J. Reprod. Fert. (1992) **95**, 567–575

Printed in Great Britain

Influence of a gonadotrophin-releasing hormone agonist and gonadotrophins on morphometric characteristics of the population of small ovarian follicles in cynomolgus monkeys (*Macaca fascicularis*)

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Summary. Small follicles, $\leqslant 100\ \mu m$, in monkey ovaries were divided into four types based on the morphological characteristics of the granulosa cells that surrounded the oocyte: primordial, intermediary, primary and secondary follicles. The proportion of primordial follicles positively correlated, whereas those of intermediary, primary and secondary follicles negatively correlated, with the total number of follicles $\leqslant 100\ \mu m$. There was no relationship between the population of nongrowing follicles (primordial and intermediary) and that of early-growing follicles (primary and secondary). Administration of exogenous gonadotrophins did not induce significant changes in the population of small follicles, whereas there was a significant increase in the number of intermediary follicles when gonadotrophins were associated with a gonadotrophin-releasing hormone agonist, buserelin. Buserelin can therefore partly inhibit the initiation of ovarian follicular growth in monkeys.

Keywords: small ovarian follicles; buserelin; gonadotrophin; monkey

Introduction

Ovarian stimulation by exogenous gonadotrophins in association with hypophysectomy (Kenigsberg *et al.*, 1984) induced by an agonist of gonadotrophin-releasing hormone (GnRH-a), is commonly used in human *in vitro* fertilization and embryo transfer (IVF–ET) programmes (Fleming & Coutts, 1986). GnRH-a was originally used to prevent a premature spontaneous luteinizing hormone (LH) surge, but is now also used to increase the number of recovered oocytes in response to high gonadotrophin doses. Despite some data demonstrating interference between GnRH or GnRH-a and ovarian function in mammals (Hsueh *et al.*, 1979; Knecht *et al.*, 1985) and primates (Tureck *et al.*, 1982; Wickings *et al.*, 1990), the effects of these compounds on the ovary have not been extensively studied. We reported (Lefèvre *et al.*, 1991) that in monkeys, buserelin had a deleterious effect, when used in short protocols (Barrière *et al.*, 1987), on the quality of human menopausal gonadotrophin (hMG)-stimulated follicles. However, this effect may be due to the 'flare-up' effect of gonadotrophins at the beginning of the treatment rather than to GnRH-a itself. Recently, Ataya *et al.* (1989) pointed out that GnRH-a inhibited the initiation of follicular growth from the pool of nongrowing follicles in rats.

Since GnRH-a is administered during successive IVF–ET attempts in humans, it was necessary to estimate the effect of GnRH-a on the population of small follicles. It is difficult to perform studies of the effects of various drugs on the pool of small follicles in humans, because of ethical problems in relation to experimental and surgical procedures. As the dynamics of the population of

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follicles $\leqslant 100\,\mu m$ are probably similar in monkeys (Koering, 1983) and humans (Gougeon & Chainy, 1987), monkeys can be used as a model to test the action of drugs on the ovary.

The aim of this study was to analyse the morphometric characteristics of the pool of dormant and early-growing follicles in the cynomolgus monkey ovary, under natural and experimental conditions. The respective influences of exogenous gonadotrophins and of a GnRH-a (buserelin) on these follicles were analysed and compared with the spontaneous cycle situation.

Materials and Methods

Animals and treatments

Adult cynomolgus monkeys (*Macaca fascicularis*) with regular menstrual cycles were used. These animals, of $3{\cdot}5 \pm 0{\cdot}2$ kg (mean ± SEM), were wild, purchased from Charles River France (Saint Aubin lès Elbeuf, France) ($n = 21$) or born in captivity ($n = 13$). Because of the high cost of animals, the present study has been performed on ovaries from monkeys previously used for other studies on effects of gonadotrophins, buserelin or a combination of both on large ovarian follicles.

