greenwald, 1989). Butcher and Kirkpatrick-Keller (1984) described early atresia as a very rapid process. However, only Osman (1985) gave a more detailed description of timing of the atretic process. He suggested that early 1^a plus 1^b atresia, according to his definitions, would take at least 24 h, and that at least another 24 h is needed for the 2^a phase of atresia. The time that follicles persisted in the 2^b late atretic stage was not determined because there were no criteria for the end of this stage. One way of arriving at some answer to the question of the actual duration of early atresia is to study the effects of other time values for atresia on the model presented here.

A reasonable value for the duration of the stage of early atresia was found by multiplying the number of early atretic follicles by a certain factor to simulate other durations. For instance, when atresia takes 48 h and the sample rate is 24 h, the same early atretic follicles are counted twice. This can be corrected for by multiplying the number of atretic follicles by 0.5.

Models were calculated by multiplying the number of early atretic follicles by a value of 0.2, 0.5, 0.67, 1.0, 1.2 and 1.5 to simulate a duration of the early atretic phase of 120, 48, 36, 24, 20 and 16 h (Table 2). Another group of rats killed at 17 h was included to reconfirm the results.

The longer times of atresia (36–120 h) resulted in four or more negative inflow numbers. A negative inflow means that follicles leave a healthy follicle class without reappearing in another healthy or atretic follicle class, i.e. extremely fast atresia, which contradicts the assumption of an early atretic period from 36 to 120 h. Negative inflow numbers disappeared in the group of rats killed at 10 h when early atresia was assumed to take 24 h or less. In the rats killed at 17 h, negative inflows disappeared when the assumed period of early atresia was 20 h or less. The negative inflow values were low from an early atretic period of 24 h.

When the duration of early atresia decreases, the total number of follicles which enter follicle volume class 1 increases. When the number of follicles that disappear from the ovaries in one cycle is known, it is possible to determine the lower border of the duration of the stage of early atresia.

Pedersen (1970, 1972) calculated an inflow of 96 follicles per two ovaries into his follicle class 3^b per 5-day cycle in mice. This follicle class 3^b consists of small preantral follicles with 20 or more granulosa cells (volume about 0.9×10^5 µm³). The method used by Pedersen (thymidine pulse labelling) was completely different from the method used here. Using the same technique as Pedersen, Groen-Klevant (1981) found an inflow of 144 follicles per two ovaries per 5-day cycle in our rat strain. Butcher and Kirkpatrick-Keller (1984) mentioned an inflow of 21 follicles per ovary between pro-oestrus and oestrus in the rat which is almost the same number as in the model at an early atretic period of 24 h (23.6). Butcher and Kirkpatrick-Keller used histological examination of rat ovaries during the various days of the cycle. In a recent experiment we calculated the number of follicles in our rats that disappear from the ovary between day 40 and day 90 of life, by counting all follicles (preantral and antral healthy and early atretic) in each fifth serial section (Meijs-Roelofs *et al.*, 1990). This number amounted to 115 follicles per two ovaries per 5-day cycle. Thus the number of follicles disappearing from the pool of healthy follicles every cycle may be between 115 and 144 in our rat strain. The period of early atresia could not be shorter than 20 h in the group of rats killed at 10 h in this study. If the stage of early atresia takes 20 h, during young adult life, most of the follicles with a volume $\geq 100 \times 10^5$ µm³ (volume class 1) would become atretic. Occurrence of atresia was found in follicles with volumes

between 20 and 350 × 10^5 μm³ (Mandl and Zuckerman 1952, 87–200 × 10^5 μm³; Pedersen, 1970, 1972, large follicles type 5[b] and 6 ±20–130 × 10^5 μm³; Mariana and Meyer, 1979, 88% of atretic follicles > 149 × 10^5 μm³; Butcher *et al.*, 1984, 20–155 × 10^5 μm³; Hirshfield, 1988, 41–350 × 10^5 μm³). The mentioned numbers of follicles lost per cycle (between 96, mice (Pedersen, 1970) and 144, rats (Groen-Klevant, 1981)) were all found in studies of young adult animals. When the animals were older the number of follicles that are lost per cycle will decrease (Meijs-Roelofs *et al.*, 1990). These studies indicate that there is early atresia in follicles with a volume < 100 × 10^5 μm³, but most atresia is found in the bigger antral follicles, so the stage of early atresia must take longer than 20 h. Best results were obtained when the stage of early atresia takes between 20 and 24 h.

In some cases in this study the number of follicles that leave a follicle class is bigger than the number of healthy follicles in this class. Some follicles did therefore grow more than the size of one class in 24 h. This finding is to be expected because the volume classification was not intended to create classes in which healthy follicles would stay for 24 h or more.

The differences in the data from the groups of rats killed at 10 and 17 h are small and may be partly accounted for by the difference in timing. The difference in the total number of follicles entering follicle volume class 1 was about 12%.

From the model presented here the following conclusions can be derived. First, the follicles that appear in volume class 1 during pro-oestrus and oestrus seem to be the follicles that will ovulate at the next pro-oestrus. From these class 1 follicles, a selection is made that does not depend on their size during oestrus. At dioestrus 2, the selected follicles can be pointed out as the larger follicles of the antral follicle pool (this study; see also Pedersen, 1970; Welschen, 1973; Butcher *et al.*, 1984; Osman, 1985).

Second, follicles that ovulate at pro-oestrus seem to have developed according to the following time scheme: inflow into volume class 1 during pro-oestrus and oestrus, into volume class 2 during pro-oestrus and oestrus, into volume class 3 during oestrus and dioestrus 1, into volume class 4 during dioestrus 1 and dioestrus 2 and finally, inflow into volume class 5 during dioestrus 2 and dioestrus 3.

Third, in the total process of maturation and atresia during a cycle, about 120 follicles are lost (in agreement with Pedersen (1970), mice; Groen-Klevant (1981), rats; Meijs-Roelofs *et al.* (1990), rats).

Fourth, there is a continuous inflow of follicles into volume class 1. However, this inflow is not constant: it is much higher during pro-oestrus and oestrus than during the dioestrous period. Probably there is a continuously, but slowly, growing pool of follicles that starts to grow much faster when influenced by the ovulatory FSH peak(s) (see also Welschen, 1973). Among these follicles appear the 'privileged' ones destined to ovulate.

Finally, follicle selection seems to take place in the volume classes 1 and 2 during the days of oestrus and dioestrus 1, since the follicles that eventually ovulate can be traced back to this period of the cycle.

In further studies, this model will be applied to describe the follicle dynamics in a situation of follicle stimulation with an FSH preparation. The model makes it possible to distinguish easily between reduced atresia and an increase in the follicle inflow and can also be used in other species, when numbers of healthy follicles and numbers of atretic follicles of certain stages are known.

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Chapter 5

Inhibin and oestradiol-17ß in antral follicles of various size classes of cyclic rats

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Inhibin and oestradiol-17β in antral follicles of various size classes of cyclic rats

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Abstract

On the various days of the 5-day oestrous cycle of the rat, ovarian antral follicles were dissected out and grouped in five size classes. Four follicles of the same size class were homogenized jointly in medium, after which inhibin-like bioactivity, inhibin immunoreactivity and oestradiol-17β content were measured. In general, there was a significant correlation between immunologically and biologically active inhibin levels in the different size classes; overall correlation was 0·85 (n=87, P<0·00001). In the smallest antral follicles (classes 1 and 2) inhibin bioactivity was detected only during the first three days of the cycle. With increasing follicle size, inhibin bioactivity and immunoreactivity increased, with maximal activity present in the largest, i.e. preovulatory, follicles (class 5) during the last three days of the cycle (the day of oestrus denotes day 1 of the cycle). These results indicate that only follicles which reach the antral stage at oestrus, and are known to be recruited by the periovulatory FSH peak, acquire the potency to produce biologically active inhibin. This is the cohort of follicles from which selection of ovulatory follicles will normally take place.

In contrast to inhibin, follicular oestradiol-17β concentrations were negligible until the last days of the cycle when oestradiol-17β was present in follicles larger than class 2; levels increased with increasing follicle size and a maximal level was found in preovulatory follicles at pro-oestrus.

It is concluded that (1) there is a good correlation between follicular content of inhibin-like bioactivity and inhibin immunoreactivity and (2) there are differences between patterns of follicular levels of inhibin immunoreactivity and oestradiol-17β during the ovarian cycle: follicles destined to ovulate start to produce inhibin in volume classes 1 and 2 and only thereafter also contain oestradiol-17β. Follicles entering classes 1 and 2 at dioestrus-2 and -3 or at pro-oestrus do not produce inhibin and become atretic.

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Introduction

During the 5-day ovarian cycle of the rat recruitment and selection of follicles destined to ovulate takes place at predictable moments. Entry of follicles into the smallest antral phase, where accelerated growth starts, takes place continuously, but on the days of pro-oestrus and oestrus more follicles start this antral growth under the influence of the periovulatory follicle-stimulating hormone (FSH) peak compared with the other days of the cycle (Peters & Levy 1966, Welschen 1972, van Cappellen *et al.* 1993). Selection for continued growth, up to preovulatory size, is seen as early as the days of oestrus and dioestrus-1 (Greenwald 1974, Osman 1985, van Cappellen *et al.* 1993). The follicles recruited by the secondary FSH peak contain the cohort of follicles ovulating at the end of the cycle (Schwartz 1974, Welschen & Dullaart 1976, Hirshfield & Midgley 1978, Hirshfield & De Paolo 1981) and characteristic changes in these follicles have been reported. An increase in the expression of inhibin α- and β_A-subunit-mRNA in follicles newly recruited by the periovulatory FSH surge has been reported in the rat (Woodruff *et al.* 1988, D'Agostino *et al.* 1989). Also, an increase in the incorporation of [^{3}H]thymidine under the influence of periovulatory gonadotrophin levels has been described, indicating increased DNA synthesis in preantral follicles (in hamster and mouse, Roy & Greenwald 1986, Wang *et al.* 1991).

Since the inflow of follicles into the accelerated antral growth phase is continuous, the above data raise the question whether the follicles recruited by the periovulatory FSH peaks are qualitatively different from those entering the antral phase during other phases of the cycle. In other words, is the individual maturational path of a follicle during the cycle dependent on the hormonal conditions at the moment of entry into the antral growth phase? We studied this question by estimating the levels of inhibin and oestradiol-17β in follicles of the different antral

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size classes during the various days of the 5-day oestrous cycle. Apart from information on possible functional differences between follicles of the various size classes, these data may give insight into the relative importance of inhibin and oestradiol-17β in the regulation of FSH secretion and, therefore, in the selection and determination of numbers of ovulatory follicles during the ongoing cycle.

Materials and Methods

Animals

Female rats of a Wistar substrain (R-Amsterdam) with regular 5-day oestrous cycles were used. They were kept in conditions of controlled temperature (23 ± 2 °C) and light (on from 0500 to 1900 h) and allowed free access to standard dry pellets and tap water. Vaginal smears were taken every morning and rats were used only after at least two successive 5-day oestrous cycles. The day of vaginal oestrus was called day 1 of the cycle.

Isolation of follicles

Ovaries were removed between 0900 and 1200 h on the various days of the oestrous cycle and placed in medium F-12 (Boehringer, Mannheim, Germany) supplemented with 10 mM Hepes (Gibco, Grand Island, NY, USA.), 2 mM magnesium acetate and 50 mM D(+) glucose.

They were then incubated for 7 min at room temperature in this medium containing 5 mM EGTA and, after three washes, ovaries were dissected and antral follicles were harvested by sight (in a laminar flow cabinet in case of culture of follicles) under a dissecting microscope with sterile injection needles (19 gauge). Follicles were collected in supplemented medium F-12 in a watchglass and then selected according to their size with the aid of precalibrated micropipettes, taking care to apply minimal force when sucking a follicle into the micropipette. The calibre of the pipettes was set to the upper level of the diameter window of one of the five follicle size classes, defined before in histological studies (Sander *et al.* 1991): class 1, 260–340; class 2, 340–405; class 3, 405–460; class 4, 460–580 and class 5, >580 μm; the actual calibers were 318, 414, 484 and 574 μm. All larger follicles were categorised as class 5.

Starting with the smallest pipette, all antral follicles were divided into the five size classes. Four follicles of the same size class from one rat were put together in 1 ml medium and homogenized immediately or after 24 h of culture. Medium of the homogenized follicles was stored at −20 °C until assayed.

Culture of follicles

Follicles were cultured in 24-well culture plates (Falcon, Lincoln Park, NJ, USA). The bottom of the wells was precoated with 100 μl bacteriological agar (0·625%). Subsequently, the four follicles in the well were covered with a further 100 μl agar to prevent floating. Finally 1 ml medium was added. Follicles were cultured in sterile Eagle's minimal essential medium with Earle's salts (MEM; Boehringer), supplemented with 1% non-essential amino acids (Gibco), 1% antibiotic-antimitotic solution (Gibco; containing 100 000 units penicillin, 100 mg streptomycin and 250 μg fungizone/l medium), 1% glucose, 1% Insulin Transferrin Selenium stock solution (ITS^+; Collaborative Research, Bedford, MA, USA; final concentration 6·25 μg insulin, 6·25 μg transferrin, 6·25 μg selenium, 1·25 mg BSA and 5·35 μg linoleic acid/ml), cortisol (40 ng/ml) and 1% sodium pyruvate (Roy & Greenwald 1989). Follicles were cultured for 24 h in a moist chamber at 37 °C in a 5% CO_2/95% air atmosphere. Subsequently medium from individual wells was harvested and stored at −20 °C until assayed. The follicles were homogenized in 1 ml fresh culture medium and stored at −20 °C until assayed.

Experiment 1

Every day of the cycle 4 rats were killed, ovaries were removed and follicles were isolated and homogenized. The experiment was performed twice. In a first series inhibin bioactivity and immunoreactivity were measured in the medium of homogenized follicles. A second series was used to measure inhibin immunoreactivity and oestradiol-17β.

Experiment 2

In a control experiment the production of bioactive inhibin and oestradiol-17β during a 24-h culture period was estimated in follicles from one rat for each day of the cycle. Follicles were homogenized at the start of the culture or after 24 h as described before.

Experiment 3

An additional experiment was performed in order to study the influence of atresia on the content of bioactive inhibin in media derived from cultured follicles. On the day of pro-oestrus a number of follicles of class 4, which were expected to be mostly atretic on the basis of routine histology, and of class 5, which should be mostly healthy, were harvested and cultured separately for 24 h; the medium from individual wells was harvested and assayed for inhibin bioactivity.

Hormone determinations

Inhibin bioactivity was estimated using a dispersed pituitary cell culture bioassay (Sander *et al.* 1984). Inhibin immunoreactivity was estimated by RIA according to

Robertson *et al.* (1988), using a bovine follicle fluid preparation with an arbitrary potency of 1 U/μg protein as a standard (Grootenhuis *et al.* 1989). Within assay variation was 12%.

Oestradiol-17β was estimated by RIA using kits provided by Diagnostic Products Corporation (Los Angeles, CA, USA.). Within assay variation was 5% and the limit of detection was 15 pmol/l.

Statistical procedures

All data were analyzed using the Systat computer program (Systat Inc, Evanston, IL, USA). The linear correlation coefficient R was calculated after a logarithmic (base 10) transformation of the data. When the data for inhibin bioactivity were below the limit of detection (0·78 U/ml or approximately 0·2 U/follicle, Sander *et al.* 1985), a value of 0·2 was substituted. The correlation coefficient was regarded as significant if the two-tailed P value was $\leq 0{\cdot}05$. When results from more than two groups were compared, the data were analyzed by the Tukey multiple comparison test. Values are shown as hormone content/follicle. For measurement of the difference in inhibin immunoreactivity between the first and second series, a three way analysis of variance was used (variables: series, day of cycle, volume class). The levels of inhibin bioactivity and oestradiol-17β before and after 24 h of culture were analyzed by a two way analysis of variance (variables: time and day).

Results

Experiment 1

Figure 1 shows the content of inhibin bioactivity and immunoreactivity in isolated follicles of the various size classes during the five days of the cycle. Class 1 and class 2 follicles contained detectable amounts of inhibin bioactivity only during the first three days of the cycle. The same pattern was paralleled by the inhibin immunoreactivity. The bioactive inhibin content increased with follicle size with maximal content in the largest follicles, those of ovulatory size (class 5) present during the last three days of the cycle. Inhibin immunoreactivity levels showed the same pattern.

Figure 2*a* shows the relationship between the levels of inhibin immunoreactivity and inhibin bioactivity. Correlations between these levels for the various follicle classes are shown in Table 1; the overall correlation coefficient (R) was 0·85 ($P<0{\cdot}0001$; $n=87$). There was a significant correlation between inhibin bioactivity and immunoreactivity for follicle volume classes 2, 3 and 4.

