AN INDIVIDUALIZED TECHNIQUE FOR INDUCING OVULATION IN THE BONNET MONKEY, *MACACA RADIATA*

JARDENA OVADIA,* JANET W. McARTHUR, OLIVE W. SMITH
AND JALEH BASHIR-FARAHMAND

*Vincent Memorial Hospital (Gynecological Service of Massachusetts General Hospital)
and Department of Obstetrics and Gynecology, Harvard Medical School, Boston,
Massachusetts, U.S.A.*

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Summary. A staircase method for the administration of HMG and HCG was employed for the induction of ovulation in anovulatory bonnet monkeys newly introduced into a primate colony. Clinical and chemical indices were utilized to monitor the ovarian response. Although the HMG dosage was found to vary over a ten-fold range, the indices enabled the treatment to be adapted to the requirements of the individual animal, and single ovulation to be dependably induced.

INTRODUCTION

The successful induction of ovulation with gonadotrophins in the cycling monkey, *Macaca mulatta* (van Wagenen & Simpson, 1957a, b), was soon repeated in the hypophysectomized rhesus monkey (Knobil, Kostyo & Greep, 1959) and in the human female with anovulatory sterility (Gemzell, Diczfalussy & Tillingier, 1958). Multiple ovulation was the rule in the experiments of Simpson & van Wagenen (1958, 1962, 1963) and van Wagenen (1968), who foresaw its importance as a clinical problem. However, the consequences of hyperstimulation of the ovary have proved to be more serious than was anticipated from animal studies, and have prompted much clinical research. This has proceeded along two general lines: (1) repeated testing of ovarian sensitivity before definitive therapy to determine the correct dose of gonadotrophin for initiating follicular growth, and (2) continuous monitoring of the patient's response during an extended course of gonadotrophin treatment by means of clinical or chemical indices. Although foetal wastage and maternal complications have been materially reduced by these means, they have not been eliminated. We have therefore sought to develop a primate model in which the mode of ovulating a single follicle could be explored systematically, and the effects of gonadotrophins upon the ovary observed at will.

* Present address: Beilinson Hospital, University of Tel Aviv Medical School, Petah-Tikva, Israel.

MATERIALS AND METHODS

The primate chosen for these studies was the bonnet monkey, *Macaca radiata*. Mature females ranging in weight from 3.8 to 4.5 kg were procured from a variety of dealers and from primate centres. During a control period extending over several months, they were examined daily by (1) quantitative aspiration of cervical mucus, and (2) vaginal irrigation for cytological studies (see below). All of the animals exhibited sporadic fluctuations in cervical mucus secretion and five, which remained amenorrhoeic, were allocated to this project.

Initially, tests were made of the classic HMG + HCG regimens which had been developed in the rhesus monkey. It seemed likely that the same dosage and time schedules would obtain, since the body weights of the two species are similar, and the cycle lengths in both are of the 4-week type. The following methods were employed:

(a) Simpson & van Wagenen (1962):

FSH 120 i.u. + LH 120 i.u. daily for Days 5 to 9 inclusive.

FSH 60 i.u. + LH 60 i.u. + HCG 2000 to 3000 i.u. daily for Days 10 to 12 inclusive.

(b) Mastroianni, Suzuki, Manabe & Watson (1967) and Wan & Balin (1969), Schedule 1:

FSH 75 i.u. + LH 75 i.u. daily for Days 5 to 10 inclusive.

FSH 38 i.u. + LH 38 i.u. + HCG 2000 i.u. daily for Days 11 to 13 inclusive.

(c) Dede & Plentl (1966):

FSH 8 i.u. + LH 9 i.u. daily for Days 3 to 10 inclusive.

FSH 8 i.u. + LH 9 i.u. + HCG 2000 i.u. daily for Days 11 and 12.

A total of four trials of Method (a), three of Method (b) and three of Method (c) were made. The results are summarized in Table 1A. It can be seen that no response occurred in the animals given the lowest dosage, and that, in those receiving higher doses, polycystic ovary formation rather than ovulation resulted in all instances.

From this disappointing outcome, it appeared that our monkeys were too heterogeneous physiologically to respond like the animals in the stable inbred colonies of van Wagenen, Mastroianni and Dede & Plentl. They resembled the clinical population with suspended menstruation which, on the basis of gonadotrophin assays, can be classified as 'just amenorrhoeic' or 'very amenorrhoeic'. Thus, the measure of control over the endogenous gonadotrophin supplement which Simpson & van Wagenen (1962) had imposed by beginning HMG administration on Day 5 of the menstrual cycle was impossible to achieve.