Ten animals were ovariectomized ($n = 20$ ovaries) during a spontaneous menstrual cycle. Thirteen animals were hemi-ovariectomized or castrated on day 8 after intramuscular gonadotrophin administration from day 1 to day 8 (day 1: first day of menstruation); three of them ($n = 5$ ovaries) received hMG (75 iu daily) (Neopergonal, Serono France, Paris, France); seven ($n = 9$ ovaries) received hMG (50 iu daily) and follicle-stimulating hormone (FSH) (25 iu daily) (Metrodin, Serono France, Paris, France) and three ($n = 3$ ovaries) received FSH (75 iu daily). On day 1, eleven animals received a polyactide–glycolide (PLG 50:50) implant containing 3·3 mg GnRH-a (D-Ser-[TBu]^6-des-Gly-$(NH_2)^{10}$-LHRH-ethylamide (buserelin Hoechst AG, Frankfurt, Germany)) with a lifespan of 1 month. This implant showed similar release profiles in rats and monkeys; the excretion rate of buserelin in the rat decreasing from 60 µg in 24 h at day 1 to 30 µg in 24 h at day 15 (Sandow *et al.*, 1989). Among these eleven females, two were hemi-ovariectomized after treatment for 20 days with buserelin alone ($n = 2$ ovaries) then castrated, after a further similar treatment, twenty days later ($n = 2$ ovaries). The last nine females were hemi-ovariectomized ($n = 5$) or castrated ($n = 4$) at various times (9–16 days) after a 'short protocol' treatment with hMG (75 iu daily) beginning at the time of GnRH-a administration.

The animals from which only one ovary was removed had been hemi-ovariectomized some months earlier. Since no difference has been noticed in the morphometric characteristics of the populations of follicles $\leqslant 100\,\mu m$ in humans, between long-term hemicastrated women (although the number of ovaries studied was low, $n = 2$) and normal women of similar age (Gougeon and Chainy, 1987), we considered it valid to use ovaries from previously hemi-ovariectomized monkeys.

Histological methods and analysis

After removal, the ovaries were immediately fixed in Bouin's fluid for 3 days, then processed by routine histological methods and serially sectioned at 10 µm and stained with haematoxylin–Masson blue.

Small follicles have been classified into four types as proposed for human follicles (Gougeon & Chainy, 1987): (i) primordial follicles in which oocytes are surrounded by flattened granulosa cells, (ii) intermediary follicles in which oocytes are surrounded by a mixture of flattened and cuboidal granulosa cells, (iii) primary follicles in which oocytes are surrounded by a single layer of cuboidal granulosa cells and (iv) secondary follicles in which oocytes are surrounded by more than one layer of cuboidal granulosa cells with no epithelioid cell in the theca layer.

Follicles were observed and counted in every one hundredth section, the first observed section being the fiftieth. Atretic follicles, i.e. shrunken in shape or exhibiting an oocyte, either shrunken or showing a pycnotic germinal vesicle, were not considered. Between 300 and 5900 healthy follicles were counted per ovary.

To correct the observed numbers of follicles, germinal vesicle diameters were measured, in the section where the nucleolus was observed, using a microscope with an ocular micrometer at ×400 magnification. For primordial, intermediary, primary and secondary follicles the mean (±SEM) diameters of the germinal vesicles, calculated on 100 follicles of each type, were $18{\cdot}2 \pm 0{\cdot}10$, $17{\cdot}9 \pm 0{\cdot}13$, $19{\cdot}0 \pm 0{\cdot}19$ and $24{\cdot}0 \pm 0{\cdot}27\,\mu m$, respectively.

The total number of each type of follicle was estimated for each ovary using the following correction factor (Block, 1951):

$$Nt = \frac{No \times St \times ts}{So \times d_o}$$

where Nt is the total corrected number of follicles of one type, No the number of follicles observed in the analysed sections, St the total number of sections in the ovary, ts the thickness of the section (10 µm), So the number of observed sections and d_o the mean diameter of germinal vesicles according to the follicle type.