Figure 3 shows the levels of inhibin immunoreactivity and oestradiol-17β in follicles of the various size classes. Inhibin immunoreactivity, found in this second series of

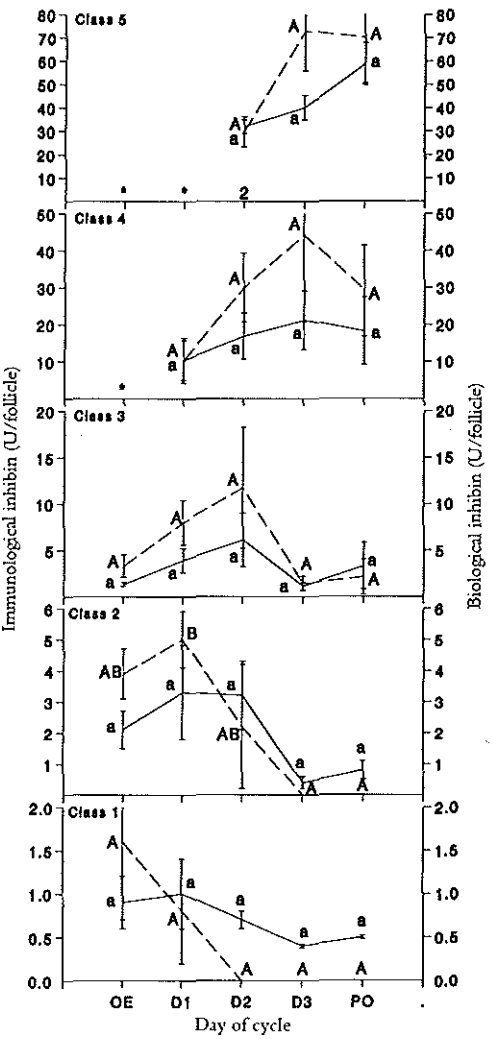
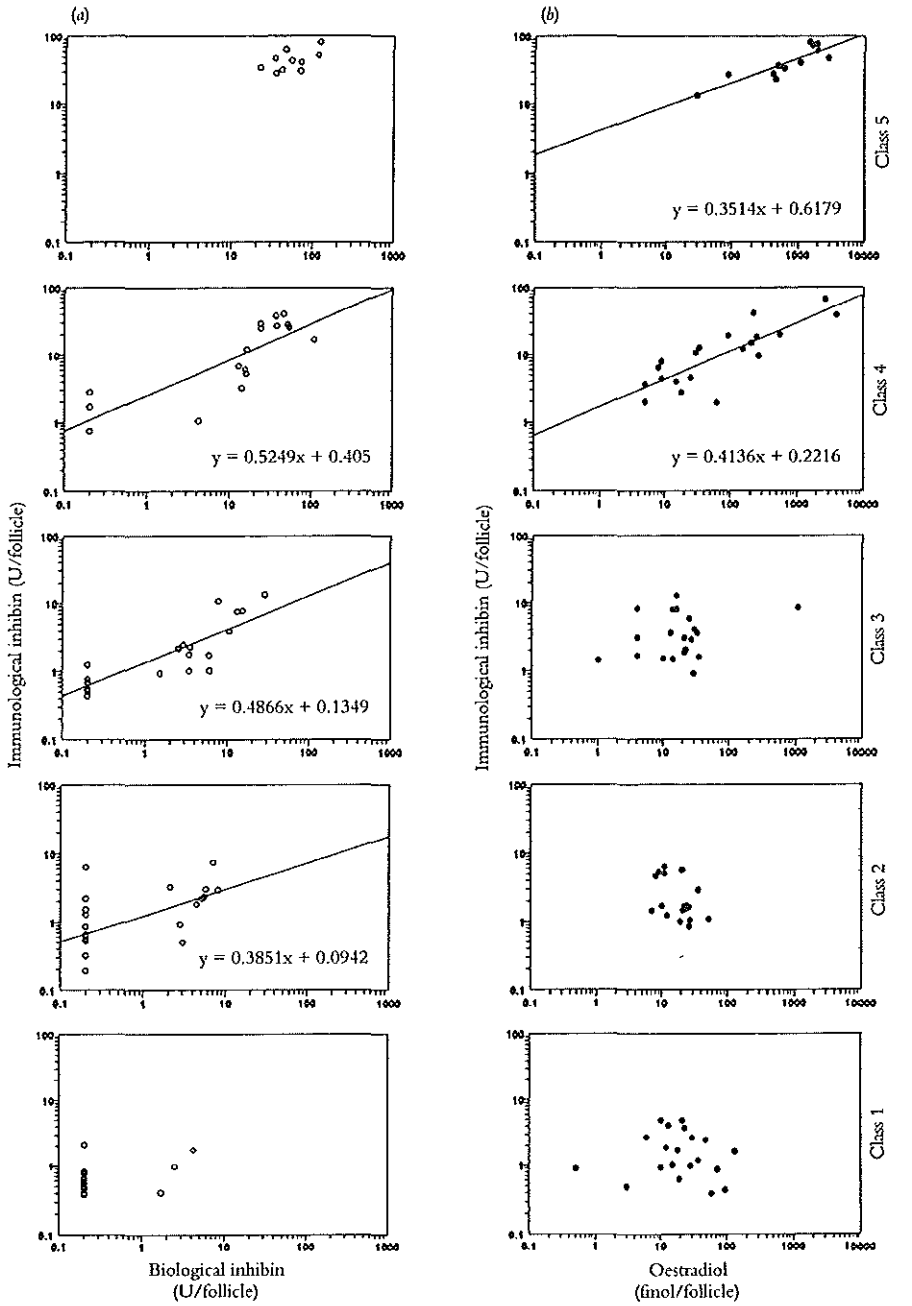


FIGURE 1. Inhibin bioactivity (broken lines, capital letters) and immunoreactivity (solid lines, small letters) in the media of homogenized follicles of various size classes (see Materials and Methods for explanation of class size). Values are means ± S.E.M. and are expressed per follicle (mean of 4 rats=4 wells of 4 follicles). Different letters represent a significant difference between the days of the cycle (PO, pro-oestrus; OE, oestrus; D1, dioestrus-1; D2, dioestrus-2; D3, dioestrus-3). *, no follicles present. 2, follicles of two rats ($n=2$).


(a)
(b)
y = 0.3514x + 0.6179
y = 0.5249x + 0.405
y = 0.4136x + 0.2216
y = 0.4866x + 0.1349
y = 0.3851x + 0.0942
Class 5
Class 4
Class 3
Class 2
Class 1
Immunological inhibin (U/follicle)
Immunological inhibin (U/follicle)
Biological inhibin (U/follicle)
Oestradiol (fmol/follicle)

samples, did not differ significantly from that found in the first series. Oestradiol-17β levels were generally below detection level until the last days of the cycle, when oestradiol-17β could be detected in follicles larger than volume class 2. The oestradiol-17β level increased with increasing follicle size with maximal levels in preovulatory (class 5) follicles on pro-oestrus. Significant correlations between oestradiol-17β and inhibin immunoreactivity were only found in class 4 and class 5 follicles (Fig. 2*b*; Table 2). The overall correlation coefficient for the relationship between immunoreactive inhibin and oestradiol-17β was 0·70 ($P<0·0001$; $n=91$).

Experiment 2

There was no difference in the inhibin bioactivity and oestradiol-17β concentration between the media of the homogenized follicles at 0 h and the media after 24 h of culture (data not shown). Follicles homogenized in fresh medium after 24 h of culture did not contain detectable amounts of inhibin bioactivity or oestradiol-17β (data not shown). The data indicate that inhibin bioactivity and oestradiol-17β values do not change between 0 and 24 h of culture.

Experiment 3

Only class 4 and class 5 follicles were collected at pro-oestrus and cultured individually. On the basis of numbers of follicles normally present at pro-oestrus (Osman 1985) it may be expected that 5% of class 5 follicles (0·3 out of 5·9) and 81% of class 4 follicles (1·3 out of 1·6) are atretic. Inhibin bioactivity (27·7 ± 13·0 units/ml) was found in the media of 22 out of 24 class 5 follicles; no activity was seen in the remaining 2 (8% of the follicles). In class 4 follicles ($n=14$), 1 follicle showed inhibin bioactivity (21·0 units/ml) and 13 (93%) showed no detectable activity.

Discussion

In this study, follicle classification was carried out by means of glass pipettes with openings of fixed diameters. Since histological procedures will give rise to a certain shrinkage of the tissue, histological follicle classes would need to have

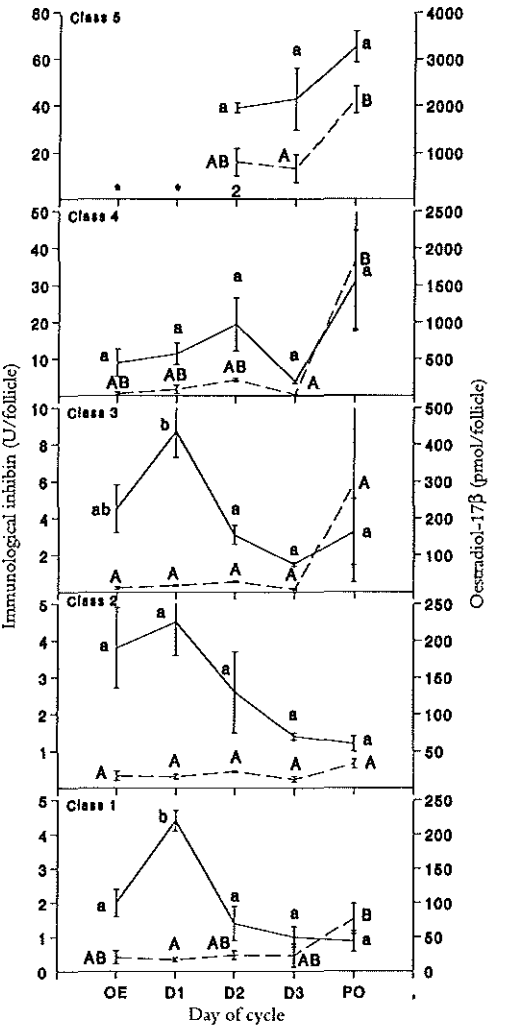


FIGURE 3. Inhibin immunoreactivity (solid lines, small letters) and oestradiol-17β (broken lines, capital letters) in the media of homogenized follicles of various size classes (see Materials and Methods for explanation of class size). Values are means ± S.E.M. and are expressed per follicle (mean of 4 rats=4 wells of 4 follicles). Different letters represent a significant difference between the days of the cycle (PO, pro-oestrus; OE, oestrus; D1, dioestrus-1; D2, dioestrus-2; D3, dioestrus-3). *, no follicles present. 2, follicles of two rats ($n=2$).

FIGURE 2. Relationships between *a*) inhibin immunoreactivity and bioactivity and *b*) inhibin immunoreactivity and oestradiol-17β in follicles of various size classes obtained during the oestrous cycle (see Materials and Methods for explanation of class size). Data of inhibin bioactivity below the limit of detection were substituted by 0·2 U/follicle. Regression lines were only drawn when there was a significant correlation ($P\leq 0·05$).

TABLE 1. Correlation between levels of bioactive and immunoreactive inhibin. (R = correlation coefficient). Regression coefficients were calculated after a logarithmic transformation. Values of bioactive inhibin below the limit of detection were substituted by 0·2

	n	R	*P*	Regression coefficient	Intercept
Follicle class					
1	20	0·39	0·09	0·19	−0·98
2	20	0·52	0·02	0·39	0·09
3	20	0·83	<0·01	0·49	0·14
4	17	0·82	<0·01	0·53	0·41
5	10	0·56	0·09	0·35	1·0
Day of cycle					
Oestrus	13	0·46	0·12	0·22	0·04
Dioestrus-1	16	0·70	<0·01	0·42	0·27
Dioestrus-2	18	0·88	<0·01	0·55	0·37
Dioestrus-3	20	0·95	<0·01	0·77	0·08
Pro-oestrus	20	0·98	<0·01	0·74	0·32

n = number of follicles in one group.
The correlation is regarded as significant when $P \leqslant 0{\cdot}05$.

TABLE 2. Correlation between levels of immunoreactive inhibin and oestradiol. (R = correlation coefficient). Regression coefficients were calculated after a logarithmic transformation

	n	R	*P*	Regression coefficient	Intercept
Follicle class					
1	20	0·05	0·83	−0·08	1·29
2	19	0·41	0·08	−0·32	1·36
3	20	0·27	0·25	0·48	0·98
4	20	0·82	<0·01	1·61	0·25
5	12	0·87	<0·01	2·13	−0·62
Day of cycle					
Oestrus	17	0·24	0·36	0·26	0·96
Dioestrus-1	17	0·67	<0·01	1·09	0·49
Dioestrus-2	18	0·91	<0·01	0·92	1·16
Dioestrus-3	19	0·82	<0·01	1·17	0·68
Pro-oestrus	20	0·92	<0·01	0·95	1·65

n = number of follicles in one group.
The correlation is regarded as significant when $P \leqslant 0{\cdot}05$.

somewhat lower size limits per class for comparison with fresh material. On the other hand, sucking fresh follicles into the pipettes may have slightly deformed the shape of the follicle and thus a somewhat larger follicle may have entered a pipette with a certain diameter. It was assumed that these two factors are likely to balance out. Therefore follicle sizes at the various days of the cycle, as used in the present study, were directly compared with those observed previously in histological studies (Osman 1985).

During the cycle only follicles reaching the antral stage (class 1) during oestrus and dioestrus-1 (i.e. recruited by the periovulatory FSH peak) contain detectable amounts of inhibin bioactivity. This happens to be the cohort of follicles which normally contains the group of follicles that will ovulate at the end of the cycle (Welschen 1972, van Cappellen *et al.* 1993). This is in agreement with data of D'Agostino *et al.* (1989) who found, by *in situ* hybridisation, an increase in inhibin α- and β_A-mRNA levels in newly recruited follicles after unilateral ovariectomy, but in contrast with data of Meunier *et al.* (1988), who reported that the α-chain of inhibin is expressed in follicles at all stages of maturation, but that inhibin β_A-mRNA is seen only in large tertiary follicles immediately before ovulation.

A significant overall correlation was found between the results of the two procedures for inhibin estimation. This significance for the relationship between bioactive and immunoreactive inhibin levels persisted in follicles of the separate volume classes except for classes 1 and 5. The class 1 follicles only contain small amounts of biologically active inhibin at the start of the cycle (oestrus and dioestrus-1) and no detectable amounts thereafter. This may explain the poor correlation for this class. Production of free α-inhibin subunits could be the reason for the lack of correlation in follicle class 5. The antibody used in the RIA was raised against purified 31 kDa inhibin, which represents only one of the biologically active forms of inhibin in the female rat (Grootenhuis *et al.* 1989).

The finding that a maximal content of biologically active inhibin is seen in the large, preovulatory follicles is in good agreement with earlier observations by Sander *et al.* (1984), who showed maximal inhibin-like bioactivity in cultured granulosa cells obtained on the day of pro-oestrus. Similarly and again for the day of pro-oestrus, Meunier *et al.* (1988) and Woodruff *et al.* (1988) found a dramatic increase in inhibin-subunit mRNAs in preovulatory Graafian follicles.

Various authors have suggested an inverse relationship between circulating FSH and inhibin levels during the rat oestrous cycle (DePaolo *et al.* 1979, Welschen *et al.* 1980, Hasegawa *et al.* 1989, Watanabe *et al.* 1990) and in pubertal rats a role for inhibin in the regulation of FSH secretion and thus, indirectly, in the regulation of follicle growth has been suggested (Sander *et al.* 1986). Watanabe *et al.* (1990) suggested changes in circulating inhibin levels to be a precise indicator for events in follicular development such as recruitment, selection and ovulation. Accordingly, in the rat, inhibin would be a more important factor than oestradiol-17β in determining follicle growth and number of ovulations, via regulation of FSH secretion. The present results support this suggestion, since oestradiol-17β concentration in the follicles started to increase only at the very end of the 5-day cycle, when not only recruitment of new antral follicles but also selection of follicles to ovulate has already taken place (van Cappellen *et al.* 1993). Only in follicles with high oestradiol-17β and inhibin levels (classes 4 and 5) was a good correlation between oestradiol-17β and immunoreactive inhibin found.

Of the small antral follicles only those that were recruited by the periovulatory FSH peaks contained detectable levels of biologically active inhibin. This could indicate that these follicles are more privileged in terms of inhibin production and in their chances of surviving up to the ovulatory stage than follicles entering the antral growth phase at other times. It would be interesting to study other possible qualitative differences, as for example the presence of various growth factors, between these 'privileged' and other follicles. Variation in the process of initiation of antral follicle growth leading to 'privileged' and 'non-privileged' follicles, was an idea put forward by Gougeon in 1986 for follicle growth in the human.

It has been reported in the rat that once the follicle has become atretic inhibin as well as oestradiol-17β production declines to a very low level within 24 h (Kaneko *et al.* 1987). The finding that the levels of inhibin and oestradiol-17β after 24 h of culture were not significantly different from those before culture, indicates that during *in vitro* culture only very small amounts of hormones were produced. This may be due to the absence of hormones in the culture system.

It is not possible to discern (early) atretic follicles during dissection of the ovaries and thus accidental presence of an atretic follicle in a well containing four follicles cannot be excluded. The additional experiment in which individual class 4 and class 5 follicles were harvested and cultured on the day of pro-oestrus, suggests that hormone levels in atretic follicles are low. This indicates that combined results of 4 follicles are an underestimation of actual amounts of hormones in healthy follicles.

From the current experiments it can be concluded that inhibin immunoreactivity is a good indication for inhibin bioactivity in follicles of classes 2, 3 and 4. The follicles which enter the antral follicle stage during oestrus and dioestrus-1 contain bioactive and immunoreactive inhibin and almost no oestradiol-17β. Follicles with these characteristics seem to constitute the cohort of follicles from which the follicles for ovulation are selected.

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Chapter 6

Recombinant FSH (Org32489) induces follicle growth and ovulation in the adult cyclic rat

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Recombinant FSH (Org32489) induces follicle growth and ovulation in the adult cyclic rat

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Abstract

The effects of a single injection of recombinant human FSH (rhFSH; Org32489) on ovulation rate and timing and on antral follicle growth were studied in adult 5-day cyclic rats. Rats injected at 1700 h on dioestrus-2 with a dose of 10 IU rhFSH showed, on average, no increase in ovulation rate on the day of expected oestrus. However, an additional, precocious ovulation resulting in a normal number of corpora lutea (13·3 ± 0·4, n=6) was found to take place on the night after injection, i.e. dioestrus-3. No mating behaviour, as shown by the absence of vaginal plugs the next morning, was observed at this ovulation. Follicle counts showed a loss of large antral follicles due to ovulation and increased numbers of healthy small antral follicles at 17 and 41 h after injection, indicating a decrease of atresia of growing follicles as well as additional recruitment of new antral follicles. The endogenous serum FSH concentration on the subsequent day of oestrus (65 h after the rhFSH injection) as well as recruitment of small antral follicles were lower in the rhFSH-treated rats than in saline-treated controls. The ovulation at oestrus, 48 h after the precocious, rhFSH-induced ovulation showed large differences in the number of oocytes between the rats in one treatment group.

Similar results in terms of immediate ovulation induction were obtained by using a highly purified human urinary FSH preparation (i.e. metrodin). Furthermore, the direct induction of ovulation by rhFSH or metrodin could not be prevented by the injection of an LHRH antagonist.

It was concluded that rhFSH can induce acute ovulation in rats, and stimulates follicular development directly or indirectly through increased FSH levels after ovulation. It induces antral follicle growth and decreases early atresia in small antral follicles.

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Introduction

The availability of recombinant human follicle-stimulating hormone (rhFSH) with a high specific FSH bioactivity and negligible intrinsic luteinizing hormone (LH) bioactivity (Mannaerts *et al.* 1991) provides a challenge for reinvestigating the actions attributed specifically to FSH during the ovarian cycle.