It appeared that the fixed-dose regimens should be supplanted by one which would (1) possess the flexibility required to induce ovulation with equal effectiveness in animals with gonadotrophin deficiencies of varying degree, and (2) imitate the response pattern characteristic of the normal menstrual cycle in this species. Ideally, that amount of exogenous gonadotrophin would be administered which, when added to the endogenous level, would provide

precisely the total quantity needed to initiate follicular growth and to ovulate the mature follicle.

A variable-dose regimen for inducing single ovulation was developed and, in the absence of direct measurements of circulating gonadotrophin levels, was monitored by means of the following indices of ovarian response (McArthur, Ovadia, Smith & Bashir-Farahmand, 1971): (1) cervical mucus weight, spinnbarkeit and ferning, (2) vaginal cytology, with collection of cells by the irrigation technique of Davis (1962a, b), millipore filtration and staining by the Harris-Shorr method, and classification of cell-types according to criteria defined by the International Academy of Gynecological Cytology, Terminology

TABLE 1

RESPONSE OF ADULT FEMALE BONNET MONKEYS TO HMG+HCG TREATMENT FOR INDUCING OVULATION

A. Fixed-dose regimens

Methods	Trials	Ovulation		Failures	
		Single	Multiple	Polycystic ovaries	No response
Simpson & van Wagenen (1962)	4	0	0	4	0
Mastroianni <i>et al.</i> (1967), Wan & Balin (1969)	3	0	0	3	0
Dede & Plentl (1966)	3	0	0	0	3
Totals	10	0	0	7	3

B. Variable-dose regimen

Methods	Trials	Ovulation		Failures	
		Single	Multiple	Polycystic ovaries	Persisting follicular cyst
Present authors	12	10	0	1	1

Subcommittee (1958), (3) sex skin reddening, graded from 0 to 5, (4) urinary excretion of 'total oestrogens' by the rapid method of Brown, MacLeod, Macnaughtan, Smith & Smyth (1968), with creatinine correction for variation in the number of hours' urine collected and (5) laparoscopy and laparotomy. The ovaries were inspected before the initiation of gonadotrophin treatment, and at selected times thereafter to ascertain its effects. Drawings were made of ovaries examined laparoscopically and photographs were taken of ovaries exposed at laparotomy. Fresh corpora lutea with stigmata and well-formed boutons were thus readily distinguishable from regressing corpora lutea, the position of which had been previously determined. Transillumination proved to be exceedingly useful for differentiating the corpus luteum, with its characteristic density, from the transparent follicular cyst (see below).

Treatment was begun with HMG in a small sub-threshold dose which was gradually increased according to a staircase pattern. Pergonal Lot No. 2267, each ampoule of which contained 75 i.u. of FSH and 75 i.u. of LH, was employed. (Since, in this particular Lot, the dosage of FSH and LH was equal, we will

hereafter refer interchangeably to i.u. of HMG, FSH or LH.) The hormone was dissolved in 10% gelatin to delay absorption and was injected once daily by the subcutaneous route. If, after 5 days' administration, no increase in urinary oestrogen had occurred, the dose was increased by a factor of 1.3 and continued for an additional 5 days. Progressive increases by the same factor were made at 5-day intervals until the follicle had entered upon the phase of pre-ovulatory swelling. This stage is recognizable by a rapid, but not precipitous, increase in urinary oestrogen excretion (Townsend, Brown, Johnstone, Adey, Evans & Taft, 1966). The threshold dose was then maintained until the urinary oestrogen had risen to the normal peak level of 6 to 8 $\mu\text{g}/24\text{ hr}$, after which the gonadotrophin treatment was suspended for 1 to 2 days and the oestrogen level allowed to fall. A single intravenous injection of HCG, in a dose of approximately 500 i.u., was then given to simulate the LH surge. This dose, which is much smaller than that administered hitherto by investigators employing the rhesus monkey, is based upon the observation of Brown, Evans, Adey, Taft & Townsend (1969) that 3000 i.u. of HCG ordinarily suffices to induce ovulation in the human female. This dosage was adapted to the animals in the present study by utilizing their relative surface area as the basis for calculation. Since the average human female has a surface area of 1.5 m^2 and the bonnet monkey one of 0.24 to 0.28 m^2 , approximately one-sixth of the human dose was administered.