Statistical analyses

Regression analyses were performed on a STAT VIEW II (Alpha Système diffusion, Meillan, France) statistical software package on a MacIntosh IIx microcomputer (Apple Computer Inc., Cupertino, CA). The statistical significance of the observed differences between slopes of regression was determined by the Student's *t* test and that of the follicular populations between left and right ovaries, by the paired *t* test. The differences between populations were tested by analysis of variance (ANOVA), with differences between groups tested by two-sample Scheffé F-test.

Results

Unstimulated females

The populations of the various types of small follicle have been analysed (Table 1). Primordial follicles constitute the greatest part of the population of small follicles and showed large variations in number from one subject to another (range 21 200–230 300). No differences were found between right and left ovaries of each animal. The variations in distribution of types of follicle with age were not analysed because age was not known for sufficient animals.

Table 1. Mean numbers of various types of follicle $\leqslant 100\,\mu m$ in the cynomolgus monkey ovary

Treatments	Number of ovaries	Total number of follicles	Primordial	Intermediary	Primary	Secondary
Control	20	90 100 ± 14 200	72 000 ± 13 600	11 800 ± 1200[a]	4600 ± 710	1610 ± 290
Gonadotrophins	17	107 400 ± 22 000	91 000 ± 20 800	11 200 ± 1400[b]	3800 ± 470	1430 ± 200
Buserelin with and without gonadotrophins	17	101 100 ± 10 700	78 900 ± 9950	16 200 ± 1400[c]	4350 ± 400	1670 ± 180

All values are means ± SEM.
[a] and [b] are significantly different from [c] ($P = 0{\cdot}02$ and $P = 0{\cdot}01$, respectively).

The total number of follicles $\leqslant 100\,\mu m$ correlated with that of primordial ($r = 0{\cdot}99$, $P = 0{\cdot}0001$) or intermediary follicles ($r = 0{\cdot}63$, $P = 0{\cdot}003$), but did not correlate with that of primary or secondary follicles (Fig. 1). The population of early-growing follicles (primary and secondary) did not correlate with that of nongrowing follicles (primordial and intermediary) (Fig. 2).

The total number of follicles $\leqslant 100\,\mu m$ positively correlated ($r = 0{\cdot}77$, $P = 0{\cdot}0001$) with the percentage of primordial follicles and negatively correlated with the percentages of intermediary, primary and secondary follicles ($r = -0{\cdot}80$, $P = 0{\cdot}0001$; $r = -0{\cdot}72$, $P = 0{\cdot}0003$ and $r = 0{\cdot}64$, $P = 0{\cdot}002$, respectively) (Fig. 3). Thus, fewer follicles $\leqslant 100\,\mu m$ were associated with higher proportions of intermediary, primary and secondary follicles.

Treated females

The populations of various follicle types did not show significant differences between females treated with buserelin alone and with buserelin plus hMG. All data from animals treated with buserelin were therefore pooled. A similar situation was observed in the group of monkeys treated only with various gonadotrophins, and these data were also pooled.

The characteristics of the populations of various types of follicle of animals treated with buserelin ± hMG or with gonadotrophin alone did not differ greatly from those observed in unstimulated ovaries. A similar range of inter-individual variations in follicle numbers was observed, from 32 500 to 199 000 and from 8300 to 270 200 for monkeys treated with buserelin ± hMG and with gonadotrophin alone, respectively.

