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Is there a role for estrogen in follicular maturation in the primate?

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The present study focusses attention on the effects of blocking estrogen synthesis, during follicular phase, on follicular maturation in the adult female bonnet monkey (*Macaca radiata*). Administration of cycling females ($n = 4$) with an aromatase inhibitor CGS 16949A (AI) by Alzet mini-pump (2.5 mg/day) from day 3 of cycle resulted in significant reduction in basal (by 53%) and surge levels of estrogen (by 70%) but this had no effect on follicular maturation, ovulation and luteal function as assessed by serum hormone profiles as well as laparotomy. This lack of need for estrogen for completion of follicular maturation process was confirmed by administering cycling monkeys hFSH (25 IU/day) from day 3 till day 8 of the cycle along with (5 mg AI/day) or without AI ($n = 3$/group). Administration of AI resulted in suppression of FSH induced increase in serum estrogen (by 100%) and elevation in circulating androstenedione. Aromatase inhibitor treatment had no effect on either the number of follicles developed or their size relative to control. Testing the ability of both granulosa and thecal cells, removed on day 9 of treatment cycle, to respond to gonadotropins *in vitro* showed no change indicating that cellular development and maturation of follicular cells had occurred normally. It is concluded that follicular maturation in the primate can occur even when increase in estrogen synthesis is blocked.

Introduction

Follicular maturation in the primate involves recruitment of a cohort of follicles to grow, selection of a single follicle among the cohort to mature leading to production of large quantities of estrogen and formation of a ovulable follicle (Goodman & Hodgen, 1983). These events are primarily regulated by FSH and LH. Granulosa and thecal cells of ovarian follicles undergo development related changes which include proliferation of granulosa cells, production of growth factors, secretion of estrogen etc. (Hillier, 1990). Although the thecal cell of the primate is endowed with aromatase activity (Channing, 1980) increased expression of aromatase is a function of the differentiating granulosa cell and this cell type appears to be largely responsible for increase in estrogen production seen during follicular maturation. As the follicle matures the granulosa cell becomes increasingly responsive to FSH and LH (Zeleznik *et al.*, 1977; Zeleznik & Kubik, 1986; Shaw *et al.*, 1989).

Estradiol has been shown to augment FSH-stimulated aromatase activity and induction of gonadotropin receptor expression in the rat granulosa cell (Sheela Rani *et al.*, 1981: Veldhuis *et al.*, 1982; Adashi & Hseuh, 1982; Vidyashankar & Moudgal, 1984). It has also been demonstrated to be a potent mitogen with respect to the rat granulosa cells (Sheela Rani *et al.*, 1977; Richards, 1980). These observations in the rodent have led to the belief that estrogen could, even in the primate, be modulating granulosa cell functionality and as such follicular maturation. There has, however, been no direct studies to confirm this hypothesis. In the present study, we have attempted to delineate the role of estrogen, if any, in regulating follicular maturation in the adult bonnet monkey (*Macaca radiata*) by employing Fadrozole, a new generation highly potent aromatase inhibitor (AI CGS 16949A) to selectively block estrogen synthesis (Hausler *et al.*, 1989) during follicular phase.

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Results

Effect of blocking estrogen synthesis during normal cycle: Continuous infusion of the aromatase inhibitor CGS 16949A from day 3 onwards at a dose of 2.5 mg/day resulted in a significant reduction of E secretion (Figure 1). Total E produced between days 3 to 14 in the control and experimental monkeys were (area under the curve) 16.74 ± 0.93 and 8.87 ± 0.41 cm^2 respectively ($P < 0.005$). Cervical mucus discharge, a parameter dependent on E level was markedly reduced in animals treated with the aromatase inhibitor. Estrogen levels between days 8-11 coinciding with the normal E surge of the cycle, was reduced by 70% (area under the curve: cm^2-control: 7.92 ± 0.35; experimental 2.16 ± 0.47 $P < 0.005$). Despite this, LH surge levels as measured by a