It has been generally accepted that FSH stimulates follicles to grow (see Schwartz 1974, Greenwald & Terranova 1988) and may, via this action, regulate the number of ovulations at the end of the cycle (see Richards 1980). On the basis of these observations, various more or less purified FSH preparations have been widely used to induce superovulation, i.e. ovulation of a supranormal number of ova, both in immature and in adult rodents (Wilson & Zarrow 1962, (immature mouse and rat), Lostroh & Johnson 1966 (immature rat), Greenwald 1974 (adult hamster), Nuti *et al.* 1974 (immature rat), Chiras & Greenwald 1978 (adult hamster), Miller & Armstrong 1981 (immature rat), Armstrong & Opavsky 1988 (immature rat)). The FSH preparations used showed varying low degrees of LH contamination. Numbers of ovulations per ovary can also be increased via a transient rise in endogenous FSH secretion, induced by either unilateral ovariectomy (Welschen 1970, Welschen & Dullaart 1974, Meijs-Roelofs *et al.* 1982, Osman *et al.* 1982) or by the administration of an inhibin-neutralizing antiserum (Sander *et al.* 1991*a*,*b*). On the basis of follicle counting it was concluded that the increased number of ovulations in the latter two conditions was caused by a combination of increased follicular recruitment and reduction of atresia of antral follicles already recruited. The aim of the present study was to investigate whether similar mechanisms play a role during stimulation of follicle growth and ovulation rate following rhFSH treatment. During these investigations direct ovulation-inducing properties of the rhFSH were found; this was also studied in more detail.

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Materials and Methods

Animals

Female rats of a Wistar substrain (R-Amsterdam) with a 5-day oestrous cycle and body weight of 170–210 g were used. They were kept in conditions of controlled temperature (23 ± 2 °C) and light (lights on from 0500 to 1900 h) and allowed free access to standard dry pellets and tap water. Adult littermates were randomly divided over the treatment groups. Vaginal smears were taken every morning and rats were used only after at least two successive 5-day oestrous cycles. To test mating behaviour female rats were caged together with males of proven fertility and checked the next day for vaginal plugs.

Experiment 1

Rats received one intramuscular injection with rhFSH (Org32489; batch IP 190/0824; Organon, Oss, The Netherlands) or solvent (0·9% NaCl) at 1700 h on dioestrus-2 (the second day after the day of oestrus). International Units (IU) refer to bioactivity (assay method of Steelman & Pohley 1953) in terms of IS 71/223. Different amounts of rhFSH were used in small groups of rats to find the optimal dose of rhFSH for induction of follicle growth.

Rats injected with saline (n=5), or 2·5 (n=5), 5 (n=5) or 10 (n=6) IU rhFSH were killed 65 h later, i.e. at 1000 h on the subsequent day of oestrus. Other groups of rats, injected with saline (n=6), 2·5 (n=5), 5 (n=7) or 10 (n=5) IU rhFSH, were bled and killed 41 h after injection, i.e. at 1000 h on the day of expected pro-oestrus. A final group of rats, injected with saline (n=7) or 10 (n=6) IU rhFSH, was killed at 17 h after injection, i.e. at 1000 h on dioestrus-3, the day after injection.

At necropsy, blood was sampled, tubal ova were counted whenever present and ovaries and uteri were excised and weighed. Ovaries were fixed in Bouin's fluid, embedded in paraffin wax, serially sectioned (10 μm) and stained with haematoxylin and eosin. Ovulation rate was confirmed histologically by counting the number of fresh corpora lutea (CL) in the ovaries. Differential counts of antral, healthy and atretic follicles were made according to Meijs-Roelofs *et al.* (1982). Volume classes and diameters for antral follicles were defined as follows: class 1, $100–200 \times 10^5$ μm^3 and 260–340 μm; class 2, $200–350 \times 10^5$ μm^3 and 340–405 μm; class 3, $350–500 \times 10^5$ μm^3 and 405–460 μm; class 4, $500–1000 \times 10^5$ μm^3 and 460–580 μm; class 5 $>1000 \times 10^5$ μm^3 and >580 μm. Atretic follicles were designated as 'early atretic' if local or widespread pycnosis and cell shrinkage was present in the granulosa cell wall and as 'late atretic' if the oocyte was degenerating and either surrounded by degenerating cumulus cells or naked (Osman 1985).

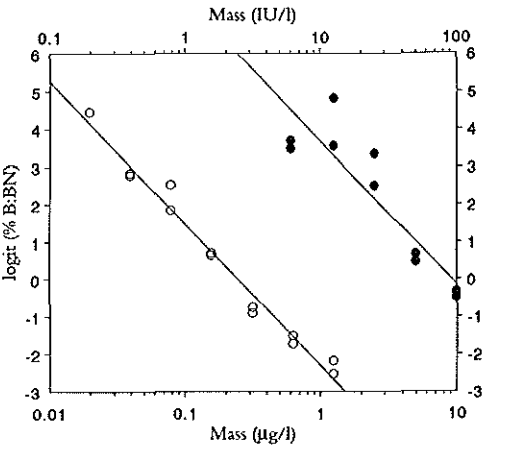

FIGURE 1. Dose–response curves for rat FSH-RP-3 (○) and rhFSH (●) in the rat FSH RIA after log–logit transformation. Points were assayed in duplicate; individual results are shown. BN, normalized binding.

Experiment 2

Rats received intramuscular injections of 50 μg of a luteinizing hormone-releasing hormone antagonist (LHRH-a; Org30276, Organon, Oss, The Netherlands), a dose which has been proven to suppress the ovulatory LH peak (Meijs-Roelofs *et al.* 1990), or solvent (0·9% NaCl) at 1700 h on dioestrus-2. Thereafter rats received either 10 IU rhFSH or 10 IU highly purified human urinary FSH (hFSH; Metrodin, Serono, Rome, Italy, 75 IU/ampoule declared *in vivo* bioactivity). Rats were killed 17 h later, i.e. at 1000 h on dioestrus-3 and the number of tubal ova was counted.

Hormone determinations

Concentrations of endogenous FSH and LH were estimated by radioimmunoassay (RIA) with anti-ovine FSH and LH as antiserum and rat FSH/LH as tracer, and expressed as μg NIADDK-rat FSH-RP-3/I and μg NIADDK-rat LH-RP-3/I (Sander *et al.* 1986). Within-assay variations were 4% (FSH) and 5% (LH). Parallel dose–response curves for rat FSH-RP-3 and rhFSH were found after log–logit transformation (Fig. 1) indicating that cross-reactivity of rhFSH in the rat FSH assay amounted to 2·6% on the basis of weight: since 1 μg of rhFSH is equivalent to 10 IU, 40 IU/l of 71/223 is equivalent to 0·1 μg/l of rat RP-3. Finally, the immunopotency of IS 71/223 is 1·5 times higher than that of IS 78/549 in the human FSH immunoassay. This results in equivalence of 0·1 μg rat FSH-RP-3 to 27 IU of IS 78/549. Immunoreactive inhibin was estimated by RIA according to

Robertson *et al.* (1988), using a bovine follicle fluid preparation with an arbitrary potency of 1 U/μg protein as a standard (Grootenhuis *et al.* 1989). Within-assay variation was 12%. Oestradiol-17β was estimated by RIA using kits provided by Diagnostic Products Corporation (Los Angeles, CA, USA); intra- and interassay variations were less than 5 and 8%. Progesterone was estimated by RIA using an antibody described by de Jong *et al.* (1974). Intra- and interassay variations were 16 and 17%.

Serum rhFSH concentrations (IU/l) were determined using a two-site FSH time-resolved fluoroimmunoassay (Delfia, Pharmacia, Woerden, The Netherlands) for human serum. Rat serum samples of untreated animals caused only background signals indicating that rat FSH is either not recognized or below the detection limit of the assay. When rat serum was spiked with rhFSH a linear log-dose–response curve parallel to the FSH kit standard (IS 78/549) was obtained. The detection limit of the assay applied for rat serum was 0·2 IU/l. The intra-assay variation was 4·3%.

Statistical procedures

Data are presented as mean ± S.E.M. Student's independent *t*-test was used to detect differences between saline-treated controls and rhFSH-treated groups. When all values in one group were zero or below the detection limit of the assay, a *t*-test against zero was performed. A difference was considered significant if the double-tail probability (P) was $\leq 0{\cdot}05$.

Results

Experiment 1

Effects of rhFSH on ovulation After injection of rhFSH on dioestrus-2, oestrus was found at the expected time in 19 out of 20 rats; in one rat treated with 2·5 IU rhFSH oestrus occurred 1 day early. Data from this rat were excluded from the study.

The effects of a single injection of rhFSH (dose 2·5, 5 or 10 IU) on dioestrus-2 at 1700 h on the number of ovulations observed on the subsequent day of oestrus are given in Table 1. The mean number of fresh CL in rhFSH-treated rats was not different from that in saline-treated rats; however, variation in the number of ovulations increased with increasing doses of rhFSH: superovulation but also partial ovulation occurred in a number of rats treated with a 5 IU or 10 IU rhFSH dose.

Ovarian histology on the day of oestrus revealed that in rhFSH-treated rats, apart from fresh CL and unluteinized or partly luteinized follicles with fragmented trapped ova, older CL and older CL with trapped ova were also present. Based on morphology, these older CL were more than 1 but less than 5 days old, i.e. they were generated during the previous cycle. When rats treated on dioestrus-2 at 1700 h with 10 IU rhFSH were caged together with males of proven fertility on the day of pro-oestrus, vaginal plugs were found in all rats the next morning, i.e. the day of oestrus.

TABLE 1. Ovulation rate (number of corpora lutea; CL) per two ovaries in rats on the day of oestrus, 65 h after an injection of recombinant human FSH (rhFSH) on dioestrus-2 at 1700 h. Control rats received saline. Values are means ± S.E.M.

	No. of rats	No. of fresh CL (range)
Dose of rhFSH (IU)		
Saline	5	13·0 ± 0·4 (12–14)
2·5	4	14·3 ± 0·6 (13–16)
5	5	12·0 ± 2·5 (6–21)
10	6	10·5 ± 4·3 (4–31)

In the group of rats killed at pro-oestrus, i.e. 41 h after injection, no signs of recent ovulation were found in any of the six saline-treated rats; however, in rhFSH-treated rats, less than 4-day-old CL, presumably resulting from ovulation during the night after injection, were often seen (Table 2). Low numbers of trapped ova were also found after all doses of rhFSH.

The group of rats injected with 10 IU rhFSH on dioestrus-2 and killed at 1000 h on dioestrus-3, showed a mean number of 13·3 ± 0·4 (12–14, n=6) fresh CL compared with the absence of ovulation in saline-treated controls (n=7). When these rats were caged together at dioestrus-2 with males of proven fertility, no vaginal plugs were found the next morning (dioestrus-3).

Follicle counts after injection of 2·5–10 IU of rhFSH at 1700 h on dioestrus-2 On dioestrus-3, 17 h after injection of 10 IU rhFSH, a significant increase ($P \leq 0{\cdot}01$) in the number of healthy class 1, 2 and 3 follicles was seen compared with saline-treated rats; class 5 follicles were absent (Fig. 2*a*). The total number of early atretic follicles

TABLE 2. Numbers of fresh[a] corpora lutea (CL) present in the ovaries of rats 41 h after injection of recombinant human FSH (rhFSH) at 1700 h on dioestrus-2. Values are mean ± S.E.M.

	No. of rats ovulation/ total no.	No. of fresh CL
Dose of rhFSH (IU)		
Saline	0/6	—
2·5	2/5	2·5 ± 1·5
5	7/7	9·1 ± 1·7
10	5/5	13·0 ± 0·6

[a]More than 1 but less than 4 days old.

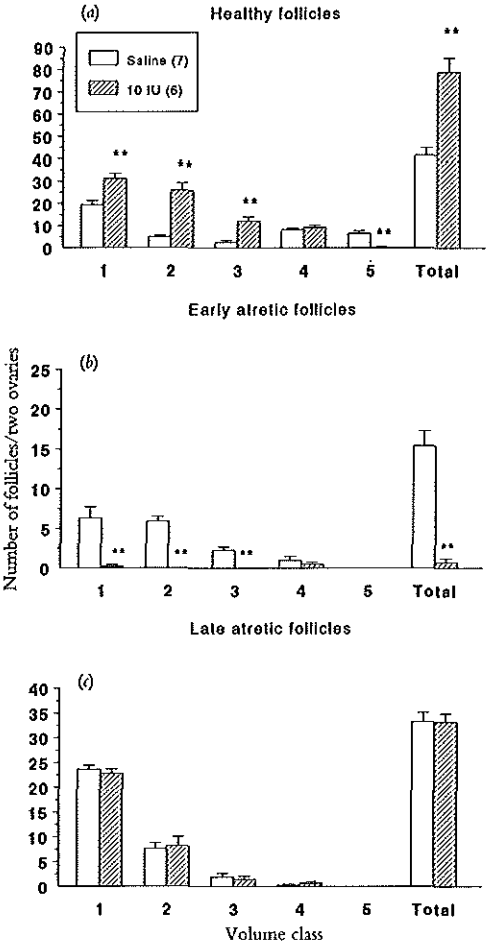

FIGURE 2. Mean ± S.E.M. number of antral follicles at 1000 h on dioestrus-3, 17 h after treatment of rats with saline or 10 IU recombinant human FSH (rhFSH) at 1700 h on dioestrus-2. (*a*) Number of healthy follicles, (*b*) number of early atretic follicles, and (*c*) number of late atretic follicles. $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ compared with saline-treated controls (Students *t*-test).

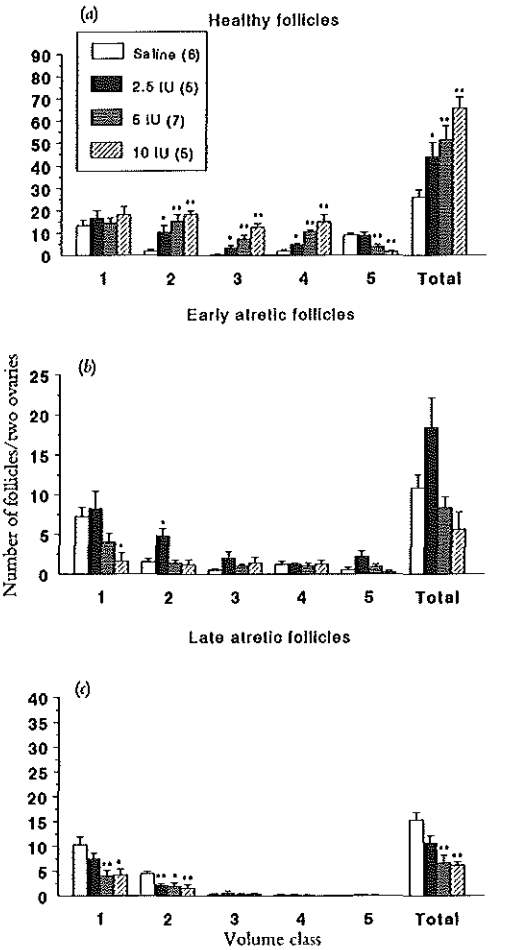

FIGURE 3. Mean ± S.E.M. number of antral follicles at 1000 h on pro-oestrus, 41 h after treatment with saline or 2·5, 5 or 10 IU recombinant human FSH (rhFSH) at 1700 h on dioestrus-2. (*a*) Number of healthy follicles, (*b*) number of early atretic follicles, and (*c*) number of late atretic follicles. $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ compared with saline-treated controls (Students *t*-test).

was significantly lower ($P \leq 0{\cdot}01$) and the number of late atretic follicles was not different in the rhFSH-treated rats, when compared with numbers in saline-treated controls (Fig. 2*b*,*c*). The number of early atretic follicles was on average 14·7 lower and the number of healthy follicles was 37·5 higher in the rhFSH-treated rats compared with saline-treated control rats, representing an extra recruitment of 22·8 follicles.

Follicle counts at pro-oestrus, i.e. 41 h after injection of 2·5, 5 or 10 IU rhFSH showed a dose-dependent increase ($P \leq 0{\cdot}05$) in the number of healthy class 2, 3 and 4 follicles compared with saline-treated controls; numbers of class 5 follicles decreased dose-dependently (Fig. 3*a*). There was no significant difference in the total number of early atretic follicles (Fig. 3*b*). The number of late atretic follicles was

reduced after treatment with 5 or 10 IU rhFSH (class 1 and 2) compared with the saline-treated group (Fig. 3*c*, $P \leq 0{\cdot}05$).

At oestrus, i.e. 65 h after injection of rhFSH (2·5, 5 and 10 IU) the number of healthy class 1 follicles (newly recruited) was lower after 5 ($P \leq 0{\cdot}05$) and 10 ($P \leq 0{\cdot}01$) IU rhFSH when compared with numbers in saline-treated controls. However, the number of healthy class 3 and 4 follicles was higher ($P \leq 0{\cdot}01$) after these doses of rhFSH (Fig. 4*a*). Early atresia after rhFSH treatment was generally higher, but late atresia (class 1 and 2) was lower than in controls ($P \leq 0{\cdot}05$, Fig. 4*c*).

Serum concentrations of FSH, LH, rhFSH, oestradiol-17β, inhibin and progesterone after injection of rhFSH Data on serum concentrations of rhFSH at various times after injection of 2·5, 5 or 10 IU rhFSH at 1700 h on dioestrus-2 are given in Fig. 5*a*. rhFSH concentrations were undetectable (<0·2 IU/l in terms of IS 78/549) in saline-treated control rats, were highest at 1000 h on dioestrus-3 in rhFSH-treated rats, and low on the days of pro-oestrus and oestrus. A dose–response relationship between the concentrations of rhFSH and the injected dose of rhFSH was found.

At 1000 h on dioestrus-3, higher ($P \leq 0{\cdot}05$) serum concentrations of rat FSH were found in rats injected with 10 IU rhFSH, compared with those in saline-treated controls (Fig. 5*b*). On the mornings of pro-oestrus (10 IU dose) and oestrus (5 and 10 IU doses) FSH concentrations were lower ($P \leq 0{\cdot}05$) in rats that had been treated with rhFSH than those in saline-treated controls.

A decrease ($P \leq 0{\cdot}01$) in serum oestradiol-17β concentration was found at pro-oestrus in the groups of rats treated with 5 and 10 IU rhFSH (Fig. 5*d*). At oestrus no significant difference was found between oestradiol levels in saline- and rhFSH-treated rats. The oestradiol-17β concentration was significantly higher in rats treated with 10 IU rhFSH (dioestrus-2 at 1700 h) at dioestrus-3 compared with saline-treated rats at oestrus. Both groups ovulated the night before.