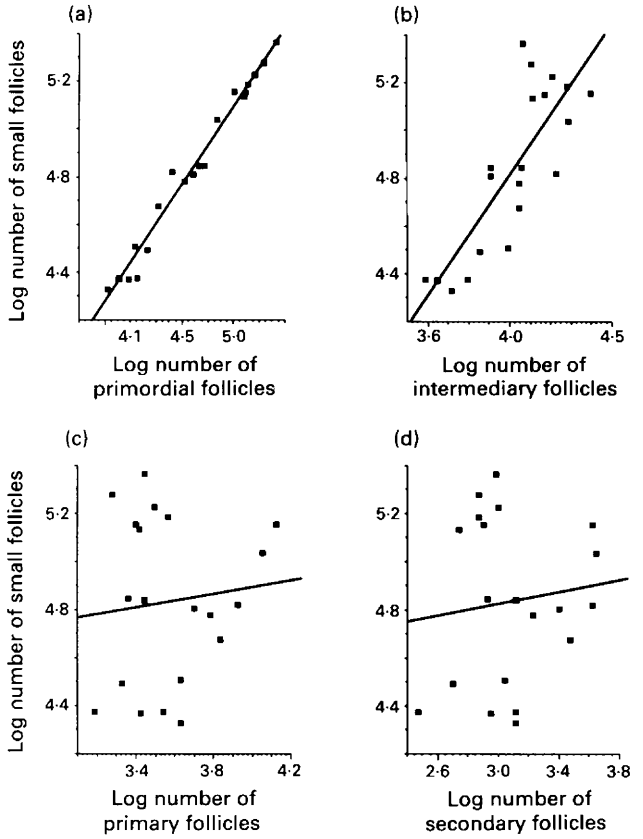

Fig. 1. Analysis of correlation between total numbers of follicles $\leqslant 100$ μm and numbers of type of follicle in unstimulated monkey ovaries: (a) primordial follicles; (b) intermediary follicles; (c) primary follicles and (d) secondary follicles.

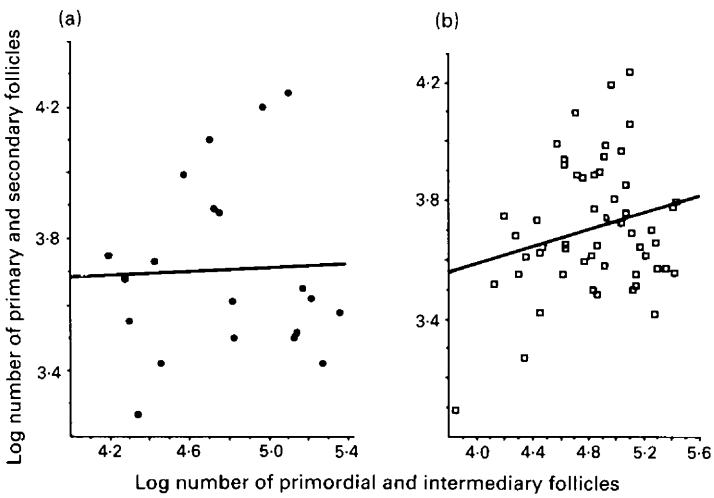

Fig. 2. Analysis of correlation between numbers of nongrowing (primordial plus intermediary) follicles and numbers of early-growing (primary plus secondary) follicles in (a) unstimulated monkeys and (b) unstimulated and treated monkeys.