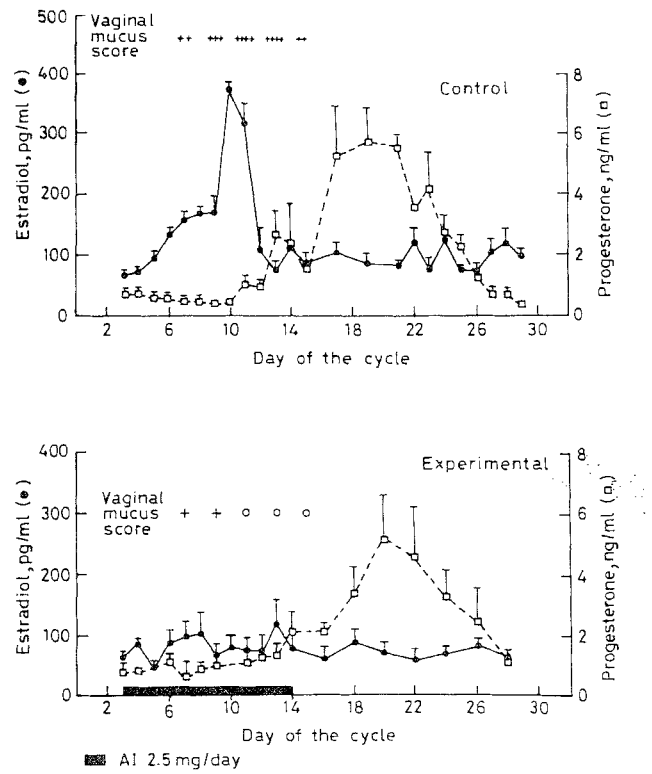

Figure 1 Effect of blocking estrogen synthesis during follicular phase on serum steroid levels in normal cycling female bonnet monkeys. Aromatase inhibitor CGS 16949A (AI) was administered by Alzet mini-osmotic pump between days 3–14 of the cycle at a dose of 2.5 mg/day

radioreceptor assay (control: 50.6 ± 4.2; experimental: 46.6 ± 8.1 ng/ml) was normal and ovulation had occurred. The corpus luteum formed was functionally normal as evidenced by luteal progesterone profile (P levels from day 14–28 of the cycle area under the curve: cm^2-control: 67.6 ± 5.23 *vs* Exptl: 58.3 ± 2.76). The incidence of ovulation was confirmed in two of the monkeys by examining the ovarian surface on day 10 of the cycle for the presence of follicle with stigmata. The cycle length of the experimental animals (30.5 ± 0.5 days) was not significantly different from that of the controls (28.2 ± 2.2 days).

Effect of blocking estrogen synthesis during exogenous FSH induced super follicular maturation: Two set of experiments were done using this model system. In the first experiment FSH alone (Metrodin 25 iu/day) or FSH + AI (5 mg/day) was administered over a 8 day period to two groups of monkeys (n of each group = 3) from days 3–10 of cycle and the ovarian surface examined on day 11 for the presence of mature follicle(s). Both FSH and FSH + AI treated animals showed the presence of 3–4 hyperimic follicles in each of the ovaries (Figure 2 a,b). Treatment with FSH alone caused an increase in serum E which reached a peak by day 9 but declined by day 10 and 11. Serum E remained totally suppressed in the FSH + AI treated animals (data not shown). The above experiment was repeated in toto in a fresh set of animals (*n* of each group = 3) but the FSH and FSH + AI treatment was restricted to 6 days only (from days 3–8 of cycle).