Serum inhibin concentrations were increased ($P \leq 0{\cdot}01$) in the rats treated with 10 IU rhFSH compared with saline-treated rats at dioestrus-3 (Fig. 5*f*). No difference was found between saline- and rhFSH-treated rats at pro-oestrus. At oestrus the inhibin levels were increased ($P \leq 0{\cdot}05$) in rats treated with 2·5–10 IU rhFSH when compared with levels in saline-treated control rats.

Serum LH concentrations were lower ($P \leq 0{\cdot}01$) in rats treated with 10 IU rhFSH and killed at pro-oestrus when compared with saline-treated controls (Fig. 5*c*).

Finally, serum progesterone concentrations were increased ($P \leq 0{\cdot}01$) at pro-oestrus in rats treated with 10 IU rhFSH (Fig. 5*e*). At oestrus higher ($P \leq 0{\cdot}01$) serum concentrations of progesterone were seen in rats treated with either 5 or 10 IU rhFSH.

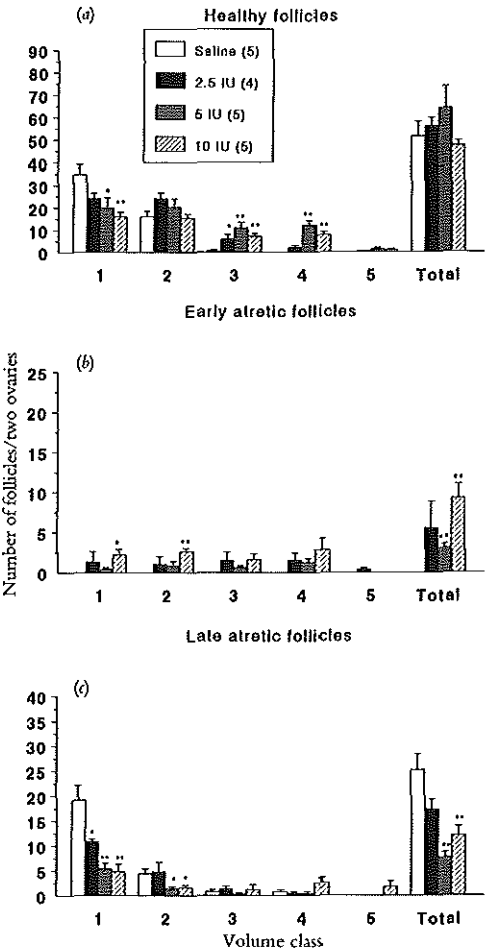

FIGURE 4. Mean ± S.E.M. number of antral follicles at oestrus 1000 h, 65 h after treatment with saline or 2·5, 5 or 10 IU of recombinant human FSH (rhFSH) at 1700 h on dioestrus-2. (*a*) Number of healthy follicles, (*b*) number of early atretic follicles, and (*c*) number of late atretic follicles. $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ compared with saline-treated controls (Students *t*-test).

Experiment 2

After injection with combinations of LHRH-a and either 10 IU rhFSH or 10 IU hFSH at dioestrus-2 the same number of tubal ova were found as with the FSH preparations alone at 1000 h on dioestrus-3. No tubal ova were found in the rats treated with saline (Table 3). No LHRH-a-treated control group was added because it is

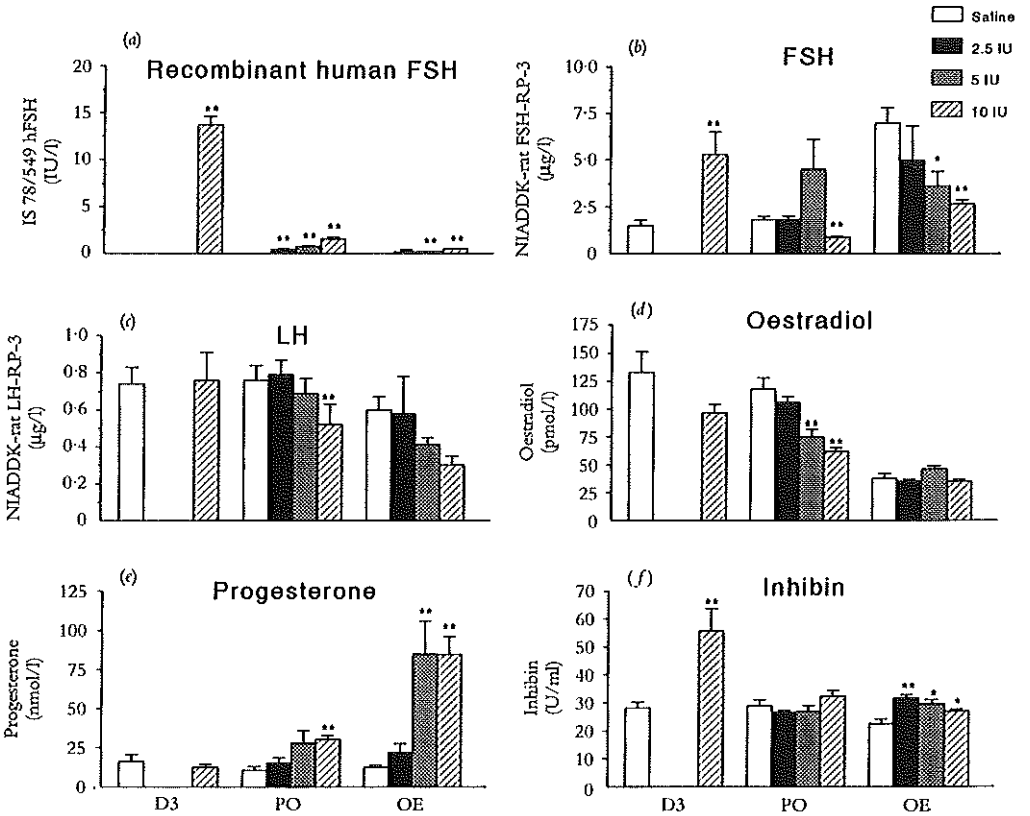

FIGURE 5. Mean ± S.E.M. hormone concentrations at 1000 h on the various days of the cycle after treatment with saline or 2·5, 5 or 10 IU recombinant human FSH (rhFSH) at 1700 h on dioestrus-2. (*a*) Concentration of rhFSH, (*b*) concentration of rat FSH, (*c*) concentration of LH, (*d*) concentration of oestradiol-17β, (*e*) concentration of progesterone, and (*f*) concentration of inhibin. D3, dioestrus-3; PO, pro-oestrus; OE, oestrus. $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ compared with saline-treated controls (Students *t*-test). Absence of bars means that no data were obtained, except for rhFSH in saline-treated controls.

known that administration of this amount of LHRH-a given on the morning of pro-oestrus blocks ovulation completely (Meijs-Roelofs *et al.* 1990).

Discussion

Recombinant human FSH, which increased ovarian weight and aromatase activity in hypophysectomized immature rats (Mannaerts *et al.* 1991), also provoked biological effects during the cycle of the intact rat. The effects depended on the dose and time after administration of rhFSH. A single injection of 10 IU rhFSH given at 1700 h on dioestrus-2 caused a significant increase in the number of small (class 1, 2, 3) antral follicles 17 h later. These follicles are apparently seen as class 2, 3 and 4 follicles at pro-oestrus; the non-ovulating smaller follicles are still present at oestrus. The absence of class 5 follicles, the largest antral follicles of ovulatory size, at 17 h after injection of rhFSH may be explained by the finding that a dose of 10 IU rhFSH acutely induced ovulation.

The results of the follicle measurements indicate that the increase of healthy class 1, 2 and 3 follicles 17 h after injection of rhFSH was partly due to a prevention of atresia in already developing healthy follicles, but also to increased inflow of follicles into the antral stage. The number of late atretic follicles was not different between the saline- and the rhFSH-treated group 17 h after

TABLE 3. Number of oocytes present the next morning (dioestrus-3) at 1000 h in the tubae of rats injected with 50 μg LHRH antagonist (LHRH-a) and either 10 IU recombinant human FSH (rhFSH) or purified human urinary FSH (hFSH) at 1700 h on dioestrus-2. Values are means ± S.E.M.

Treatment	No. of rats ovulating/ total no.	No. of tubal oocytes
Saline	0/7	—
rhFSH	6/6	11·3 ± 1·0
hFSH	7/7	11·0 ± 0·6
LHRH-a+rhFSH	7/7	10·7 ± 0·7
LHRH-a+hFSH	7/7	10·1 ± 0·7

injection. Since the stage of early atresia takes about 24 h (Osman 1985, van Cappellen *et al.* 1993), this means that follicles that were early atretic the day before were not rescued from atresia. This confirms the earlier findings of Hirshfield (1989).

Induction of ovulation by repeated administration of more or less pure FSH has been reported previously (Lostroh & Johnson 1966, Harrington & Elton 1969, Harrington *et al.* 1970, Tsafriri *et al.* 1976, Greenwald & Papkoff 1980, Taya & Greenwald 1980). In a recent study Galway *et al.* (1990) reported induction of ovulation with rhFSH in hypophysectomized immature rats, pretreated with pregnant mare serum gonadotrophin (PMSG) or rhFSH. Ovulation induction with a dose of 1 IU of an rhFSH preparation was also found in cyclic mice which were pretreated with rhFSH or PMSG (Rice *et al.* 1993). In the present study, acute ovulation induction by single doses of 5–10 IU rhFSH was found 48 h before the normal LH and FSH peaks, during the cycle of adult non-pretreated rats. Even with a dose as low as 1·25 IU some rats would ovulate (R. de Leeuw, unpublished data). During this precocious ovulation observed here there was a dissociation between ovulation and behaviour shown by the absence of vaginal plugs in rats caged with males. This dissociation is probably due to different hormonal conditions during this precocious ovulation compared with a normal ovulation and was not seen in FSH-pretreated cyclic mice in which ovulation was induced by rhFSH (Rice *et al.* 1993). The decrease of the oestradiol concentration normally seen between pro-oestrus and oestrus does not seem to take place between dioestrus-2 and dioestrus-3 when the precocious rhFSH-induced ovulation takes place. In spite of the observed differences between the normal and the precocious ovulation, the CL formed during the latter ovulation are morphologically normal and produce progesterone concentrations comparable with the normal pattern (Toorop *et al.* 1982).

The mechanism of the precocious ovulation induced by rhFSH remains unclear. Induction of an LH peak very quickly after the injection of FSH is not likely in view of the short interval between the FSH injection and ovulation; induction of an LH peak by treatment with oestradiol takes at least 24 h (Everett 1947, Brown-Grant 1969). The non-involvement of an LH surge was ascertained in experiment 2 where an LHRH-a preparation was used to suppress the possible occurrence of a precocious LH peak. This did not influence the number of ovulated oocytes at dioestrus-3. The recombinant nature of the FSH used was not a contributing factor in the precocious ovulations, since there was no difference in number of oocytes found after treatment with either rhFSH or a purified human urinary FSH preparation. The question remains as to whether this ovulation-inducing effect is typical for human FSH preparations used in rat studies, or whether it is merely due to the pattern of FSH concentrations after injection of an exogenous FSH preparation. This problem can only be resolved when rat recombinant FSH becomes available.

An interpretation of the inter-relationships between the data on ovulation and follicle growth and the observed hormone concentrations can only be tentative, since the hormone values were obtained at intervals of 24 h and because of the partial or complete absence of advanced ovulation in the groups treated with 2·5 and 5 IU rhFSH. Therefore, only the group of rats treated with 10 IU rhFSH will be considered. The higher concentration of endogenous serum FSH 17 h after injection could be explained by the disappearance of the largest follicles due to ovulation, because these large follicles are the main source of the FSH-secretion inhibitors oestradiol and inhibin. These high levels of endogenous FSH after ovulation and the still high circulating levels of rhFSH probably stimulated the inflow of small antral follicles. These newly recruited follicles together with the remaining class 3 and 4 follicles would then start to produce inhibin in response to the high exo- and endogenous FSH levels (Lee *et al.* 1982), causing the increased levels of inhibin seen at 1000 h on dioestrus-3. Because immunologically active inhibin was measured it is not clear whether these higher inhibin levels could have had a biological effect or were due to free α-inhibin subunits. The discrepancy between the high circulating levels of endogenous FSH and the high levels of inhibin could also be a question of timing. Because no intermediate samples were taken it cannot be ruled out that the serum FSH concentration is decreasing while serum inhibin and oestradiol concentrations are increasing. Finally, rhFSH could be metabolized in rat serum to a form that may cross-react in the rat FSH assay. The induction of ovulation and the extra recruitment of follicles seen at dioestrus-3 probably resulted at pro-oestrus in a very high number of intermediate (especially class 2 and 3)-sized follicles and a severely decreased number of class 5 antral follicles. This may account for the lower concentration of oestradiol which, in turn, may have caused a subnormal LH peak, much lower in fact than that normally seen at

this stage of the cycle. Also the increased progesterone concentration, seen at pro-oestrus and probably due to the steroid production of the CL formed at dioestrus-3, may have caused the decreased serum LH concentrations at pro-oestrus. This combination of altered hormonal circumstances and the lack of viable follicles of the correct size caused an abnormal ensuing ovulation at oestrus in the rhFSH-treated rat, as reflected by the wide range of numbers of CL. In view of earlier findings (for review see Rothchild 1965) it is surprising that these animals ovulate at all since the progesterone concentration is significantly increased. After ovulation, a significantly higher number of class 3 and 4 follicles remained in the ovaries. This could explain the higher serum inhibin concentration found at oestrus. This higher inhibin concentration may finally have caused the low serum FSH values seen at oestrus which may, in turn, have resulted in a lower number of newly recruited class 1 follicles.

On the basis of the data on occurrence of ovulation and follicle growth, it is concluded that in the rat the administration of 5–10 IU rhFSH on dioestrus-2 has an acute ovulation-inducing effect. Apart from this effect, recruitment of new antral follicles takes place and atresia of antral follicles is inhibited. However, follicles that are already atretic will not reverse this process after a single injection of rhFSH. Finally, the acute ovulation-inducing effects of rhFSH or purified human urinary FSH do not result from a stimulation of LH concentrations.

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Chapter 7

Induction of superovulation in cyclic rats by administration of decreasing dosis of recombinant follicle stimulating-hormone (Org32489)

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Induction of superovulation in cyclic rats by administration of decreasing doses of recombinant follicle stimulating hormone (Org32489)

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The objective of this study was to set up a superovulation protocol in adult cyclic rats by using recombinant human follicle stimulating hormone (rhFSH; Org32489). Good results were obtained by treatment with decreasing doses of rhFSH (2.5 to 0.5 IU) during the dioestrus period. The number of corpora lutea (CL) found in rats treated with this protocol was 43.5 ± 3.4; this is more than three times the number in saline-treated control rats (13.0 ± 0.4). Fertilization of oocytes after superovulation was as good as after normal ovulation in terms of number of 2-cell stage embryos found 2 days after mating. The absolute number of implantations was twice the number observed in saline-treated control rats (23.3 ± 1.8 versus 10.6 ± 0.5); therefore the number of implantations per CL was lower in superovulated rats. The serum concentrations of luteinizing hormone (LH), endogenous FSH and oestradiol-17β were decreased during rhFSH treatment, while the inhibin serum concentration was increased. The progesterone serum concentration was increased on the days of pro-oestrus and oestrus after treatment. No difference was observed in the testosterone serum concentration. Pretreatment with 10 IU rhFSH at oestrus before giving the decreasing doses of rhFSH during dioestrus reduced the ovulatory response. Finally, treatment with a constant low dose of rhFSH instead of a decreasing dose of rhFSH did not result in spontaneous ovulation. However, ovulation induction by means of a human chorionic gonadotrophin bolus resulted in superovulation in six out of eight rats. It is concluded that superovulation in cyclic rats can be achieved using rhFSH treatment. However, it was found that the type of rhFSH regimen was very important to achieve appropriate stimulation. The optimal protocol was treatment with decreasing doses of rhFSH during dioestrus. The oocytes retrieved could be fertilized as well as oocytes of saline-treated control rats. The results also indicate that treatment with higher doses of rhFSH might induce a desensitization for FSH and LH.

Key words: adult rat/follicle development/FSH/recombinant FSH/superovulation

Introduction

Many studies have been performed in the rat on the subject of superovulation, defined as ovulation of more than twice the normal number of oocytes, but very few studies have been performed in the adult rat (Sameshima *et al.*, 1982; Sander *et al.*, 1991; Akira *et al.*, 1993; Hayes *et al.*, 1993; Szoltys *et al.*, 1994). Most studies were based on treatment with a combination of pregnant mare's serum gonadotrophin (PMSG)/human chorionic gonadotrophin (HCG). One of the disadvantages of this treatment is the long half-life of PMSG, which interferes with normal embryo formation (Miller and Armstrong, 1981). As treatment of the infertile human patient can now be performed with recombinant human follicle stimulating hormone (rhFSH), we have tried to develop a superovulation protocol in adult cyclic rats using rhFSH in order to study the hormonal and morphological development of follicles. In addition, we have studied the effect of this treatment on early embryonic development.

In an earlier study (van Cappellen *et al.*, 1995a), it was found that injection of 10 IU rhFSH in cyclic rats at 17.00 h on dioestrus-2 resulted in a precocious ovulation during the following night. This model was used in this study to test for changed sensitivity of developing follicles towards rhFSH.

Materials and methods

Animals

Female rats of a Wistar (R-Amsterdam) strain with a 5-day oestrous cycle and a body weight of 170–210 g were used. They were kept under conditions of controlled temperature (23 ± 2°C) and light (lights on from 05.00 to 19.00 h) and had free access to standard dry pellets and tap water. Adult littermates were randomly divided over the treatment groups. Vaginal smears were taken every morning and rats were used only after at least two successive 5-day oestrous cycles. For all experiments, approval was obtained from the Animal Welfare Committee (DEC) of the Erasmus University.

Histology and counting of follicles

Rats were killed by ether anaesthesia after blood sampling by puncture of the ophthalmic venous plexus. Serum samples were stored at −20°C until assayed. Tubal ova or implantation sites were counted macroscopically and ovaries and uteri were excised and weighed. Ovaries and uteri were fixed in Bouin's fluid, embedded in paraffin wax, serially sectioned (10 mm) and stained with haematoxylin and eosin. Ovulation rate was confirmed histologically by counting the number of fresh corpora lutea (CL) in the ovaries. The numbers of implantation sites were confirmed by histological examination of the uteri.