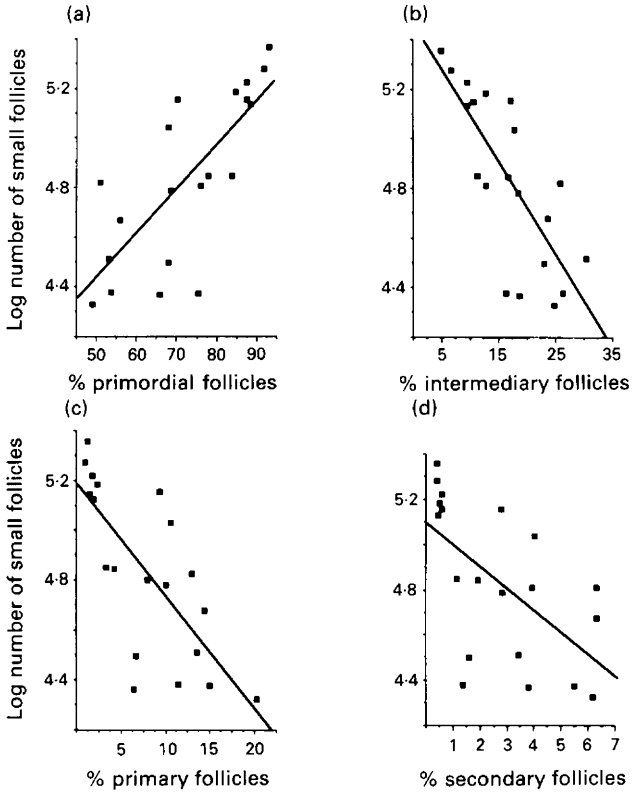

Fig. 3. Analysis of correlation between total numbers of follicles $\leqslant 100$ μm and proportions of each type of follicle in unstimulated monkeys. (a) Primordial follicles; (b) intermediary follicles; (c) primary follicles and (d) secondary follicles.

As observed in unstimulated animals, the total number of follicles $\leqslant 100$ μm correlated with that of primordial follicles ($r = 0{\cdot}99$, $P = 0{\cdot}0001$) or intermediary follicles in monkeys treated with buserelin ± hMG ($r = 0{\cdot}68$, $P = 0{\cdot}003$) and with gonadotrophin alone ($r = 0{\cdot}74$, $P = 0{\cdot}0007$). No correlations were found between the total number of follicles $\leqslant 100$ μm and that of either primary and secondary follicles in ovaries treated with gonadotrophin alone or that of primary follicles in ovaries treated with buserelin ± hMG. However there was a slight correlation between the total number of small follicles ($r = 0{\cdot}50$, $P = 0{\cdot}04$) and that of secondary follicles in animals treated with buserelin ± hMG.

The mean numbers of primordial, primary and secondary follicles were not influenced by the treatment. However, there were more intermediary follicles in ovaries treated with buserelin ± hMG than in unstimulated ovaries or ovaries treated with gonadotrophin alone (Table 1). The females treated with buserelin alone for 40 days exhibited more intermediary follicles (19 600 ± 3300) than did females treated for 20 days (15 400 ± 1400) although the difference was not significant because of the small numbers of ovaries. In buserelin ± hMG-treated animals there were significantly ($P < 0{\cdot}05$) fewer primary follicles per intermediary follicle (0·28 ± 0·02) compared with unstimulated animals (0·41 ± 0·04) and animals treated with gonadotrophin alone (0·38 ± 0·04).

These data suggest that buserelin could alter the follicular maturation process during the transitional stage between primordial and primary follicles. To confirm the existence of a buserelin

effect on early folliculogenesis, we calculated correlations between the populations of each follicular type for unstimulated ovaries, ovaries treated with buserelin ± hMG and ovaries treated with gonadotrophin alone, and the slopes of the regression lines were compared.

In cycles from animals either unstimulated or treated with gonadotrophin alone, the number of intermediary follicles correlated with that of primary ($P = 0{\cdot}001$ and $0{\cdot}007$, respectively) and secondary follicles ($P = 0{\cdot}009$ and $0{\cdot}008$, respectively) (Fig. 4) and the number of primary follicles correlated with that of secondary follicles ($P = 0{\cdot}0001$ and $0{\cdot}004$, respectively) (Fig. 4). However, in animals treated with buserelin, the number of intermediary follicles did not correlate with that of primary or secondary follicles (Fig. 4) and no significant correlation was found between the numbers of primary and secondary follicles.

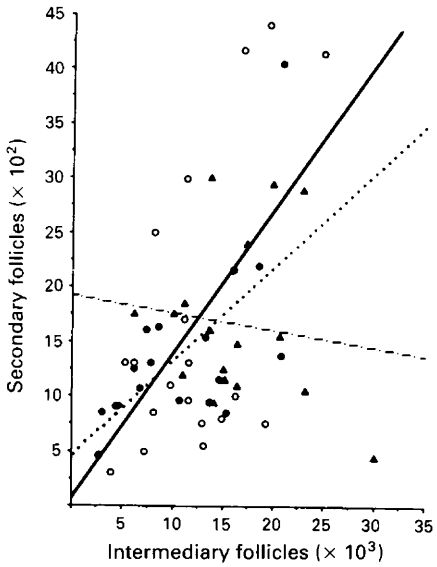

Fig. 4. Analysis of correlation between numbers of intermediary follicles and numbers of secondary follicles in unstimulated (○), gonadotrophin-treated (●) and buserelin ± human menopausal gonadotrophin treated (▲) monkeys.