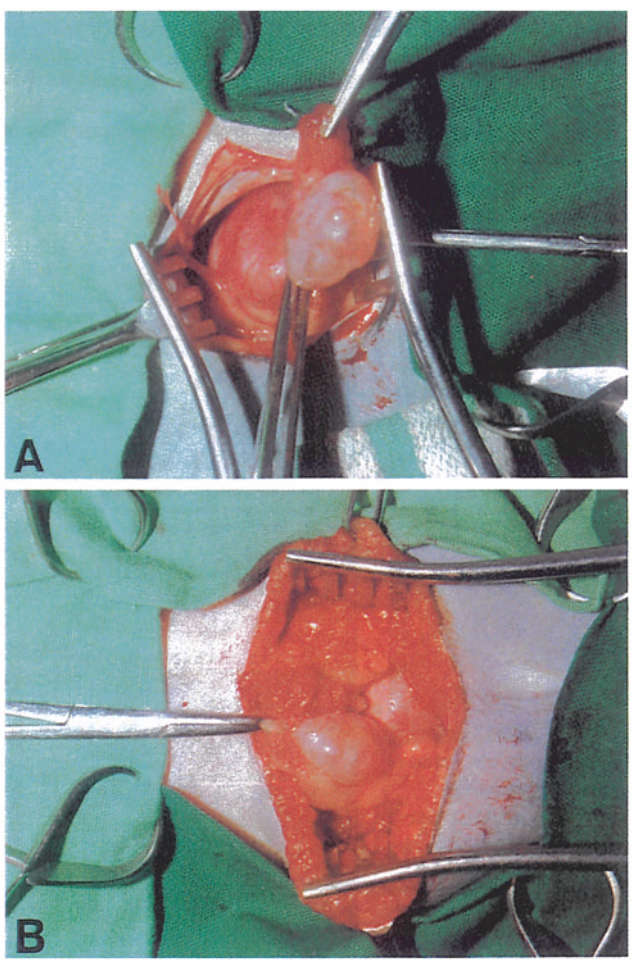

Figure 2 Photograph of the ovarian surface of female bonnet monkey on day 11 of the cycle administered hFSH (25 IU/day) from day 3–10. Note the presence of multiple follicles in both control treated with FSH alone (A) and the animals treated with FSH + AI from day 3–11 (B)

From the serum estradiol and androstenedione profile of both control (*n* = 3) and aromatase inhibitor treated animals (*n* = 3) that received FSH (25 iu/day) from day 3 to 8 of the cycle (Figure 3) it is evident that circulating E_2 gradually increased in controls to a peak level by day 9 of the cycle. Treatment with AI, however, markedly inhibited this increase in serum E_2 and on day 9 it was only 82 ± 0 pg/ml compared to the control value of 598 ± 2 pg/ml ($P < 0.05$). Androstenedione (the substrate for the enzyme aromatase), in contrast was significantly elevated ($P < 0.05$) in the experimental compared to the control animals (Figure 3). The above observations clearly indicated that growth and maturation of multiple follicles had occurred despite blockade of E synthesis. Ovulation had not occurred in these animals as no exogenous LH/hCG was administered.

The granulosa and thecal cells isolated from the mature follicles on day 11 of the cycle from both the control and experimental animals of the first set (FSH and AI regimen on from days 3–10 of cycle) of experiment (data not shown) failed to respond to added gonadotropins *in vitro*. Assuming this perhaps is due to atresia that had already set in because of lack of ovulation, in the next experiment wherein exogenous FSH or FSH + AI was administered only between days 3–8 of the cycle the granulosa and thecal cells were isolated on day 9 from the multiple mature follicles of both groups of animals and tested *in vitro* for responsivity to added gonadotropins. The ability of granulosa cells to produce progesterone and estrogen (Figure 4) and that of thecal cells to produce progesterone and testosterone (Figure 5) upon incubation with different doses of oFSH or hCG remained essentially the same (statistically not significant) for both control (FSH alone) and experimental (FSH + AI) animals.

Discussion

The role of estrogen as a neuroendocrine signal to release the pre-ovulatory surge of gonadotropins as well as its need for the preparation of endometrium are well established phenomenon. Besides this, increment in serum estrogen has generally been used as a marker to follow the progression of follicular maturation leading to formation of an ovulable follicle. In the current study we have been able to show in the cycling monkey that administration of CGS 16949A, a specific aromatase inhibitor as a continuous infusion at a dose of 2.5 mg/day during the follicular phase leads to marked inhibition in both the basal (by 53%) and the preovulatory E surge (by >70%) levels. This compound has earlier been observed to specifically inhibit estrogen but not progesterone synthesis of monkey luteal cells under LH stimulation *in vitro* (Selvaraj, 1993). The decrease in the circulatory E level following AI treatment during the follicular phase also resulted in a marked drop in the cervical mucus score and this concurs with the observation that E stimulates the cervical mucus secretion in both the human and macaque (Holt *et al.*, 1979; Nasir-ud-din *et al.*, 1983). The fact that despite marked reduction in E synthesis, normal luteal function (progesterone secretion during luteal phase) was observed indicates that a) overall follicular development was normal; b) the developed follicle was able to mature to a Graafian follicle stage and undergo ovulation and c) the ovulated follicle luteinized to a healthy corpus luteum capable of producing normal quota of progesterone. This questions the role of E in the process of follicular development as such in the primate. However, we have to be cautious in our conclusion as blockade of E synthesis was not complete and it is possible that low concentration of E in the presence of high levels of androstenedione is capable of discharging the physiologic functions associated with estrogen surge. An example of this is the occurrence of normal pre-ovulatory LH surge and ovulation despite significant reduction in estrogen.