RESULTS

The outcome of twelve treatment courses employing the staircase method in five animals is summarized in Table 1B. Ten ovulations, all single, were induced and two failures resulted. Two examples of the successful use of the staircase procedure are shown in Text-figs. 1 and 2. During the first of these, which was begun in early summer, all of the indices remained stationary until a daily HMG dose of 103 i.u. was reached. The cervical mucus weight, vaginal cornification and urinary oestrogen level then commenced a gradual increase. Under the stimulus of increments which brought the daily dose to 174 i.u., the urinary oestrogen level reached 7.2 $\mu\text{g}/24\text{ hr}$ and HMG treatment was suspended. The oestrogen level then began to fall, whereupon HCG was administered. A laparotomy performed 9 days later revealed normal-sized ovaries containing a single corpus luteum (Pl. 1, Figs. 1 and 2). The second example shows the reaction of the same animal to a second course of treatment, given during the following winter in the months of February and March. On this occasion, there was a surprisingly rapid response which again culminated in the formation of a single corpus luteum.

The threshold dose of HMG which initiated the preovulatory surge of oestrogen in the ten successful cycles is summarized in Table 2. It is evident that, although the maximum dose is more than ten times the minimum, the majority fall within a narrow range. The six-fold difference between the summer and winter threshold doses in the animal whose treatment courses have been illustrated is an isolated finding. However, the result appears to warrant a systematic study of seasonal differences in threshold dosage.

PLATE 1

1

2

The internal genitalia as observed at laparotomy. Monkey, Jean, 9 days after injection of nccg, following treatment course detailed in Text-fig. 1.

FIG. 1. Single corpus luteum in left ovary.

FIG. 2. Transilluminated left ovary; dark area corresponding to corpus luteum.

3

4

The internal genitalia as observed at laparotomy, Monkey Bonnie, 11 days after injection of ncc, following treatment course detailed in Text-fig. 4.

FIG. 3. Persisting follicular cyst in right ovary.

FIG. 4. Transilluminated right ovary.

The failures merit particular attention. The first, in which polycystic ovary formation took place (Text-fig. 3), was marked by periodic indications of an oestrogenic effect upon the sex skin and cervical mucus secretion. However, on only one of these occasions (*circa* Day 30 of treatment) was there a significant rise in urinary oestrogen excretion, and that to values below the normal pre-ovulatory peak range. Such a dissociation of the indices connotes an as yet

TEXT-FIG. 1. The urinary excretion of total oestrogens, the cervical mucus weight, spinnbarkeit (SBK) and ferning, the degree of clumping of the vaginal epithelial cells and the percentage of parabasal (B), intermediate (I), anucleate squamous (AnS) and superficial (S) cells in the vaginal smear, and the intensity of reddening of the sex skin in a female bonnet monkey (Jean) during HMG + HCG treatment administered in the months of June, July and August.

poorly characterized metabolic abnormality (Boquoi & Hammerstein, 1969). Conceivably, one of the known oestrogens which is not measurable by the Brown method was secreted and exerted the physiological effects.

The second failure differs fundamentally from the first, in that a single follicle was developed to the preovulatory stage by HMG treatment but that, for some reason, ovulation failed to result from HCG administration. Persistence of an unovulated follicle was recognizable from the pattern of response even before laparotomy was performed (Text-fig. 4 and Pl. 2, Figs. 3 and 4). The

TEXT-FIG. 2. The urinary oestrogen excretion and clinical indices (as in Text-fig. 1) in the same monkey during HMG+HCG treatment administered in the following winter, during the months of February and March.

TABLE 2

THRESHOLD DOSE OF HMG
FOR INITIATION OF PREOVU-
LATORY SWELLING DURING
TEN TRIALS CULMINATING
IN A SINGLE OVULATION

FSH-LH (i.u.)	No. of trials
16.0	2
21.0	4
28.0	3
174.0	1

TEXT-FIG. 3. The urinary oestrogen excretion and clinical indices (as in Text-fig. 1) in a monkey (April) who developed polycystic ovaries in response to HMG + HCG treatment.

urinary oestrogen excretion and cervical mucus weight continued to be inordinately high after HCG administration and the regression of reddening of the sex skin was minimal. The timing of the HCG dose may have been defective in that the injection was delayed too long.

TEXT-FIG. 4. The urinary oestrogen excretion and clinical indices (as in Text-fig. 1) in a monkey (Bonnie) who developed a persisting follicular cyst in response to HMG + HCG treatment.