Correlations between intermediary, primary and secondary follicles were observed in unstimulated ovaries and ovaries treated with gonadotrophin alone, whereas they were not in ovaries treated with buserelin ± hMG. This observation was reinforced by the existence of a significant difference in the slopes of the regression lines between unstimulated ovaries and ovaries treated with buserelin ± hMG (intermediary versus primary: $P < 0{\cdot}04$; intermediary versus secondary $P < 0{\cdot}02$ and primary versus secondary follicles: $P < 0{\cdot}02$, respectively). From these data, we conclude that (i) for a given population of intermediary follicles there were fewer primary and secondary follicles, and (ii) for a given population of primary follicles, there were fewer secondary follicles in buserelin ± hMG-treated animals than in unstimulated animals or in animals treated with gonadotrophin alone.

Discussion

The general characteristics of the population of follicles $\leqslant 100$ μm in *Macaca fasicularis* were quite similar to those previously reported for *Macaca mulatta* (Green & Zuckerman, 1954; Koering, 1983). In these species, the total number of small follicles were similar and the primordial follicles

constituted the main part of the population of ovarian follicles. Moreover, as previously reported for *Macaca mulatta* (Koering, 1983), there was no difference between numbers of follicles from the left and right ovary of a pair.

Our classification of follicles was slightly different from that of Koering (1983). Assuming that primordial and intermediary follicles corresponded to Koering's 'primordial class', i.e. nongrowing follicles, and that primary and secondary follicles, corresponded to Koering's 'a' class, i.e. early-growing follicles, the observed ratios of these types of follicle were similar. Koering (1983) suggested that there was a positive correlation between the number of primordial follicles in an ovary and the number of developing follicles. Koering's assumption implies a constant recruitment rate to explain how the progressive decrease in the number of primordial follicles is followed by a decrease in the number of growing follicles. In the present study, we failed to find such a correlation, either in the whole population studied or in the untreated ovaries or ovaries treated with gonadotrophin alone. From this observation, it can be concluded that the recruitment rate increases as the number of primordial follicles decreases. Whether this increase can be attributed to intra-ovarian regulation or to high circulating gonadotrophins remains to be determined, since the short duration of the gonadotrophin treatment in the present study does not exclude the possible involvement of pituitary hormones.

This observation can be explained by changes in the proportions of the various types of follicle. As the total population of follicles $\leqslant 100\,\mu m$ decreased, the percentage of primordial follicles decreased while percentages of primary and secondary follicles increased, as previously observed in humans (Gougeon & Chainy, 1987). When the population of ovarian follicles is low, owing to age or to genetic factors, the percentage of early-growing follicles is higher than in animals with a large number of follicles $\leqslant 100\,\mu m$. Consequently, the early-growing follicles represent a larger part of the population of follicles $\leqslant 100\,\mu m$ and become similar in number to those observed in animals showing a large number of primordial follicles. This change can be considered as an adaptive process: an intra-ovarian mechanism may be operating to regulate the initiation of follicular growth and perhaps to maintain a constant sufficient number of growing follicles for further follicular development and ovulation.