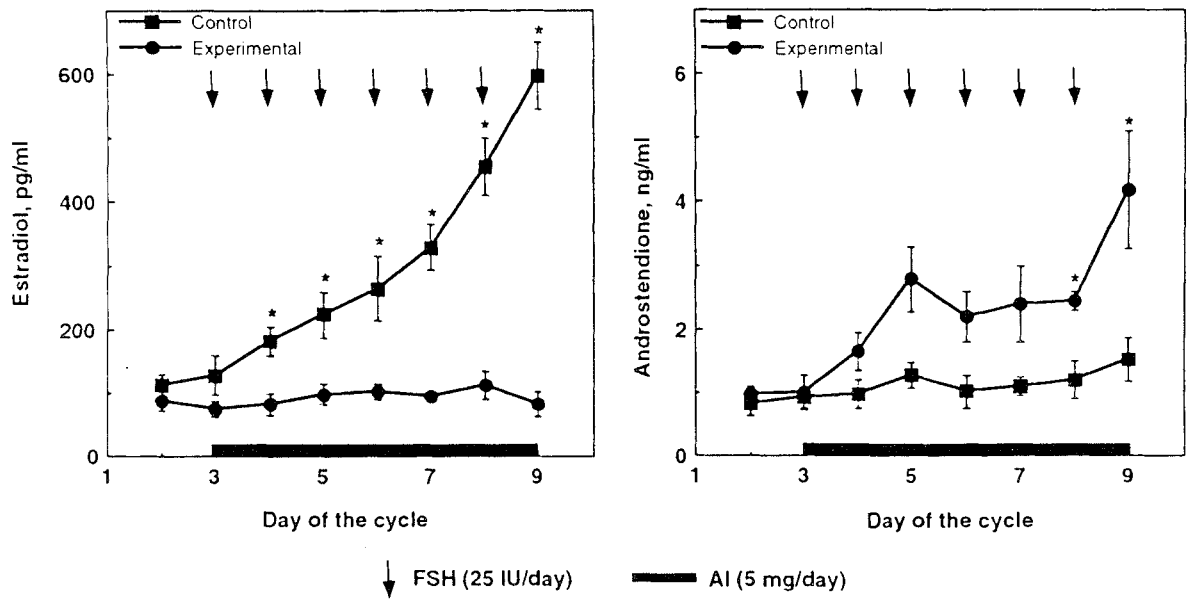

Figure 3 Effect of AI on serum estrogen and androstenedione levels in female bonnet monkeys treated with hFSH (Metrodin, 25 IU/day, arrow) from days 3–8 of the cycle. Controls ($n = 3$) received only hFSH while the experimentals ($n = 3$) were implanted with Alzet pump containing AI with a release rate of 5 mg/day from day 3–9 in addition to hFSH administration. *Significant difference $P < 0.05$ between groups (Scheffe F-test after ANOVA)

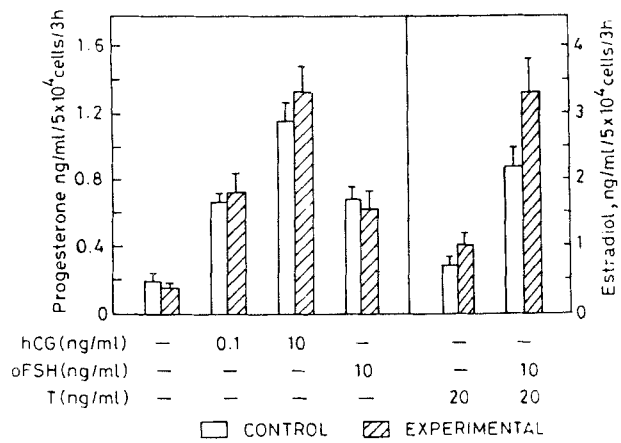

Figure 4 Effect of estrogen deprivation *in vivo* on the *in vitro* responsiveness of granulosa cells to gonadotropins. Granulosa cells (5×10^4 cells/tube) isolated from animals administered FSH alone (control $n = 3$) or from animals administered FSH and AI (experimental, $n = 3$) were incubated in triplicate at 37°C for 3 h in the presence or absence of gonadotropins and steroid secreted into the supernatant was estimated by appropriate RIA. Each group comprised of 3 animals and the values represent mean + SEM of 9 samples (3 replicates of each animal)