DISCUSSION

It would appear that in the bonnet monkey, as in the human female, the key point in achieving single ovulation is to begin treatment with a subthreshold quantity of HMG; the effective dose is then attained by small increments, as recommended by Brown *et al.* (1969). The dose is increased only until it stimulates the follicles to secrete oestrogen at a rate characteristic of the pre-

ovulatory phase of the normal cycle. Significant as is the absolute level of oestrogen achieved, the rate of rise is even more important. Studies of the spontaneous cycle reveal that a single follicle growing normally secretes oestrogen at a rate which increases no more than two-fold within a 24-hr period. If a rate greater than this is observed, it is likely that more than one follicle is responding to gonadotrophin stimulation. The HMG dosage increment factor of 1.3 acts as a safeguard against overstimulation of the follicles. In repeated tests of ovarian sensitivity before definitive therapy, Crooke, Butt & Bertrand (1966) have shown that the human ovary is also capable of responding differentially to increments of this magnitude, and Brown *et al.* (1969) have confirmed the observation of Crooke *et al.* (1966) during extended courses of gonadotrophin treatment.

Another factor which merits emphasis is the timing of the HCG injection. Since the oestrogen peak normally precedes the LH surge (Burger, Catt & Brown, 1968; Corker, Naftolin & Exley, 1969; Dufau, Dulmanis, Hudson, Catt, Fullerton & Burger, 1970), HMG and HCG should be administered sequentially. There should be no overlapping of HMG and HCG treatment; rather, a pause should be introduced between them. The correctness of this procedure is evident from the clinical studies of Brown *et al.* (1969), which demonstrate that the follicles are maximally sensitive to HCG stimulation when the rate of oestrogen secretion is increasing slowly or has begun to decline.

These studies facilitate the interpretation of previous studies concerned with the induction of ovulation in rhesus monkeys. Simpson & van Wageningen (1962), working with a long-established colony of normally menstruating animals, desired to obtain an unequivocal effect and began treatment with large doses of HMG. Presumably, the FSH/LH ratio of the preparation they employed (Pergonal 23) approximated to that of the plasma gonadotrophins during the normal follicular phase, so that an additive effect of endogenous and exogenous gonadotrophin was obtained and multiple ovulation resulted. Reduction of the gonadotrophin dosage did not abolish multiple ovulation (van Wageningen, 1968), a finding compatible with subsequent clinical studies which have demonstrated the narrowness of the margin between under- and over-stimulation. Since, in the Yale colony, the subthreshold dose of HMG was zero, any increment, however small, necessarily resulted in overstimulation of the ovarian follicles.

Duplication of the Simpson-van Wageningen regimen in our animals resulted in polycystic ovary formation rather than multiple ovulation. Being amenorrhoeic, the monkeys were not in the early follicular phase at the time HMG treatment was begun, either with respect to pituitary gonadotrophin secretion or to ovarian structure, and must be presumed to differ qualitatively as well as quantitatively from those in the Yale colony.

The magnitude of the dosage excess employed by Simpson & van Wageningen (1962) is evident from the results of Mastroianni *et al.* (1967). The latter desired to induce multiple ovulation for studies of tubal ovum transport and succeeded by administering approximately two-thirds of the dose given by Simpson & van Wageningen (1962), beginning on Day 5 of the cycle. Schedule 1 of Wan & Balin (1969) is similar to that of Mastroianni, and their Schedule 2 differs only in minor particulars. However, Wan & Balin (also Balin & Wan,

1969) noted a decrease in the incidence of multiple ovulation to 46%, a result which they attributed to dealer procurement, with a presumed reduction of endogenous gonadotrophin levels as a reaction to the stress of capture and shipment. Dede & Plentl (1966), working in a domiciliary breeding colony akin to that of van Wagenen, attempted to improve the pregnancy rate by inducing follicular growth with one-sixteenth of their HMG dosage. Thirty-one instances of ovulation, for which the criteria were ovarian enlargement and reddening of the sex skin, are claimed but only five pregnancies, all single, resulted.

As regards HMG, the dosage required by our monkeys more closely resembles that of Dede & Plentl (1966) than that employed in any other study. However, as previously noted, the use of their fixed dose of 8 i.u. failed to initiate follicular maturation in any of our animals, in which the gonadotrophin deficiency was too great to be repaired by such a small quantity of HMG.

It is clear that the induction of single ovulation in animals with intact hypophyses by means of gonadotrophin treatment requires hypogonadotrophic subjects, like the clinical population of women with anovulatory sterility. In such subjects, the quantity of HMG needed for correct supplementation of the endogenous gonadotrophin secretion can be readily determined by adjustment of the dose to the individual. That the staircase method for achieving such individual adjustment not only possesses the flexibility required to induce single ovulation in a heterogeneous population but that it does so in quasi-normal fashion is indicated by the similarity of the ovarian response pattern to that of the spontaneous ovulatory cycle (McArthur *et al.*, 1971). With the aid of this primate model system, it should be possible in future to isolate the various components of the process that result in single ovulation, to analyse them and ultimately to subject them to control.

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