The study of the effects of treatments upon the populations of small follicles has shown that the GnRH-a, buserelin, significantly increased the mean numbers of intermediary follicles. Whether the increasing number of intermediary follicles was related to accelerated departure of follicles from the primordial stage or to inhibition of transition of intermediary follicles into the primary stage remains uncertain. No significant changes in mean populations of either primordial, primary or secondary follicles were observed in buserelin $\pm$ hMG-treated females compared with other females. If there was an accelerated transformation of primordial into intermediary follicles, there should be fewer primordial follicles for similar numbers of intermediary follicles in buserelin $\pm$ hMG-treated animals than in unstimulated animals or in animals treated with gonadotrophin alone. This was not the case and consequently, the hypothesis that GnRH-a affects the transformation of primordial into intermediary follicles can be discarded. The hypothesis that buserelin partially inhibits follicular growth appears most likely because there were fewer primary and secondary follicles per intermediary follicle in ovaries treated with buserelin $\pm$ hMG than in unstimulated ovaries and ovaries treated with gonadotrophin alone. Despite these data, the mean populations of primary and secondary follicles did not show any changes.

Ataya *et al.* (1989) have shown that long-term administration of a GnRH-a inhibited the follicular loss in rats, allowing a significant increase in the number of follicles $\leqslant 35\,\mu m$ and a significant decrease in the number of growing follicles. In a previous report, Ataya *et al.* (1985) stated that short-term administration of GnRH-a had similar results, but these changes were not significant. In our study it could be assumed that the populations of primary and secondary follicles did not exhibit significant changes in buserelin $\pm$ hMG-treated animals because of the short duration of the treatments. Even though the transition from intermediary to primary and then to secondary follicles was inhibited, the growth rate of primary and secondary follicles could be too slow to

exhibit some changes in these populations after only two weeks. For longer treatments, secondary follicles would have entered the further stages in development, changing from early-growing to growing follicles and subsequently the number of secondary follicles would decrease.

The mechanism of action of GnRH-a on the process of follicular recruitment remains unknown. It has been previously shown (Reddy *et al.*, 1980) that a GnRH-a directly suppresses LH/hCG receptors in ovaries of rats, resulting in functional deprivation of follicles from gonadotrophins. Since LH and FSH have been immunohistochemically detected in oocyte and granulosa cells of primordial, intermediary and primary follicles of rats (Mulheron *et al.*, 1989), receptors to gonadotrophins are probably present in these follicles. It is possible that in monkeys, as in rats, buserelin could suppress LH/hCG receptors of small follicles, inhibiting entrance of small follicles into the proliferating pool. However, the factors governing the initiation of the follicular growth are not known in mammals. Although gonadotrophins have been suggested to control the entrance of small follicles into the pool of early-growing follicles (Lintern-Moore, 1977a; Arendsen de Wolff-Exalto, 1982), the initiation of follicular growth should be independent of gonadotrophins (Eshkol *et al.*, 1970; Peters *et al.*, 1973) or inhibited by other substances (Lintern Moore, 1977b; Lintern Moore *et al.*, 1979) or under the control of other mechanisms (Krarup *et al.*, 1969; Peters, 1973). As gonadotrophins are not necessarily involved in development into the early-growing stage, GnRH-a treatment might modify some factors in the ovarian microenvironment and thus alter the process of folliculogenesis.

The results of the present study showed that a potent GnRH-a, buserelin, when used during the follicular phase of one cycle, did not induce follicular loss. Nevertheless, GnRH-a increased the frequency of intermediary follicles, between primordial and primary stages. In conclusion, short-term use of GnRH-a alters the first steps of folliculogenesis. The possible inhibition of follicular growth initiation is probably a transitory phenomenon since human ovaries may be successfully superovulated during successive attempts at *in vitro* fertilization and embryo transfer. Consequently, we suggest that clinicians increase the interval between two successive treatments with GnRH-a to allow intermediary follicles to change to primary then secondary follicles and that the normal intra-ovarian regulatory processes may restore the normal ratios of small follicles of various types. Nevertheless, because of the great variability in populations of small follicles in the monkey ovaries, our suggestion that GnRH-a has a direct effect on the population of intermediary follicles remains to be confirmed.

The authors appreciate the animal husbandry of C. Genty (INSERM) and the technical assistance of Françoise Nomé (INSERM). They thank Serono France and Hoescht France laboratories for their financial support and especially J. Sandow (Hoescht AG, Frankfurt, Germany) for generous gift of buserelin implants.

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Received 29 April 1991