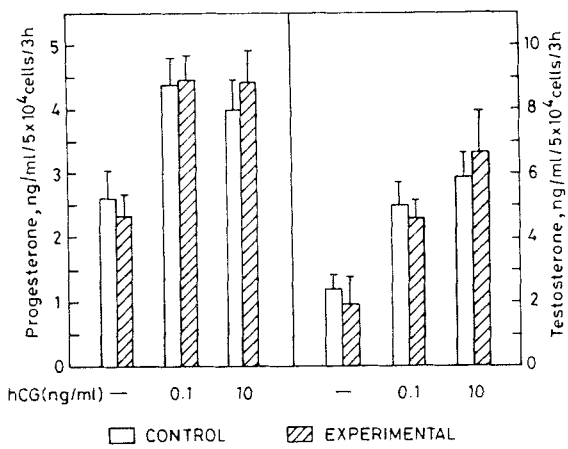

Figure 5 Effect of E deprivation *in vivo* on the *in vitro* responsiveness of thecal cells to hCG. Thecal cells isolated from control and experimental animals were incubated for 3 h at 37° C and steroid secreted was estimated. For each hormone level incubation was carried out in triplicate (5×10^4 cells/tube) and n of each group was 3. Values given are mean + SEM similar to Figure 4

Administration of FSH from the early follicular phase leads to recruitment of multiple follicles for development and maturation (Schenken *et al.*, 1984). During this process E is secreted in large quantities. If E is truely needed for follicular maturation/growth, blockade of E synthesis following FSH administration should lead to the inhibition of multiple follicular maturation. Though FSH induced E secretion was blocked by the administration of AI at a dose of 5 mg/day, we observed in contrast that the formation of multiple follicles was not inhibited and in fact equal number of follicles were formed in both control and experimental monkeys. Recently Zelinski-Wooten *et al.* (1994) using trilostane, a 3β-hydoxysteroid dehydrogenase inhibitor in cycling rhesus monkeys have also observed that follicular growth is not dependent on high levels of estrogen. Rabinovici *et al.* (1989) were able to induce multiple follicular development by administration of FSH to a woman with congenital 17 *alpha* hydroxylase deficiency. They were able to aspirate oocytes from three follicles following hCG injection. In this patient both serum and follicular fluid E and androgen levels were very low, indicating that despite the extremely low follicular fluid estrogen level development of follicles was not impaired. In another study, Schoot *et al.* (1992) demonstrated that administration of human recombinant FSH induced growth of preovulatory follicles without concomittant increase in androgen and estrogen biosynthesis in a woman with isolated gonadotropin deficiency. Since FSH alone in the absence of LH and estrogen could induce follicular growth and maturation they raise the question whether estrogen biosynthesis is mandatory for the growth of preovulatory follicles in the

human. These studies besides lending support to our observation in the bonnet monkey essentially indicate that high amount of E is not required for regulating follicular maturation in primates. Though Hild-Petito *et al.* (1988) and Wu *et al.* (1993) were not able to detect E receptors in the granulosa cells of macaque and human respectively, Billiar *et al.* (1992) have immunohistochemically localized estrogen receptors in about 30–40% of the granulosa cells of the antral follicles of the baboon thus reopening the question of existance of estrogen receptors in primate granulosa cells.

Since evidence in literature (mostly derived from the rodent study) suggests that E augments/facilitates ovarian cell proliferation and responsivity to gonadotropins, the granulosa and the thecal cells isolated from FSH and FSH + AI treated animals were analysed for their responsiveness to added gonadotropins *in vitro*. The results of this study clearly indicate that the functionality of these cells were not altered by the lack of E *in vivo*. Pariente *et al.* (1990) observed that the granulosa-lutein cells obtained after induction of ovulation in the 17 alpha hydroxylase deficient patient were able to convert testosterone to estrogen in a 24 h *in vitro* culture indicating thereby that aromatase activity was found in these cells despite the follicle being developed in an extremely hypoestrogenic environment. Progesterone secretion during the 24 h culture was markedly higher when compared to the granulosa-lutein cells obtained from normal women undergoing IVF programme. Similarly, Zelinski-Wooten *et al.* (1991), have made a preliminary report on the ability of luteal cells aspirated 27 h after hCG injection from monkeys treated with androstatrien-3-17-dione between days 8–10 of cycle to behave similar to the control in terms of both basal and hCG stimulated progesterone production *in vitro*. Our observation on the granulosa and thecal cell *in vitro* responsiveness from E deprived animals complements the above in that functionally these cells were normal prior to exposure to ovulating dose of hCG. It can thus be concluded that in the primate, the requirement of E to promote follicular growth and maturation as well as individual cell functionality is questionable.