Differential counts of antral, healthy and atretic follicles were made according to the method of Meijs-Roelofs *et al.* (1982). Volume

classes and diameters for antral follicles were defined as follows: small antral follicles, $100–500\times10^5$ μm^3 and 260–450 μm; large antral follicles, $>500\times10^5$ μm^3 and >450 μm respectively. Atretic follicles were designated as 'early atretic' if local or widespread pyknosis and cell shrinkage were present in the granulosa cell wall and as 'late atretic' if the oocyte was degenerating and either surrounded by degenerating cumulus cells or no cumulus cells at all (Osman, 1985).

Hormone determinations

Concentrations of endogenous FSH and luteinizing hormone (LH) were estimated by radioimmunoassay with anti-ovine FSH and LH as antisera and rat FSH/LH as tracer, and expressed as mg NIADDK-rat FSH-RP-3/l and mg NIADDK-rat LH-RP-3/l (Sander *et al.*, 1986). Within-assay variations were 4% (FSH) and 5% (LH). Cross-reactivity of rhFSH in the rat FSH assay amounted to 2.6% on basis of weight; this results in equivalence of 0.1 mg of rat FSH RP3 to 27 IU of IS 78/549 (van Cappellen *et al.*, 1995a).

Immunoreactive inhibin was estimated by radioimmunoassay according to Robertson *et al.* (1988), using a bovine follicle fluid preparation with an arbitrary potency of 1 U/mg protein as a standard (Grootenhuis *et al.*, 1989). Within-assay variation was 12%. Oestradiol-17β was estimated by radioimmunoassay using kits provided by Diagnostic Products Corporation (Los Angeles, CA, USA); intra- and inter-assay variations were <5 and 8% respectively. Progesterone was estimated by radioimmunoassay as described by de Jong *et al.* (1974). Intra- and inter-assay variations were <16 and 17% respectively. Testosterone was measured by radioimmunoassay as described by Verjans *et al.* (1973). The intra-assay variation was 3%.

Serum rhFSH concentrations (U/l) were determined using a two-site FSH time-resolved fluoroimmunoassay (Delfia, Pharmacia, Woerden, The Netherlands) for human serum. Rat serum samples of saline-treated animals caused only background signals, indicating that rat FSH is either not recognized or below the detection limit of the assay. When rat serum was spiked with rhFSH, a linear log dose–response curve parallel to that of the human FSH standard (IS 78/549) was obtained. The detection limit of the assay applied for rat serum was 0.2 IU/l. The intra-assay variation was 4.3%.

Early and mid-cycle treatment (experiment 1)

Following the protocol used earlier to induce superovulation in cyclic rats by means of PMSG (Welschen and Rutte, 1971), rats were injected i.m. with 10 IU of rhFSH [Org32489; batch IP 190/0824 from Organon, Oss, The Netherlands; International units (IU) refer to in-vivo bioactivity in terms of IS 71/223] at oestrus at 17.00 h and/or dioestrus-2 at 17.00 h to induce ovulation. Rats in experiment 1a were injected with 10 IU rhFSH at oestrus, 17.00 h and killed 17 h later at dioestrus-1, 10.00 h (see Table I). Rats in experiment 1b were injected at dioestrus-2, 17.00 h with either 10 IU rhFSH or saline and killed 17 h later at dioestrus-3, 10.00 h. Animals in experiment 1c were injected at oestrus, 17.00 h with 10 IU rhFSH and at dioestrus-2, 17.00 h either with 10 IU rhFSH or 10 IU HCG and killed 17 h later at dioestrus-3, 10.00 h. After routine histology, the number of follicles and the number of fresh CL were counted at the day of ovariectomy for all these groups.

Dioestrus treatment with decreasing rhFSH doses (experiment 2)

Because the first treatment did not result in superovulation, a second treatment protocol was set up. The aim of this protocol was to rescue follicles from atresia by giving repeated injections of rhFSH. Treatment was started at dioestrus-1 with a low dose of 2.5 IU rhFSH to prevent precocious ovulation, and this dose was decreased even further thereafter for the same reason. In a first experiment (experiment 2a, Table I) groups of rats were treated with i.m. injections of rhFSH using the following scheme: dioestrus-1, 10.00 h (2.5 IU), 17.00 h (1 IU); dioestrus-2, 10.00 h (1 IU), 17.00 h (0.5 IU) and dioestrus-3, 10.00 h (0.5 IU), 17.00 h (0.5 IU) or saline and were killed at 10.00 h on dioestrus-1 (controls $n = 6$), dioestrus-2 ($n = 7$, controls $n = 6$), dioestrus-3 ($n = 6$, controls $n = 7$), pro-oestrus ($n = 6$, controls $n = 6$) or oestrus ($n = 8$, controls $n = 5$). Rats killed at dioestrus-1, -2 or -3 did not receive the rhFSH injection of that morning. Ovaries were fixed and the follicles counted. Blood samples were taken at the time of ovariectomy and hormone concentrations were measured.

Additional groups of rats, treated according to this protocol, were placed with males of proven fertility on the day of pro-oestrus. These rats were checked for the presence of vaginal plugs the next morning. If a plug was present, rats were allowed to live for 2 ($n = 7$, controls $n = 7$), or for 7 ($n = 7$, controls $n = 7$) more days. Both oviducts from the rats killed 2 days after mating were flushed with saline and the flushings were examined for the presence of fertilized ova (2-cell stage embryos). The ovaries were fixed for histological examination of the number of CL. Rats that were killed 7 days after mating were examined for the number of implantation sites in both uterine horns. Ovaries were fixed for histological examination of the number of CL.

To test the effect of pretreatment with rhFSH on superovulation, a second group of rats (experiment 2b) was first treated with 10 IU of rhFSH at oestrus, 17.00 h and thereafter with the decreasing dosage scheme as described for experiment 2a. These rats were killed 5 days after the first injection on the expected day of oestrus at 10.00 h ($n = 5$). Ovaries were dissected and fixed and the numbers of CL were counted.

To investigate the sensitivity of the developing follicles (treated with decreasing rhFSH doses) to the ovulation-inducing effect of a large dose of rhFSH, a third group of rats (experiment 2c) was treated with decreasing doses of rhFSH using the following scheme: dioestrus-1, 10.00 h (2.5 IU), 17.00 h (1 IU); dioestrus-2, 10.00 h (1 IU). These rats received an injection of 10 IU rhFSH at 17.00 h at dioestrus-2 to induce ovulation and were killed at dioestrus-3, 10.00 h ($n = 6$). Ovaries were dissected and fixed and the number of CL were counted.

Dioestrus treatment with a constant rhFSH dose (experiment 3)

To investigate if decreasing doses of rhFSH are necessary to induce superovulation, rats were treated from dioestrus-1 until dioestrus-3, at 10.00 h and 17.00 h with 2.5 IU rhFSH. At the expected day of pro-oestrus at 17.00 h, rats were injected with saline (experiment 3a, $n = 8$, Table I) or with 10 IU HCG (experiment 3b, $n = 8$). The rats were killed 17 h later, and the ovaries were dissected and fixed for counting of the number of CL.

Statistical procedures

Data are presented as means ± SEM. Student's independent *t*-test was used to detect statistically significant differences between the control and rhFSH-treated groups. When all values in one group were zero or below the detection limit of the assay, a *t*-test against zero was performed. When results from more than two groups were compared, the data were analysed by the Tukey multiple comparison test. A difference was considered statistically significant if the double tail probability (P) was ≤ 0.05

Results

Early and mid-cycle treatment (experiment 1)

None of the rats in experiment 1a ($n = 4$) ovulated on dioestrus-1 after an injection of 10 IU rhFSH at oestrus,

Table I. Treatment protocols of the different groups. Saline-treated controls were injected at the same time points whenever relevant

Experiment no.	Oestrus		Dioestrus-1		Dioestrus-2		Dioestrus-3		Pro-oestrus		Oestrus	
	10.00 h	17.00 h	10.00 h	17.00 h	10.00 h	17.00 h	10.00 h	17.00 h	10.00 h	17.00 h	10.00 h	17.00 h
1a		F_{10}	O									
1b						F_{10}	O					
1c	F_{10}					F_{10}	O					
1c	F_{10}					H_{10}	O					
2a			F_2^1	F_1	O							
2a			F_2^1	F_1	F_1	F_2^1	O					
2a			F_2^1	F_1	F_1	F_2^1	F_2^1	F_2^1	O			
2a			F_2^1	F_1	F_1	F_2^1	F_2^1	F_2^1			O	
2b		F_{10}	F_2^1	F_1	F_1	F_2^1	F_2^1	F_2^1			O	
2c			F_2^1	F_1	F_1	F_{10}	O					
3a			F_{22}^1	F_2^1	F_{22}^1	F_2^1	F_{22}^1	F_{22}^1			O	
3b			F_{22}^1	F_2^1	F_{22}^1	F_2^1	F_{22}^1	F_{22}^1		H_{10}	O	

F = Recombinant human follicle stimulating hormone; H = human chorionic gonadotrophin; O = ovariectomy; subscript is dose of recombinant human follicle stimulating hormone used (IU).

Table II. Numbers of ovulating rats, of corpora lutea (CL) and of healthy and early atretic follicles on dioestrus-3 in rats treated with combinations of saline (Sal), 10 IU of recombinant human follicle stimulating hormone (rhFSH) and 10 IU of human chorionic gonadotrophin (HCG). Values are means ± SEM

Experiment no.	Treatment day		No. of ovulating rats total	Follicle counts on the day of ovariectomy			No. of CL
	Oestrus	D2		Healthy follicles		Early atretic	
				Small antral	Large antral	Total antral	
1b	Sal	Sal	0/7	26.7 ± 3.1[a]	15.0 ± 0.8[a,b]	15.4 ± 1.9[a]	0[a]
	Sal	rhFSH	6/6	69.5 ± 6.2[b]	9.7 ± 1.2[a]	0.7 ± 0.5[b]	13.3 ± 0.4[b]
1c	rhFSH	rhFSH	0/4	58.5 ± 8.7[b]	25.0 ± 9.9[b,c]	23.0 ± 5.9[a,c]	0[a]
	rhFSH	HCG	0/5	53.2 ± 5.5[b]	29.4 ± 0.7[c]	33.8 ± 5.6[c]	0[a]

[a,b,c]Statistical significance was determined by analysis of variance. Values differing significantly ($P < 0.05$) do not share a common superscript.
D2 = dioestrus-2.

17.00 h (data not shown). All rhFSH-treated rats in experiment 1b ovulated precociously in response to the rhFSH injection at dioestrus-2, 17.00 h (Table II). In addition, there was a significantly increased number of small antral healthy follicles in the rhFSH-treated rats when compared to saline-treated controls. After pretreatment with 10 IU rhFSH at oestrus, no ovulations were observed after a second rhFSH injection at 17.00 h on dioestrus-2. Also, an injection of 10 IU HCG at 17.00 h on dioestrus-2 did not result in ovulation in the rhFSH-pretreated rats (Table II). Both groups had a significantly larger ($P \leq 0.05$) number of small antral follicles compared to saline-treated control rats (Table II).

Dioestrus treatment with decreasing rhFSH doses (experiment 2)

Numbers of ovulations

Rats treated with decreasing doses of rhFSH (experiment 2a) ovulated spontaneously at the expected day of oestrus with a significantly increased number of ovulations when compared to saline-treated controls (Table III). Pretreatment with 10 IU rhFSH at oestrus (experiment 2b) resulted in significantly lower ($P \leq 0.05$) numbers of ovulations when compared to rats that were not pretreated (experiment 2b compared to experiment 2a). However, the number of ovulations was higher than in saline-treated control rats (Table III, controls compared to experiment 2b). Injection of 10 IU rhFSH at dioestrus-2, 17.00 h in the group of rats treated with decreasing doses of rhFSH resulted in absence or very low number of ovulations at dioestrus-3, 10.00 h (experiment 2c, Table III).

Numbers of follicles

Because of the effectiveness of the induction of ovulation with decreasing doses of rhFSH, numbers of follicles and hormone concentrations in experiment 2a were studied in more detail by killing groups of rats on various days after the start of treatment.

Treatment with a decreasing dose of rhFSH showed a significant increase in the number of small antral follicles on dioestrus-3 and pro-oestrus compared to saline treatment

Table III. Numbers of ovulating rats and corpora lutea (CL) after treatment with decreasing doses (see text) of recombinant human follicle stimulating hormone (rhFSH). Values are means ± SEM

Experiment	rhFSH treatment	Day of ovariectomy	No. of rats ovulating	No. of CL
Control	—	OE	5/5	13.0 ± 0.4[a,b]
2a	decreasing from D1	OE	8/8	43.5 ± 3.4[c]
2b	10 IU OE; decreasing from D1	OE	3/4	22.7 ± 3.8[b]
2c	decreasing from D1; 10 IU D2	D3	2/6	3.0 ± 2.0[a]

[a,b,c]Statistical significance was determined by analysis of variance. Values differing significantly ($P < 0.05$) do not share a common superscript. OE = oestrus; D1–D3 = dioestrus 1–3.

Table IV. Fertilization and implantation of oocytes ovulated after treatment with decreasing doses (see text) of recombinant human follicle stimulating hormone (rhFSH), between dioestrus-1 and dioestrus-3. Values are means ± SEM

	Control	rhFSH
No. of 2-cell ova 2 days after mating	8.9 ± 0.3	34.3 ± 1.6**
No. of 2-cell ova/CL (%)	81 ± 3	83 ± 2
No. of intact, 1-cell ova 2 days after mating	1.4 ± 0.3	3.6 ± 0.5**
No. of 1-cell ova/CL (%)	13 ± 2	9 ± 1
No. of fragmented oocytes 2 days after mating	0.7 ± 0.4	3.0 ± 0.8*
No. of fragmented oocytes/CL (%)	6 ± 3	7 ± 2
No. of implantations 7 days after mating	10.6 ± 0.5	23.3 ± 1.8**
No. of implantations/CL (%)	91 ± 5	48 ± 6**

*$P \leq 0.05$; **$P \leq 0.01$, t-test. CL = corpora lutea.

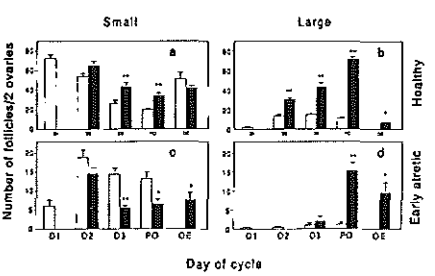

Figure 1. Numbers of healthy and early atretic follicles in rats treated with decreasing doses of recombinant human follicle stimulating hormone (rhFSH: D1, 10.00 h, 2.5 and 17.00 h, 1 IU; D2, 10.00 h, 1 and 17.00 h, 0.5 IU; D3, 10.00 h, 0.5 and 17.00 h, 0.5 IU; closed bars) and in saline-treated control rats (open bars). Small antral follicles had a volume between 100 and 500×10^5 μm^3 (diameter 265–450 μm). Large antral follicles had a volume $>500 \times 10^5$ μm^3. *$P \leq 0.05$, **$P \leq 0.01$, Student's t-test between rhFSH-treated and saline-treated control rats on the same day. D1–D3 = Dioestrus 1–3; PO = pro-oestrus; OE = oestrus.

(Figure 1a). The number of large antral follicles was significantly increased from dioestrus-2 to pro-oestrus and on oestrus (Figure 1b). The number of small early atretic follicles was significantly lower on dioestrus-3 and pro-oestrus and oestrus (Figure 1c). The number of large early atretic follicles was significantly higher on pro-oestrus and oestrus (Figure 1d).

Oocyte quality

The absolute number of fertilized ova (2-cell stage) was almost 4-fold higher ($P \leq 0.01$) in rhFSH-treated rats (Table IV) when compared to that in saline-treated control rats. However, the ratio of fertilized ova to number of CL was not different. The absolute numbers of intact (1-cell) and fragmented oocytes were significantly higher ($P < 0.01$ and $P < 0.05$ respectively) in rhFSH-treated rats when compared to saline-treated control rats, whereas the ratios of ova to number of CL and fragmented oocytes to number of CL were not different. The ratio of number of implantation sites to number of CL in rhFSH-treated rats was lower compared to that in saline-treated control rats ($P < 0.01$, Table IV), but the absolute number of implantation sites was significantly higher ($P < 0.01$, Table IV).

Hormone concentrations

The serum concentration of endogenous FSH was decreased ($P \leq 0.05$) in rhFSH-treated rats (experiment 2a) from dioestrus-2 until pro-oestrus (Figure 2a) when compared to that in saline-treated control rats. The rhFSH serum concentration found at dioestrus-2 was significantly higher ($P < 0.01$) than that found on subsequent days of rhFSH treatment. At oestrus the rhFSH concentration found in rhFSH-treated rats was not significantly different from the detection limit of the assay (Figure 2c). The LH concentration was significantly decreased from dioestrus-2 until pro-oestrus in rhFSH-treated rats when compared to saline-treated control rats (Figure 2b). At oestrus the concentrations were no longer significantly different. Progesterone concentrations were not different between rhFSH-treated and saline-treated control rats until pro-oestrus, when progesterone was increased in the rhFSH-treated rats (Figure 2d). The serum oestradiol concentration was decreased ($P \leq 0.05$) on dioestrus-2 and -3 in rhFSH-treated rats (Figure 2e), but at pro-oestrus and oestrus no significant difference was found. Serum inhibin was increased ($P \leq 0.01$) from dioestrus-2 until pro-oestrus in rhFSH-treated rats (Figure 2f). At oestrus the inhibin concentrations were no longer significantly different. No differences were found in the testosterone concentration between rhFSH-treated and saline-treated control rats (Figure 2g).