The present study brings out the existence of clear cut differences in the regulation of follicular development between the rat and the monkey. In the rat, blockade of E synthesis does not have any affect on the functionalilty of the granulosa cell in terms of responsiveness to gonadotropins *in vitro* and it also has no effect on cell proliferation (^{3}H thymidine incorporation). Despite this there appears to be a reduction in the number of ovulable follicles produced and the mature follicles fail to respond to the ovulatory dose of hCG as evidenced by the lack of oocytes in the oviduct and the drastic reduction in the formation of corpora lutea (Selvaraj *et al.*, 1994). The possible reasons why these events do not seem to be blocked in the non-human primate is currently not known. Similarly, administration of DES to immature female rats led to the growth of large number of preantral follicles and priming with DES was required for these follicles to respond to FSH (Richards, 1980). However, Koering (1987) had demonstrated that DES had no effect on follicular maturation in the prepubertal rhesus monkey. Furthermore, Hillier *et al.* (1987) observed that estradiol had no effect on the enhancement of FSH action on aromatase activity and progesterone synthesis in cultured granulosa cells from the marmoset.

Materials and methods

Materials

PMSG (2,940 IU/mg) and hCG (CR127, 14,900 IU/mg) were provided by NICHD, Bethesda, USA (courtesy Dr Bialy G). oFSH (1 ng oFSH corresponds to 1.46 ng hFSH, NIH AFP 4822B) was purified in our laboratory (Moudgal *et al.*, 1992), hFSH (Metrodin) used was a product of Serono, Italy. Tritiated progesterone (88 Ci/mmol), testosterone (97 Ci/mmol), estradiol (90 Ci/mmol) and androstenedione (97 Ci/mmol) were purchased from Amersham, UK. Aromatase inhibitor CGS 16949A, Fadrozole hydrochloride (AI) was a gift from the Ciba-Geigy Ltd., Basel, Switzerland (Bhatnagar *et al.*, 1989). Mini osmotic pumps were procured from Alza Corp, Palo Alto, CA, USA. DMEM was from GIBCO, USA, HEPES and all other chemicals were obtained from Sigma Chemical Co, St. Louis, MO, USA.

General methodology

Healthy adult regularly cycling female bonnet monkeys (*Macaca radiata*) 8–12 years of age were used. The details of animal husbandry, monitoring of cycle, procedure for collecting blood samples have been previously described (Prahalada *et al.*, 1975). All the animal experiments were cleared by our Institutional ethical committee for use of laboratory animals for biomedical research. The day of onset of menstrual bleeding was considered as day 1 of the cycle. Blood samples were collected at 0930 h from unanesthetised monkeys by venipuncture using vacutainer tubes (Becton-Dickinson, USA) as described earlier (Ravindranath *et al.*, 1989). Following processing of the blood for serum the samples were stored at −20°C till analysed for steroid hormone levels. The amount of mucus in the vagina of each animal was subjectively scored from the beginning of the cycle and the number of + signs denote the quantity of mucus secreted. Since only from day 6 of the cycle notable amount of mucus secretion could be observed the score (+ signs in increasing progression) becomes significant between day 6 and the expected day of ovulation.

In the first set of experiments, normally cycling monkeys ($n = 4$) were implanted each with an Alzet mini-osmotic pump (model 2002 containing aromatase inhibitor-release rate 2.5 mg/day) on day 3 and these were removed on day 14 of the cycle. This enabled us to study the effect of blockade of estrogen synthesis during the follicular phase on cyclic follicular maturation. The dose AI to be administered was determined by undertaking pilot experiments in a different set of bonnet monkeys (Selvaraj, 1993). The control monkeys ($n = 3$) were implanted with pumps containing vehicle (saline).

Super follicular maturation was induced in regularly cycling adult females by injecting ($n = 6$) 25 IU hFSH (Metrodin) per monkey, per day from either days 3 to 8 or 3 to 10 of cycle in split doses of 12.5 IU/inj at 0945 and 2145 h of each day. While each of the experimental animals (n = 6) was implanted with an aromatase inhibitor (release rate 5 mg/day) containing Alzet mini-osmotic pump (model 2001) on day 3 of the cycle, the control monkeys (n = 6) were implanted with pumps containing vehicle alone. Depending upon the FSH treatment protocol employed the pumps were removed either on day 9 or 11 of the cycle and laparotomy was performed under ketamine anaesthesia to isolate granulosa and thecal cells as per procedure outlined below.

Granulosa and thecal cell isolation and incubation protocol

The dominant follicle(s) was aspirated using a 24 gauge needle mounted on a tuberculine syringe. The antrum was gently flushed twice to remove the loosely held granulosa cells with DMEM containing 25 mM HEPES, 0.37% $NaHCO_3$ and 0.1% BSA pH 7.4 (Krietmann *et al.*, 1981). The follicular aspirate was centrifuged, the fluid separated and the cells suspended in DMEM. The medium obtained from flushing the follicle was also centrifuged to collect the residual granulosa cells and the cells thus obtained were all pooled and used. The thecal tissue that was excised was extensively washed to remove any contaminating granulosa cells and was subjected to collagenase digestion essentially according to Stouffer *et al.* (1976) with slight modifications to

obtain single cells. In brief, this consisted of cutting the thecal tissue into small pieces, suspending it in DMEM containing 2% BSA and 0.2% collagenase and incubating at 37°C in a shaker water bath for 15 min. This was followed by addition of 2 ml of DMEM containing 2% BSA and releasing the single cells by repeatedly aspirating and releasing the suspension with a pipette for about a minute to release the dispersed cells. After allowing the debris to settle, the supernatant containing the cells was aspirated and subjected to a wash step with the medium. The debris was further digested twice and the supernatant from different digests pooled and the cells collected by centrifugation washed thrice to remove excess collagenase prior to suspending the final cell pellet in a fixed volume of medium.

The granulosa and thecal cells in the DMEM medium were pre-incubated at 37°C for 30 min in a shaker water-bath followed by washing and the cells suspended in a known volume of medium were counted in a haemocytometer and 5×10^4 viable cells per tube (as determined by trypan blue exclusion test) were incubated in triplicates in the presence and absence of the gonadotropins (hCG and oFSH) in a total volume of 0.5 ml (in 22 × 75 mm glass tubes) for 3 h at 37°C in a shaker water bath with 60 oscillations per minute. After incubation the tubes were kept frozen at −20°C until they were assayed for appropriate steroid hormones.

Hormone assays

Estradiol (E), progesterone (P), androstenedione (A) and testosterone (T) assays were carried out as described elsewhere (Prahalada *et al.*, 1975). The serum samples were ether extracted twice and suspended in gelatin phosphate buffered saline (GPBS). The *in vitro* incubates were assayed for P without any processing. Progesterone and T antisera used were kind gifts of Dr. Chandana Das, AIIMS, New Delhi and Dr. Usha Joshi, IRR, Bombay respectively. Characteristics of the P and T antisera used have been provided earlier by Ravindranath *et al.* (1989) and Moudgal *et al.* (1992) respectively. The antibody to estradiol which was raised in our laboratory cross reacted with estrone and estriol to an extent of 3.7% and 1.1% respectively and for P and T the cross-reactivity was less than 0.1%. Antiserum to A used was a kind gift of Dr. S.G. Hillier (Edinburgh, UK). Serum LH was estimated using a radio-receptor assay as described by Selvaraj and Moudgal (1993).

Statistics

Individual experiments were carried out using a minimum of 3–4 animals per group and the data presented are mean ± SEM of replicate experiments. The amount of steroid in circulation was calculated by computing the area under the curve for estradiol and progesterone in an IBM compatible computer using GP2 software and the treatment differences were analysed by student's *t* test. Differences between control and experimental serum estradiol and androstenedione in the FSH induced super follicular maturation model was compared by ANOVA followed by Schefee F-test using Macintosh based Stat View 512+ software developed by Brain Power Inc., CA, USA. In the *in vitro* incubation study the response to each level of hormone addition was determined in triplicate. Treatment differences were analysed by Student's *t*-test.

Acknowledgements

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