Dioestrus treatment with a constant rhFSH dose (experiment 3)

When rats were treated with 2.5 IU rhFSH at 10.00 h and 17.00 h from dioestrus-1 until dioestrus-3 (six injections in total), no precocious ovulations were detected. Only two out of eight rats had ovulated 2 days after the last injection on the expected day of oestrus. The numbers of ova found were 46 and 47 respectively. When ovulation was induced by 10 IU HCG given at 17.00 h 1 day after the last rhFSH injection (the expected day of pro-oestrus) in a group of rats with the

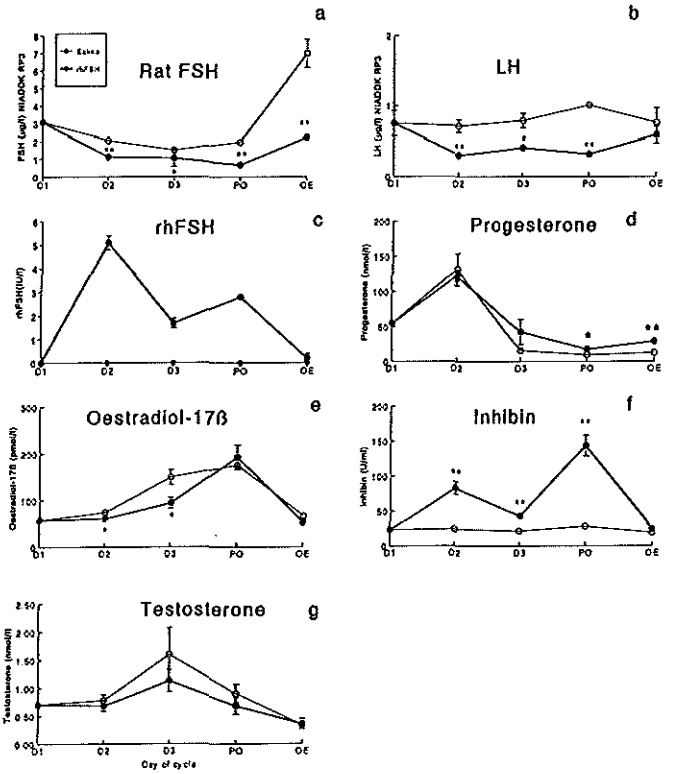

Figure 2. Hormone concentrations in serum after treatment with decreasing doses of recombinant human follicle stimulating hormone (rhFSH: D1, 10.00 h, 2.5 and 17.00 h, 1 IU; D2, 10.00 h, 1 and 17.00 h, 0.5 IU; D3, 10.00 h, 0.5 and 17.00 h, 0.5 IU rhFSH; closed circles) and saline-treated control rats (open circles). $*P \leq 0.05$, $**P \leq 0.01$, Student's *t*-test between rhFSH-treated and saline-treated control rats on the same day. D1–D3 = Dioestrus 1–3; PO = pro-oestrus; OE = oestrus.

same treatment, six out of eight rats ovulated. The mean number (±SEM) of ova found was 37.5 ± 6.3.

Discussion

Many studies on superovulation have been performed in rats. Most of these studies used a PMSG/HCG combination in prepubertal rats, where high numbers of ovulations were found. However, fertilization of the superovulated oocytes yielded rather poor results (Miller and Armstrong, 1981), probably because of adverse hormonal conditions in these rats due to the long-lasting high circulating concentrations of PMSG, resulting in high oestradiol concentrations. In contrast, it is rather difficult to obtain superovulation in adult cyclic rats due to different reactions to hormonal treatment on various days of the ovarian cycle. In this respect, the rat differs from the mouse in which (super)ovulation can be induced on random days of the ovarian cycle (Fowler and Edwards, 1957; Edwards and Fowler, 1960). The problem in rats can be overcome by synchronizing the animals and suppressing the endogenous gonadotrophin secretion by treatment with an luteinizing hormone-releasing hormone antagonist (Hamilton and Armstrong, 1991), which, however, interferes with the ovarian–pituitary feedback loop that plays a major role in the regulation of follicle development. This would make it also impossible to study the direct effects of increased amounts of FSH on follicle development, ovulation and the ovarian–pituitary feedback loop, which was the aim of this study.

In an earlier study it was found that one single injection of 10 IU rhFSH, administered at 17.00 h on the day of dioestrus-2, induced ovulation in 5-day cyclic rats during the night following treatment (van Cappellen *et al.*, 1995a), as seen in experiment 1b. No superovulation was obtained. To mimic superovulation protocols using PMSG (Welschen and Rutte, 1971), rats were treated with 10 IU rhFSH at oestrus to stimulate follicle growth and again at dioestrus-2, 17.00 h to induce ovulation (experiment

1c). However, none of these rats ovulated. Also, 10 IU of HCG given at dioestrus-2, 17.00 h failed to induce ovulation. Apparently, the large antral follicles that grew under these hormonal conditions did not develop a normal ovulatory response system. Because superovulation failed in this system no detailed hormonal and follicular measurements were made.

When 5-day cyclic rats were treated with decreasing doses of rhFSH during the period of dioestrus, spontaneous ovulation of an increased number of follicles occurred in all rats. Decreasing doses of rhFSH were chosen because of the ovulation-inducing effect of rhFSH, an effect that was already found in some rats after a single injection of 1.25 IU rhFSH at dioestrus-2 (van Cappellen *et al.*, 1995a). This ovulation-inducing effect of FSH was also found using a purified FSH preparation (NIHFSH-S6; Harrington and Elton, 1969). Treatment with decreasing doses of rhFSH resulted in an increase in the number of healthy antral follicles and a decrease in the number of early atretic follicles. However, an increase of follicle recruitment is the most important factor responsible for the increased number of healthy follicles in this superovulation cycle, because the reduction in number of atretic follicles is much lower than the increase in the number of recruited follicles (Figure 1). This is in agreement with the earlier findings of Peppler and Greenwald (1970). On pro-oestrus and oestrus the number of early atretic large follicles increased to values higher than seen in saline-treated control rats. The increase in number of large antral follicles, the follicles that contain oestradiol and inhibin (van Cappellen *et al.*, 1995b), was not reflected in an increased peripheral oestradiol concentration. This is probably due to the decreased LH concentrations and is in agreement with the two cell/two gonadotrophin hypothesis, which postulates that lowering the LH concentration lowers the androgen production by the theca cells which will decrease the production of oestrogens by the granulosa cells, even when the FSH concentration is high. The high inhibin concentrations may also have had an effect on the aromatase activity, as inhibin can reduce the FSH-stimulated aromatase activity in rat granulosa cell primary cultures (Ying *et al.*, 1986). The low LH concentration cannot be explained on the basis of increased amounts of progesterone or testosterone, but may be due to another factor secreted by the ovary under the influence of FSH, i.e. gonadotrophin surge inhibiting factor (Littman and Hodgen, 1984; de Koning *et al.*, 1989). Serum inhibin concentrations were strongly increased after rhFSH treatment; this may explain the reduced concentrations of rat FSH found in serum.

Pretreatment with rhFSH at oestrus reduced the ovulation-inducing effect of rhFSH at dioestrus-2 (experiment 1c). This effect was found again after pretreatment at oestrus with 10 IU rhFSH, followed by injection of decreasing doses of rhFSH during dioestrus. Most of the rats treated in this way ovulated, but although the number of ovulations was higher in rhFSH-treated rats than in saline-treated control rats, this number was lower than that in rats which were not treated with rhFSH at oestrus (Table III). Also, rats treated with decreasing doses of rhFSH did not ovulate precociously after a preovulatory dose of 10 IU rhFSH given at 17.00 h on dioestrus-2. Because these results indicate that pretreatment with rhFSH might result in a loss of sensitivity of follicles towards rhFSH, a group of rats was injected twice daily with 2.5 IU rhFSH during the dioestrus period. None of these rats ovulated precociously. Moreover, only two out of eight rats ovulated spontaneously at the expected time. This lack of spontaneous ovulations could be due to an insufficient LH peak, because a single dose of HCG increased the ovulatory response to six out of eight rats. Because these latter protocols did not improve the number of ovulations, no detailed hormonal and follicular profiles were measured. The reason for the reduced ovulatory response could be a reduced number of LH receptors due to the lower peripheral LH concentration (Richards, 1980). The reduced sensitivity towards FSH could be explained on the basis of a decreased number of FSH receptors due to decreased oestradiol concentrations inside the follicles (Tonetta *et al.*, 1985). In rats treated with decreasing doses of FSH an increased number of large antral follicles was seen in combination with an equal or decreased oestradiol concentration, depending on the day of the cycle when compared to controls. This means that the oestradiol secretion per large antral follicle is severely decreased. Under the experimental conditions of most protocols used in this study, treatment could result in reduction of both the numbers of LH and FSH receptors, which could lead to decreased responses to both LH and FSH. This was shown by the decreased ovulatory response after an injection of 10 IU rhFSH on dioestrus-2 in rats treated with decreasing doses of rhFSH (Table III, experiment 2c). However, the oestradiol-17β serum concentration was apparently high enough to induce a spontaneous LH peak at pro-oestrus which induced superovulation in the group of rats treated with decreasing doses of rhFSH (Table III, experiment 2a). It may be important that the amount of rhFSH used was the lowest in the latter protocol.

The quality of the oocytes found after superovulation was normal in terms of fertilization. However, problems occurred during implantation. This may be due to oocyte quality, but also to spacing problems in the uterus with this larger-than-normal number of embryos. Spacing problems have also been described in rabbits (Adams, 1960) and in rats after unilateral ovariectomy (Hirshfield, 1991).

It is concluded that the type of regimen of (rh)FSH stimulation is very important to achieve appropriate stimulatory effects on ovulatory potential. rhFSH, like rat FSH, induces follicle recruitment and decreases follicular atresia. The oocytes found after superovulation could be fertilized as well as oocytes retrieved from saline-treated control rats.

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Chapter 8

General Discussion

Introduction

Follicle development in the ovary has been and still is a fascinating subject to study. As a source of new life it has a fundamental role in the reproduction of species. Although many studies have been carried out, very little is still known about the fundamentals of follicle development.

This discussion is intended to place the studies described in chapter 2 to 7 in a larger context of follicle development as one dynamic process. From these studies it was concluded that:

1) Gonadotrophins, especially FSH, stimulate the development of preantral follicles. However, the prepubertal FSH peak is not necessary for a normal first ovulation in terms of timing and ovulation numbers (Chapter 2).
2) A quarter of the normal number of antral follicles, shortly before first ovulation, is sufficient for a normal first ovulation in terms of timing and ovulation numbers, indicating the flexibility of the system which regulates the number of ovulating follicles (Chapter 3).
3) During the 5 day ovarian cycle of the rat only 10% of the recruited antral follicles will ovulate, 90% will become atretic (Chapter 4).
4) Selection of the ovulating follicle takes place during oestrus and dioestrus 1 from the pool of small antral follicles (Chapter 4). The follicles that will ovulate already contain inhibin at the small antral stage during oestrus. This inhibin content will increase until ovulation (Chapter 5).
5) Inhibin is more important for the ovarian-hypothalamo-pituitary feedback system in the rat than oestradiol-17ß (Chapter 5).
6) FSH supplementation during the ovarian cycle will prevent atresia of antral follicles and will recruit follicles into the antral stage of development. However, dose and timing of the FSH substitution is very important to reach desirable results. Stimulation of follicle growth during the ovarian cycle with the aim to reach high ovulation numbers is optimal when decreasing doses of FSH are used (Chapter 6,7).
7) The rat is a suitable model to study initialization of the growth of primordial follicles and selection of the dominant follicle with respect to endocrine factors (Chapter 8).

Preantral follicle development

In the rat 75.000 germ cells are found at E18.5 (Beaumont & Mandl, 1962). Two days after birth the 2 ovaries of the rat do contain 27.000 oocytes mostly in primordial follicles (Beaumont & Mandl, 1962). This number of oocytes/follicles will only decrease. Some follicles from this pool, will start their development immediately while others will reside in the primordial stage until almost the end of reproductive life. The signals necessary to initiate follicle and oocyte development are not clear yet. However, there is growing evidence that gonadotrophins do play an important role. Continuous daily injections of an antiserum against gonadotrophins from birth to day 7 of life, lead to decreased follicular development at day 7 (Lunenfeld, Kraiem & Eshkol, 1975). Transplantation of ovaries of 1-day-old rats into ovariectomized or ovariectomized-hypophysectomized adult female rats resulted 15 days later in more secondary and large follicles in the ovariectomized rats compared to ovaries in the gonadotrophin poor environment (Arendsen de Wolff-Exalto, 1982). In young immature mice (Pedersen, 1969) and rats (Hage, Groen-Klevant & Welschen, 1978) more follicles start to

grow per day compared to the numbers in adult animals. This is the period with high gonadotrophin levels (rat; Uilenbroek, Arendsen de Wolff-Exalto & Blankenstein, 1976). We found that treatment with GnRH-antagonist from day 6 to day 15 of life, severely suppressed the gonadotrophin concentration in serum and decreased the number of growing follicles during the prepubertal period (Chapter 2). These findings seem to contradict the data of Peters *et al* (1973) from which it was concluded that exogenous gonadotrophins do not influence preantral, especially primary, follicle development. This conclusion was based on studies in early prepubertal mice. Later it was found that around this period the gonadotrophin serum concentrations are very high. A further increase of gonadotrophin levels will probably not have any effect. It was shown by Richards *et al* (1980) that FSH receptors exist in all types of follicles. LH receptors were only found in large preovulatory follicles. Also in humans it was suggested that FSH plays a role in the development of small preantral follicles (Gougeon, 1986). In women with hypogonadotrophic hypogonadism almost no follicles larger than the primordial stage of development are found (Goldenberg, Powell, Rosen, *et al.*, 1976) and in ovaries of most women with gonadotrophin resistant ovary syndrome, follicular development is limited to primordial and a few primary follicles (Talbert, Raj, Hammond, *et al.*, 1984). These two conditions both indicate an important role of gonadotrophins in preantral follicle development.

Prepubertal follicle development

Shortly after birth the gonadotrophin concentrations are very high in mice, rats and other species. At this time the first primordial follicles will develop towards larger stages. In the mouse it was found that the first follicles will reach the antral stage of development around day 22 (Pedersen, 1969). In the rat the highest serum concentrations of gonadotrophins are found around day 15 (Meijs-Roelofs, Uilenbroek, Osman, *et al.*, 1973). Thereafter the serum concentrations of gonadotrophins and especially of FSH decrease until shortly before the onset of first ovulation. During the prepubertal period the FSH decreases from very high levels in the first two weeks to low (cyclic) levels in the last week. Also in the prepubertal period the rate of atresia is much higher than later in life (Ingram, 1962). The appearance of atresia in preantral follicles differs morphologically from that seen in antral follicles later in life. In preantral follicles atresia is first seen as degeneration of the oocyte, i.e. germinal vesicle breakdown, followed by division of the oocyte and formation of pycnotic nuclei in the granulosa cell layer.

The appearance of antral follicles around day 22 marks the end of the high gonadotrophin concentrations. During the last 10 days before first ovulation there is a negative correlation between the serum FSH concentration and the combined volume of antral follicles $\geq 350 * 10^5\ \mu m^3$ in the rat and a positive correlation between biologically active inhibin in the ovary and the folliclular volume (Sander, Meijs-Roelofs, van Leeuwen, *et al.*, 1986). Large antral follicles of $\geq 500 * 10^5\ \mu m^3$ are only found during the last 4 days before first ovulation. The FSH serum concentration decreases at this time to the lowest values during the prepubertal period. In contrast, the biological activity of inhibin in the ovary reaches its highest value.

When prepubertal rats of 28 days of age were treated with GnRH-antagonist (500 µg/100 mg body weight at 3 day intervals), antral follicle development was arrested at volume class 2 (diameter < 400 µm, Meijs-Roelofs, van Cappellen, van Leeuwen, *et al.*, 1990). Arrest of

follicles in this situation did not stop follicle development, but follicles with volumes between 100 and 350 * 10^5 μm^3 (diameter 265-400 μm) became atretic as was shown by increased early atresia in these follicle size classes. The treatment completely blocked development of larger antral follicles. After treatment with this high dose of GnRH-antagonist it took a few days before gonadotrophin levels regained their normal values (Meijs-Roelofs, *et al.*, 1990).

Although treatment with a GnRH-antagonist from day 6 to 15 suppressed the population of growing follicles severely, first ovulation took place normally in terms of timing and number of CLs (Chapter 2). Five days before first ovulation the number of antral follicles (volume > 100 * 10^5 μm^3) was only half the number seen normally (Appendix). The number of days before first ovulation could be calculated by timing of first ovulation after unilateral ovariectomy (ULO). It has been shown before that ULO does not influence the timing of first ovulation in untreated rats (Osman, Meijs-Roelofs & Kramer, 1982). Timing of first ovulation was also not different between GnRH-antagonist-treated or untreated intact control rats. In the groups of GnRH-antagonist treated control rats and of GnRH-antagonist-treated unilaterally ovariectomized rats not only the timing of first ovulation but also the numbers of CLs found were not significantly different from those in untreated intact control rats (Chapter 3). This means that a) only one fourth of the normal antral follicle population is needed for normal ovulation, b) the mechanism which controls the number of ovulations is already active in prepubertal rats.

Follicle development during the oestrous cycle

Follicle development is regulated by endo-, para- and autocrine factors. However, the regulation of the total number of ovulating follicles in both ovaries must be regulated by interovarian communication, i.e. by endocrine factors. The endocrine factors, which play an important role in this regulation, are LH and FSH secreted by the pituitary and oestradiol and inhibin secreted by the developing antral follicles. During pro-oestrus and oestrus the high FSH concentration stimulates granulosa cell growth and in this way recruits follicles for the next cycle. Characteristic for these newly recruited small antral follicles is their relatively high content of biological active inhibin (Chapter 5) which is probably stimulated by the high FSH concentration; *in vivo* treatment of rats with 10 IU recombinant human FSH (rhFSH) at 17.00h on oestrus results in a significantly increased immunoactive inhibin concentration at dioestrus-1 on 10.00h (97 ± 17 U/ml, n=4) compared to 22.5 ± 1.8 U/ml (n=5) in saline treated control rats (unpublished results). From this pool of newly recruited follicles the ovulating follicles are selected. These selected follicles soon produce inhibin in increasing amounts until pro-oestrus (Chapter 4; Chapter 5). Follicles entering the small antral stage later during the cycle do not contain measurable amounts of biologically active inhibin (Chapter 5). However, during this period the FSH concentration is quite low due to the increasing amounts of inhibin secreted by the larger antral follicles. The oestradiol content, like the inhibin content, is strongly related to the dimension of the follicle and both hormones have their highest follicular concentrations at pro-oestrus. Only inhibin seems to be stimulated in small antral follicles. This stimulation is reflected in the peripheral concentration of immunologically active inhibin at dioestrus-1 (Watanabe, Taya & Sasamoto, 1990). For this reason inhibin seems to be the main regulator of FSH secretion during the ovarian cycle in the rat. Besides this endocrine role, inhibin may also have an auto- or paracrine function in the developing follicle. It was found that direct injection of recombinant inhibin into a rat ovary

stimulated follicle development while recombinant activin induced atresia (Woodruff, Lyon, Hansen, *et al.*, 1990). Although the role of oestradiol in the regulation of FSH secretion seems to be limited in the rat, there is evidence that oestradiol has a synergistic action on the effect of FSH on follicular growth (Lavranos, O'Leary & Rodgers, 1996; Uilenbroek, Kramer, Karels, *et al.*, 1997). The first selection takes place from the pool of follicles with a volume between 100 and 350 * 10^5 μm^3 (275-400 μm) (Chapter 4). These follicles have an increased inhibin production and in this way they keep the FSH secretion low. This results in a slower growth of the small antral follicles. Follicles entering the FSH dependent stage when FSH is low, will go into atresia (Chapter 4). The feedback balance between the inhibitory factors inhibin and oestradiol on one side and FSH/LH on the other results in a second selection between dioestrus-2 and pro-oestrus from which the twelve ovulating follicles will emerge. The model of Lacker (1987) explains this second selection on basis of small differences in the initial secretion rates of oestradiol. It was found that these selected follicles keep on producing inhibin (Chapter 5) until pro-oestrus. When the follicles reach a size of $\geq$ 500 * 10^5 μm^3, the oestradiol production increases. Oestradiol plays an important role in making the pituitary more sensitive for GnRH and in high doses also stimulates the secretion of GnRH by the hypothalamus (Döcke, Rohde, Stürzebecher, *et al.*, 1990). When FSH is artificially increased this results immediately in a decrease of atresia and an increased inflow of small antral follicles (Appendix). However, when the FSH stimulus disappears, the normal feedback mechanism starts working again. For this reason a correct timing of the FSH treatment is very important in order to achieve an increased number of ovulating follicles (Chapters 6 and 7).

Human follicle development as compared to that in rats

In women, like in female rats, all oocytes that will be available during reproductive life are formed before birth. During the prepubertal period follicle development starts and the number of oocytes decreases from 2.000.000 at birth to 400.000 (20%) at puberty (Baker, 1963). In the rat these numbers are 27.000 and 6.000 (22%) respectively (Beaumont & Mandl, 1962; Meijs-Roelofs, *et al.*, 1990). The antrum formation of the human follicle takes place around the same developmental stage as in the rat, when the follicle diameter is between 180 and 240 μm (Gougeon, 1981). The final diameter of a preovulatory rat follicle is about 1 mm whereas a human follicle reaches a size of about 20 mm.

The ovarian cycle in women and rats is regulated by the hypothalamus-pituitary-ovarian axis; the most important hormones are LH and FSH secreted by the pituitary and oestradiol and inhibin secreted by the developing follicle. The ovarian cycle in women is divided in 2 parts, the follicular phase and the luteal phase, and lasts about 28 days while in the rat the cycle consists of a follicular phase only and last 4 to 5 days. The number of ovulations in women is 1 per cycle and in the rat 12. Still there are some clear similarities between human and rat follicular development. Like in the rat the human preantral follicle seems to be influenced by FSH (Greenwald & Roy, 1994) although there is still some discussion on this point (Fauser & Van Heusden, 1997), caused by the different approaches of the studies done in this field. At a certain point the small antral follicle reaches an FSH dependent state. During this phase the choice between atresia and ovulation is made. From the class of follicles with a diameter between 2 and 10 mm the follicle destined for ovulation is selected during the first half of the follicular phase (Pache, Wladimiroff, de Jong, *et al.*, 1990). This resembles the situation in the rat where at the beginning of the cycle the follicles destined for ovulation are

selected (Chapter 4). The model developed by Lacker (1987) for the human situation can also be used to simulate the development of the larger antral follicles in the rat, which means that the hypothalamus-pituitary-ovarian axis may be regulated in a similar way. The model however is a very simplified system which does not really differentiate between the different hormones LH/FSH on one side and oestradiol/inhibin on the other. The role of FSH is very prominent in both species; its levels must reach a threshold value before follicle recruitment will takes place (Brown, 1978, human; Hirshfield & Midgley, 1978, rat). In addition to the FSH threshold the FSH concentration must be available during a long enough period. This period is described as the FSH window (Fauser & Van Heusden, 1997, human). This FSH window is also seen in rats where suppression of the second part of the pro-oestrus FSH peak leads to absence of antral follicle recruitment (Hirshfield & Midgley, 1978). The constant inflow of follicles into the FSH dependent stage is illustrated by the follicle inflow model (Chapter 4). The proposed continued inflow of human follicles into this FSH dependent stage of development can be investigated in more detail in the rat model (Appendix). From this model it appears that the inflow of follicles is influenced by hormonal conditions. This inflow also seems to be influenced by the inflow during the last few days (Appendix). Also the ovulation inducing effect of LH is clear in both species. The role of inhibin seems to be more prominent in the rat (Chapter 5) than it is in humans (Baird, 1990) although recent findings with the newly developed inhibin sandwich assay may lead to new insights (Fauser & Van Heusden, 1997).

In rats Insulin like growth factor-1 (IGF-1) mRNA is present in the granulosa cells of developing preantral and antral follicles, but not in atretic follicles or corpora lutea. In preovulatory follicles high levels of IGF-1 mRNA are confined to the antral cell layers and to the cells of the cumulus oophorus (Oliver, Aitman, Powell, *et al.*, 1989). In the presence of FSH, IGF-1 has the ability to induce both DNA synthesis and differentiation. FSH determines the action of IGF-1 on promotion of either growth or differentiation. Furthermore, priming with FSH renders granulosa cells responsive to IGF-1 in terms of DNA synthesis (Kanzaki, Hattori & Kojima, 1996). In human granulosa cells it was found that in cells from follicles of non-stimulated cycles, FSH as well as IGF-1 significantly stimulated progesterone and estradiol production. A synergistic effect of FSH and IGF-1 could be seen when low (1 and 10 µg/l) concentrations of the two hormones were used (Bergh, Olsson & Hillensjö, 1991b). It was also found that IGF-1 alone stimulated thymidine incorporation in granulosa cells (Bergh, Carlsson, Olsson, *et al.*, 1991a).

Both insulin-like growth factor binding protein-1 (IGFBP-1) and IGFBP-3 produced a dose-related inhibition of IGF-1-stimulated oestradiol accumulation in granulosa cell-conditioned medium with complete reversal of the effects of IGF-1 in the presence of a molar excess of binding protein. IGFBPs-1 and -3 also exerted a small (25-40%) but significant and consistent inhibition of oestradiol secretion in response to FSH alone. The progesterone response to IGF-1 was inhibited by IGFBPs-1 and -3 but there was no effect on FSH-stimulated progesterone production (Mason, Willis, Holly, *et al.*, 1992). IGFBP-1 and oestradiol are differentially regulated by IGF-1 in the human ovary (Mason, Margara, Winston, *et al.*, 1993). IGF-2 inhibits IGFBP-1 production in luteinized human granulosa cells in a dose-dependent manner and with potency similar to IGF-1 and higher than insulin (Poretsky, Chun, Liu, *et al.*, 1996).

Epidermal growth factor (EGF) in the rat was found to have a multi-functional nature in influencing the growth and differentiation of immature granulosa cells. EGF can inhibit or stimulate growth and differentiated functions (aromatase and 3ß-hydroxy steroid

dehydrogenase), the response depending on the context of the signals that the cell receives from its endocrine and micro environment (Bendell & Dorrington, 1990). Treatment of granulosa cells with EGF inhibited the spontaneous onset of apoptotic DNA cleavage found during culture by 40-60%. In contrast, IGF-I was ineffective (Tilly, Billig, Kowalski, *et al.*, 1992). EGF inhibits apoptosis of large granulosa cells by stimulating progesterone synthesis and regulating the distribution of intracellular free calcium (Luciano, Pappalardo, Ray, *et al.*, 1994). It was also suggested that EGF is involved in granulosa cell proliferation, irrespective of the presence of FSH, in a pathway different from that of FSH (Liang, Yano, Tsutsumi, *et al.*, 1994). Immunoreactions with anti-rat EGF monoclonal antibody were observed sparsely in the granulosa layer and antrum of follicles, but not in the theca layer or stromal tissue (Fukumatsu, Katabuchi & Okamura, 1995). Human granulosa cells produce estrogen in response to EGF, but this effect decreased with increasing follicular size (Hurst, Zacur, Schlaff, *et al.*, 1993). Human granulosa-luteal cells require the early exposure and continuous presence of EGF for the stimulatory effect of EGF on progesterone secretion. Cells not exposed initially to EGF do not respond in a similar way. EGF is capable of maintaining progesterone production for a period > 12 days (Serta & Seibel, 1993).

In general, it can be concluded that the main factors, regulating the pathway of folliclar development are not different between rat and human. The ovarian cycle differs in length and functionality (luteal phase or not), but the main hypothalamus-pituitary-ovarian axis appears to be regulated very much in the same way. This makes it possible to use the rat as a model to gain more insight in the way follicle development could be regulated in humans.

Questions and possibilities for future research

During follicle development there are two key events that still have not been described. The first event is the initialization of growth of primordial follicles. The study of these follicles is difficult because it is not easy to separate them from the ovary. However, with the new *in vitro* follicle culture techniques, *in situ* hybridization and immunohistochemistry, it must be possible to find factors which may be involved in this process.
The second event is the selection of the antral follicles that will ovulate. Using the rat as a model it should be possible to investigate what happens with the small antral follicles during the first two days of the cycle. Something in these follicles must change due to selection or atresia. Early atresia markers are needed to give more insight on this point. Also the important role of FSH in these follicles needs further investigation.

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Summary

This thesis describes the dynamics of follicle development during the prepubertal and cyclic phases of life in the rat and the influence of hormones, especially FSH, on this development. At birth all oocytes are already present, arrested in the diplotene stage of the first meiotic division. The oocytes are surrounded by a few flat granulosa cells and together they form a follicle. These primordial follicles are the basal element of follicle development. Later in life these primordial follicles start, under the influence of yet unknown factors, their development towards the primary, secondary (preantral) and tertiary (antral) stage. The initiation of primordial follicle growth starts at different times in life for the different follicles.

During prepuberty, the time before first ovulation, many primordial follicles start this development. During the first two weeks of life the concentration of the gonadotrophins LH and FSH is very high. Suppression of the gonadotrophin concentration by treatment with a GnRH-antagonist from day 6 until day 15 results in a 20% decrease in the number of growing follicles at day 22 and a 50% decrease at the day of first ovulation (Chapter 2). From this results it was concluded that gonadotrophins have a positive effect on the development of secondary and small antral follicles. In spite of the reduction of 50% of the number of growing follicles, the first ovulation took place at a normal time with a normal number of ovulating oocytes. This led to the question how flexible the regulation of follicle development is during the period before first ovulation. By removing one ovary of the GnRH-antagonist treated rats at day 15 or shortly before first ovulation, the effect of a 75% reduction of the amount of growing follicles was studied (Chapter 3). This massive reduction in the number of growing follicles could still be compensated for by the ovarian-hypothalamic-pituitary feedback system; normal numbers of ovulated oocytes were found.

During the 5 day ovarian cycle of the rat a constant number of follicles is ovulated at oestrus. In every cycle about 120 follicles are recruited, but only 12 follicles will ovulate. The ovulating follicles are selected during oestrus and dioestrus-1 from a pool of small antral follicles (volume between 100 and 350 * 10^5 μm^3, diameter between 260 and 400 μm). The growth of these follicles depends on FSH. These follicles will either become atretic or ovulate (Chapter 4). The follicles destined to ovulate already contain biologically active inhibin at oestrus. This content will increase during the cycle to reach a maximum at pro-oestrus. There is a good correlation between levels of biologically active- and immunologically active inhibin in these follicles. Only during the last 2 days of the 5 day cycle the content of oestradiol-17ß increases (Chapter 5). Small antral follicles entering the FSH dependent developmental stage later in the cycle will not contain inhibin and they will go into atresia (Chapter 4, 5).

The effect of exogenous FSH on antral follicle development was studied by treatment of rats with rhFSH at dioestrus-2. This resulted in an immediate ovulation during the following night. Besides the induction of ovulation, also recruitment of small antral follicles was observed. Some of these newly recruited follicles were able to ovulate 65h later, at the expected day of oestrus (Chapter 6, Appendix). This growth inducing effect of FSH was further demonstrated by treatment of cyclic rats with decreasing doses of rhFSH given from dioestrus-1 to dioestrus-3. Superovulation occurred at the expected day of oestrus, yielding more than three times the normal number of ovulating oocytes (Chapter 7). This large number of ovulations

was reached not only by decreased atresia, but also by a higher recruitment of small antral follicles into the FSH dependent developmental stage. This large number of ovulations can only be achieved in the rat by a very subtle treatment with rhFSH.
Follicle development and the influence of FSH appear to be very similar in rat and human. It is concluded that the rat can be used as a model to study certain aspect of follicle development in women, such as follicle selection and recruitment of primordial follicles into the pool of growing follicles (Chapter 8).

Samenvatting

In dit proefschrift wordt de dynamiek van de follikelontwikkeling beschreven gedurende de prepuberale en vruchtbare fases van het leven van de rat. Deze ontwikkeling heeft plaats onder invloed van hormonen, in het bijzonder van FSH. Bij de geboorte zijn alle eicellen al gevormd. Ze verkeren in het diplotene stadium van de eerste meiotische deling. De eicellen zijn omringd door een aantal platte granulosa cellen, waarmee ze samen de follikels vormen. Deze primordiale follikels zijn het eerste stadium van de follikel ontwikkeling. Later in het leven zullen deze primordiale follikels, onder invloed van nog onbekende faktoren, hun ontwikkeling tot primaire, secondaire (pre-antrale) en tertiaire (antrale) follikel starten. De initiatie van de groei van de primordiale follikels begint op verschillende momenten in de tijd voor de verschillende follikels.

Gedurende de prepuberale periode, de periode voor de eerste ovulatie, beginnen vele primordiale follikels hun verdere ontwikkeling. Tijdens de eerste twee weken na de geboorte is de concentratie van de gonadotrofinen LH en FSH erg hoog. Onderdrukking van de gonadotrofinen, door behandeling met een GnRH-antagonist van dag 6 tot dag 15 resulteerde in een verlaging met 20% van het aantal groeiende follikels op dag 22 en in een 50% verlaging op de dag van de eerste ovulatie (Hoofdstuk 2). Dit resultaat leidde tot de conclusie dat gonadotrofinen een positief effect hebben op de ontwikkeling van secondaire en kleine antrale follikels. Ondanks de reductie van het aantal groeiende follikels met 50%, vond de eerste ovulatie op de normale tijd plaats met een normaal aantal ovulerende eicellen. Dit riep de vraag op hoe flexibel de regulatie van de follikel ontwikkeling is gedurende de periode voor de eerste ovulatie. Door verwijdering van een ovarium bij GnRH-antagonist behandelde ratten op dag 15 of kort voor de eerste ovulatie, kon het effect van een 75% reductie in het aantal groeiende follikels worden bestudeerd (Hoofdstuk 3). Het ovarium-hypothalamus-hypofyse terugkoppelingssysteem bleek nog steeds in staat om deze massale reductie op te vangen. Er werden normale aantallen geovuleerde eicellen gevonden.

Aan het eind van iedere 5-daagse ovariële cyclus van de rat ovuleert steeds een zelfde aantal eicellen. In iedere cyclus worden 120 follikels gerecruteerd, waarvan er 12 ovuleren. De ovulerende follikels worden geselecteerd op oestrus and di-oestrus 1 uit een voorraad van klein antrale follikels (volume tussen 100 en 350 * 10^5 μm^3, diameter tussen 260 en 400 μm). De groei van deze follikels is afhankelijk van FSH. Deze follikels worden ofwel atretisch of ze ovuleren (Hoofdstuk 4). De follikels bestemd om te ovuleren bevatten op oestrus al biologisch actief inhibine. De hoeveelheid inhibine in deze follikels neemt toe gedurende de cyclus om een maximum te bereiken op pro-oestrus. Er is een goede correlatie tussen biologisch- en immunologisch actief inhibine in deze follikels. Slechts gedurende de laatste 2 dagen van de 5-daagse cyclus neemt de hoeveelheid oestradiol-17ß toe (Hoofdstuk 5). Kleine antrale follikels die het FSH afhankelijke stadium later tijdens de cyclus bereiken bevatten geen inhibine en worden atretisch (Hoofdstuk 4, 5).

Het effect van exogeen FSH op de ontwikkeling van antrale follikels werd bestudeerd door behandeling van ratten met recombinant humaan FSH (rhFSH) op di-oestrus 2. Dit resulteerde in een ovulatie in de daarop volgende nacht. Behalve de inductie van ovulatie, werd ook een recrutering van kleine antrale follikels waargenomen. Sommige van deze nieuw gerecruteerde follikels waren in staat om 65 uur later, op de verwachte oestrus dag, te ovuleren (Hoofdstuk

6, Appendix). Het groei bevorderende effect van FSH werd verder gedemonstreerd door behandeling van cyclische ratten met aflopende doses rhFSH van di-oestrus 1 tot di-oestrus 3. Na deze behandeling vond een superovulatie plaats met meer dan drie maal het normale aantal ovulerende eicellen (Hoofdstuk 7). Dit hoge aantal ovulaties werd niet alleen bereikt door beperking van de atresie, maar ook door een grotere recrutering van kleine antrale follikels naar de voorraad van FSH afhankelijke follikels. Dit grote aantal ovulaties kan alleen worden bereikt door een zeer subtiele behandeling met rhFSH.
Er lijkt veel overeenstemming te zijn in de follikel ontwikkeling en de invloed van FSH hierop in de rat en de mens. Geconcludeerd wordt dat de rat kan worden gebruikt voor de bestudering van bepaalde aspecten van follikel ontwikkeling bij de vrouw, zoals follikel selectie en de initiatie van follikelgroei in primordiale follikels (Hoofdstuk 8).

Appendix

A model for the quantification of follicle inflow applied to different physiological conditions

A model for the quantification of follicle inflow applied to different physiological conditions

The ovulatory secondary FSH peak induces recruitment of (pre)antral follicles into the pool of antral follicles from which the ovulatory follicles are selected. Inhibition of this secondary FSH peak by means of pff (a solution with a high inhibin concentration), resulted in a lack of follicle recruitment (Hirshfield & De Paolo, 1981). The number of recruited follicles (volume $\geq 100 * 10^5 \mu m^3$) in the rat was for a long time known as twice the number of follicles that would ovulate (Welschen, 1972). Calculations of the number of follicles that reach a volume $\geq 100 * 10^5 \mu m^3$ suggest that this number may even be 5 times the number of ovulating follicles (Chapter 4).

Selection of the number of ovulating follicles

Selection of the right number of follicles is very important for reproduction but is very badly understood. Keyfactor in this regulatory systems seems to be FSH. Suppression of FSH by means of pff or other inhibin rich fluids can block the secondary FSH peak, normally seen on the morning of oestrus in the rat (Hirshfield & De Paolo, 1981). As a result no recruitment of small antral follicles can be observed. Increase of the FSH concentration by means of an injection with an inhibin antiserum (Sander, Kramer, van Leeuwen, *et al.*, 1991) or injection of exogenous FSH (Chapter 6) resulted within 48h in a tremendous increase of the number of small antral follicles. In the first study (Sander, *et al.*, 1991) this increase only led to an increased number of ovulations when the animals were treated at dioestrus-2, i.e. 3 days before the day of expected ovulation. This indicates that the regulatory mechanism reduces the number of ovulating follicles when the stimulatory FSH level is discontinued. The FSH concentration is mainly regulated by the ovarian hormones oestradiol and inhibin. Based on this feedback mechanism a mathematical model was developed by Lacker (1981), which described the interaction between an ovarian hormonal factor (oestradiol) and the pituitary hormonal factors (LH and FSH). Lacker used the following idealizations to explore the mechanism for the regulation of ovulation number (Lacker, Beers, Meuli, *et al.*, 1987):

1) The oestradiol secretion rate of a model follicle is a measure of its maturity.
2) The maturation rate of each model follicle at any time depends on its maturity and on the circulating concentrations of FSH, LH and oestradiol.
3) All model follicles are assumed to inherit the same developmental program. More precisely, any two follicles with the same maturity will develop at the same rate when exposed to the same circulating hormone concentrations.
4) The circulating concentration of oestradiol at any time is the sum of the contributions made by each follicle.
5) The circulating oestradiol concentration regulates the release of FSH and LH from the hypothalamic-pituitary axis. The response of the hypothalamic-pituitary system is fast relative to the time scale of follicle development.
6) Oestradiol, FSH and LH are removed from the circulation at rates that are proportional to their concentration. The half-life of a molecule of oestradiol, FSH or LH in the circulation is short relative to the time scale of follicle development.

With these assumptions a follicle maturation table was constructed which gives a maturation value for every individual follicle depending on its oestradiol secretion in relation to the peripheral concentration of FSH, LH and oestradiol, which are the same for all follicles at a certain time point. By calculating the maturation values of the individual follicles new peripheral levels of oestradiol, FSH and LH can be obtained, after which new maturation values can be calculated again. This process will be repeated until the peripheral oestradiol value is high eneough to induce an LH surge and thus ovulation.

With this model it is possible to describe both the mono-ovular response in humans and the poly-ovular system in rats. This model predicts a constant number of ovulations at regular time intervals. Decrease of the number of interacting follicles (follicles that secrete oestradiol), results in an irregular timing of the ovulation response, but at first the number of ovulations is still constant. This result is what is observed in older animals which first show irregular ovarian cycles before the end of ovarian cyclicity occurs. Also unilateral ovariectomy which leads to compensatory follicle development in the other ovary can be explained on basis of this model (Lacker, 1989).

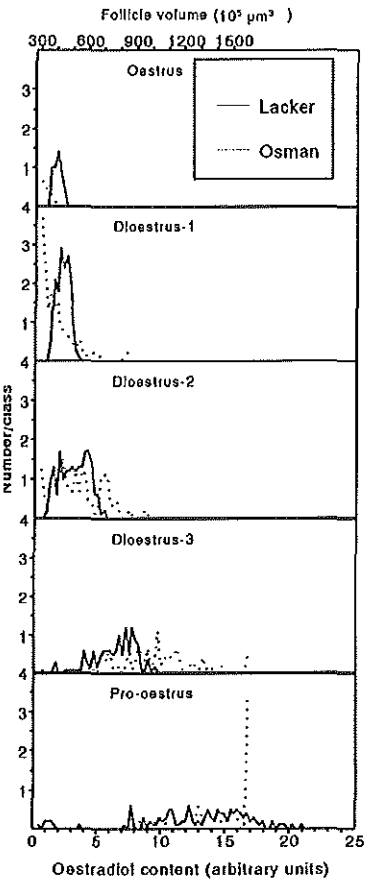

Figure 1 Profile of healthy follicles during the ovarian cycle of the rat. Solid line, follicle numbers calculated by the model of Lacker. Dotted line, follicle numbers counted by Osman (follicle volume ≥ 350 * 10^5 µm³, follicle class 20 * 10^5 µm³

From the study of Osman (1985) very detailed information about the number of antral follicles at every stage of the normal ovarian cycle of the rat was available which could be compared with predictions of the model of Lacker. Although the model of Lacker is quite stable for the number of starting follicles at the beginning of the follicular phase and/or the number of follicles which randomly start to interact at later stages (follicle recruitment throughout the cycle) we wanted to feed the model with realistic values. The number of starting follicles in an ovarian cycle of the rat can easily be counted. However, the number of follicles reaching the antral follicle stage during later stages of the cycle is unknown. For this reason a model was developed based on the follicle counts of Osman (1985) which gives more insight in the follicular dynamics during a normal ovarian cycle (Chapter 4). This model demonstrates a continuous, but not constant inflow of follicles into the antral follicle class. This inflow was fed into the model as an extra function. Probably this inflow is dependent on the FSH concentration and for this reason it should be included in the model. The data of

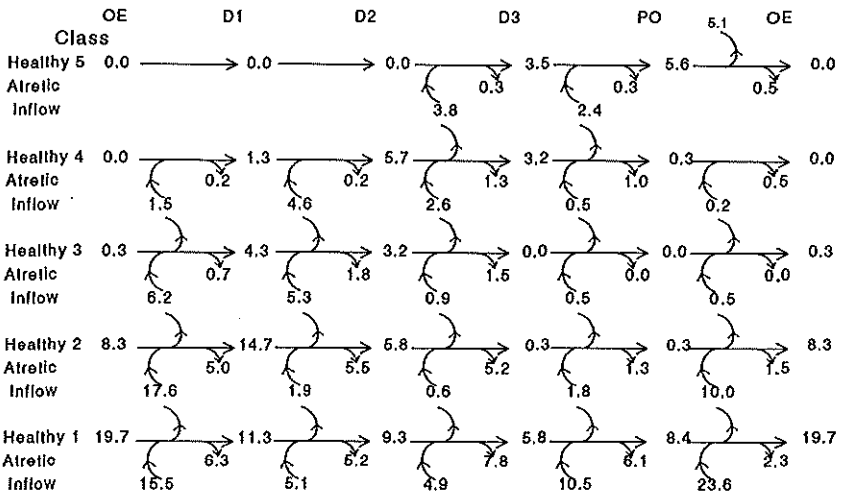

Figure 2 Inflow of antral follicles of one ovary into the different volume classes during the cycle of the rat. Upward arrow: number of follicles entering a class (inflow, calculated value). Downward arrow: number of early atretic follicles (counted). Straight arrow: number of healthy follicles (counted). OE: oestrus; D1: dioestrus-1; D2: dioestrus-2; D3: dioestrus-3; PO: pro-oestrus

Osman are divided in healthy, early atretic and late atretic follicles. For this reason also the follicles in the model of Lacker were divided in healthy and atretic follicles. Follicles were regarded as atretic when their oestradiol content decreased for a simulated period comparable of 24h. These follicles do not have any influence on the model after this period.

When the predictions of this model were fitted with the physiological data found by Osman in the rat (Osman, 1985), it was found that good similarity was only found when follicles $\geq 350 * 10^5$ μm^3 were taken into account (Figure 1). From the model of the Osman data (Figure 2) it can be calculated that during the cycle 26.8 follicles enter this developmental stage of which 12 will ovulate. From these 26.8 follicles the final selection of the ovulating follicles (the dominant follicles) will take place. Before this stage a first selection takes place in the volume classes 1 and 2, which according to the model of Lacker is not regulated by the ovarian-pituitary feedback loop.

The method developed to calculate the inflow of follicles into the different antral volume classes (Chapter 4) can also be used to study the effects of hormonal treatment during the normal ovarian cycle of the rat. After injection of 10 IU rhFSH at dioestrus-2 at 17.00h an ovulation occurs in the night following the injection and a second ovulation occurs 48h later (Chapter 6). Combination of these data with the data of the normal cycle found by Osman (Osman, 1985) shows a rapid increase of numbers of follicles into volume class 1 and an absence of follicle inflow into class 1 on the next two days (Figure 3). The inflow into volume classes 2, 3 and 4 is also increased which must be due to a very rapid increase in the

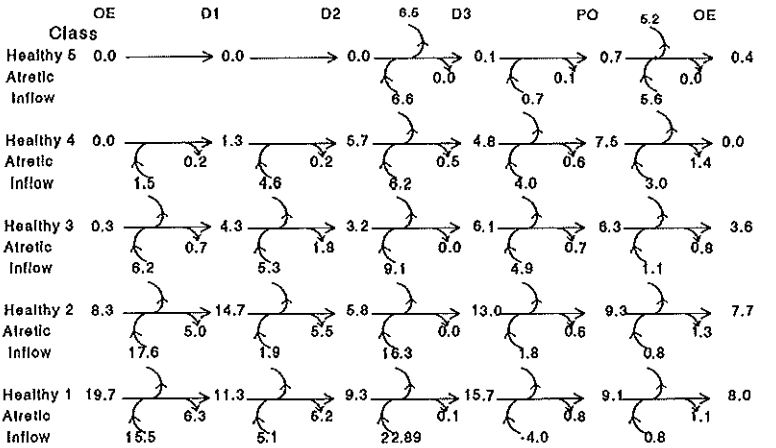

Figure 3 Inflow of antral follicles of one ovary into the different volume classes during the cycle of the rat, after treatment with 10 IU recFSH on dioestrus-2 at 17.00h. Upward arrow: number of follicles entering a class (inflow, calculated value). Downward arrow: number of early atretic follicles (counted). Straight arrow: number of healthy follicles (counted). OE: oestrus; D1: dioestrus-1; D2: dioestrus-2; D3: dioestrus-3; PO: pro-oestrus

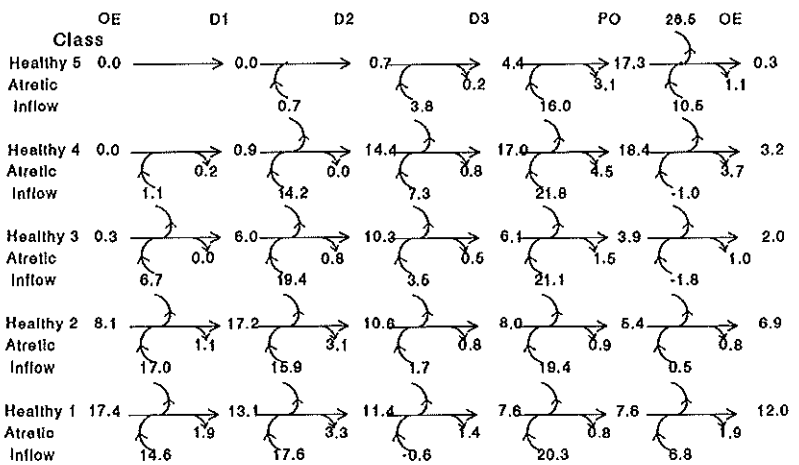

Figure 4 Inflow of antral follicles of one ovary into the different volume classes during the cycle of the rat, after treatment with decreasing doses of recFSH from dioestrus-1 to dioestrus-3. Upward arrow: number of follicles entering a class (inflow, calculated value). Downward arrow: number of early atretic follicles (counted). Straight arrow: number of healthy follicles (counted). OE: oestrus; D1: dioestrus-1; D2: dioestrus-2; D3: dioestrus-3; PO: pro-oestrus

growth velocity of the follicles, caused by the exo- and endogenous FSH. The FSH surge normally seen at the morning of oestrus is significantly decreased in this situation which is reflected in the lack of follicle inflow in the period between pro-oestrus and oestrus.

When rats are treated using decreasing doses of rhFSH from dioestrus-1 until dioestrus-3 superovulation occurs (Chapter 7; Figure 4). During this cycle the calculated inflow of follicles into volume class 1 is strongly increased at dioestrus-1. During dioestrus-2 the inflow ceases but increases thereafter at dioestrus-3. The calculated inflow at pro-oestrus seems to be lower than normal. This pattern might indicate that the inflow of follicles into volume class 1 is bound to limits. The results of the last two studies (Figure 3 and Figure 4) indicate that the FSH concentration influences the inflow of follicles into the pool of antral follicles very rapidly.

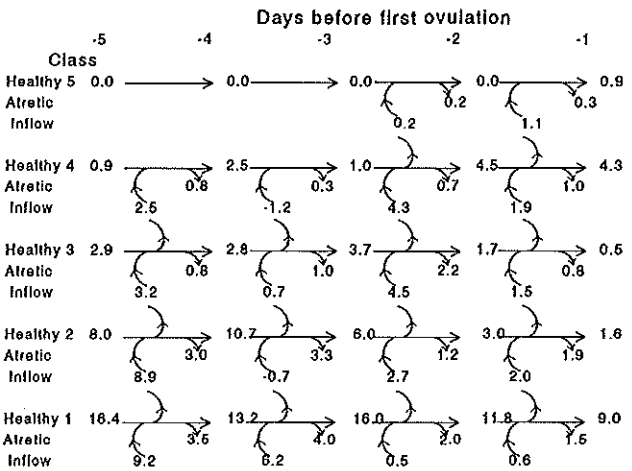

Figure 5 Inflow of antral follicles of one ovary into the different volume classes during the last 5 days before first ovulation. Upward arrow: number of follicles entering a class (inflow, calculated value). Downward arrow: number of early atretic follicles (counted). Straight arrow: number of healthy follicles (counted).

The same method can be used to describe the follicle inflow during the last 5 days before first ovulation. In the normal situation the follicle inflow decreases at day -3 and -2. The follicles destined to ovulate are already selected at day -3 (Figure 5). The number of antral follicles at oestrus is comparable with the number of follicles seen at day -5, but the inflow at oestrus seems higher. This can be expected because the FSH concentration at oestrus is still high, whereas at day -5 the FSH concentration is the lowest during the prepubertal period (Sander, Meijs-Roelofs, van Leeuwen, *et al.*, 1986). Probably the FSH concentration must be low before follicles will enter the later stages of antral follicle development. Maybe the low FSH concentration is necessary for the final development of the ovulating follicles. The inflow of follicles during the last two days before first ovulation is almost zero. In this period the ovarian inhibin secretion increases which influences the FSH secretion (Sander, *et al.*, 1986).

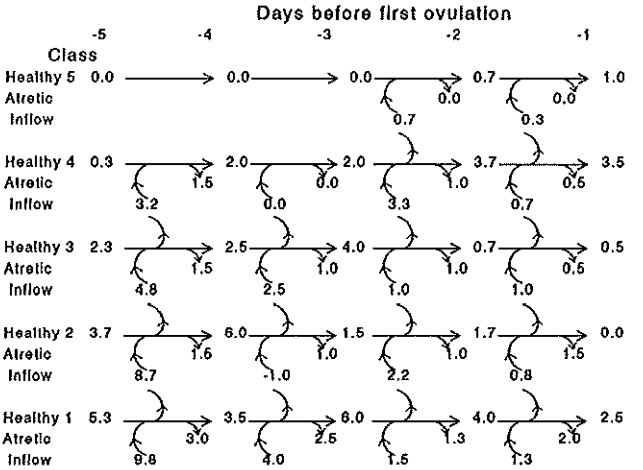

Figure 6 Inflow of antral follicles of one ovary into the different volume classes during the last 5 days before first ovulation, after treatment with a GnRH-antagonist on day 6, 9, 12 and 15 of life. Upward arrow: number of follicles entering a class (inflow, calculated value). Downward arrow: number of early atretic follicles (counted). Straight arrow: number of healthy follicles (counted).

After GnRH-antagonist treatment (Chapter 3) the prepubertal inflow pattern is almost the same as in controls although the number of antral follicles in volume classes 1 and 2 is less than half the normal number (Figure 6). The number of follicles in class 3or larger is almost the same in GnRH-antagonist treated and control rats. Apparently the ovary is still able to recruit enough follicles to reach a normal ovulation number which is reflected in the inflow pattern. This again demonstrates the overshoot in the number of follicles which might be neccesary to tightly regulate the number and timing of ovulation.

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Curriculum Vitae

Gert van Cappellen werd geboren op 8 januari 1958 te Kralingseveer (Rotterdam). In 1976 werd het HAVO diploma behaald aan de Christelijke Scholengemeenschap Comenius te Cappelle aan den IJssel. In het zelfde jaar werd de HTS studie chemie aangevangen aan het Van't Hoff Instituut te Rotterdam. Na een stage onderzoek naar de herkenning van de zwangerschap van de Okapi, Okapia Johnstoni, uitgevoerd op de afdeling Endocrinologie, Groei en Voortplanting van de Erasmus Universiteit Rotterdam (Dr. J.T.M. Vreeburg) werd in 1980 het diploma behaald. Sinds 1983 werkt de schrijver onafgebroken aan de Erasmus Universiteit Rotterdam en wel op de afdelingen Anatomie (1983-1990), waar werd meegewerkt bij het onderzoek naar geslachtsontwikkeling van de rat (Dr. H.M.A. Meijs-Roelofs) en de afdeling Endocrinologie & Voortplanting. Het promotie onderzoek dat gestart werd op de afdeling Anatomie is op de afdeling Endocrinologie & Voortplanting voortgezet en afgerond (Dr. H.M.A. Meijs-Roelofs, Prof. Dr. J. Moll, Prof. Dr. F.H. de Jong). Hiernaast werd een begin gemaakt met het onderzoek naar EDS-geïnduceerde apoptose m.b.v. de confocale laser scanning microscoop (Dr. F.G.G. Rommerts). Tevens werd de afdeling Endocrinologie & Voortplanting van de nodige computer ondersteuning voorzien.

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