Long-term contraceptive efficacy of vaccine of ovine follicle-stimulating hormone in male bonnet monkeys (*Macaca radiata*)

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Summary. A group of ten healthy fertile adult male bonnet monkeys were actively immunized using procedures acceptable for human use with pure follicle-stimulating hormone (oFSH) isolated from sheep pituitaries. The vaccine elicited an immunogenic response in all ten monkeys; the antibody-binding capacity, determined by Scatchard analysis, varied from 3 to 18 µg oFSH ml^{-1}, the binding affinity ranging from 0·13 to 2·0 × 10^{10} mol^{-1}. A substantial population of antibodies against oFSH crossreacted with ^{125}I-labelled human (h) FSH, used here as a representative ligand of primate FSH. The bioneutralization activity of the antisera assessed by a specific bioassay *in vitro*, when the antibody titre was high, was 6·9 ± 0·18 µg hFSH ml^{-1}. Immunization for 4·7–5·7 years did not affect the health and libido of the animals. Concentration of testosterone in serum remained normal throughout the study, but, within 150 days of immunization, there was a marked decrease (75–100%) in the number of spermatozoa in seminal ejaculates. Oligospermic status interspersed with azoospermia was maintained by periodic boosting. The fertility of these animals was monitored between 6 months and 2 years after primary immunization. All the ten animals proved infertile in repeated mating experiments with females of proven fertility. After stopping booster injections, nine of ten animals regained fertility, but the time taken for this depended upon the rate of decline of antibody titres. Re-boosting these monkeys with 100 µg oFSH after confirming that recovery had occurred revealed prompt increases in antibody titres followed once again by onset of oligo-azoospermia and infertility, underscoring the specificity of immunization effect. The immunized monkeys, apart from being acutely oligospermic, ejaculated spermatozoa that were markedly deficient in key acrosomal enzymes, such as acrosin and hyaluronidase, and motility as well as in their ability to penetrate a gel *in vitro*, suggesting that the infertility observed was due to gross reductions in the numbers of spermatozoa that could effectively interact with the oocyte and cause successful fertilization.

Keywords: gonadotrophins; follicle-stimulating hormone; testicular function; fertility; immunization; contraception; bonnet monkey

Introduction

Considerable emphasis has been placed on research into development of contraceptives that are effective when administered as a vaccine. Immunogens that can be used in males are currently restricted to follicle-stimulating (FSH), luteinizing (LH) and gonadotrophin-releasing (GnRH) hormones, although immunization with nonspecific proteins has also been suggested (Talwar *et al.*, 1979). The choice of oFSH as an immunogen was based on the results of an earlier study in which

we showed that chronic passive immunoneutralization of endogenous FSH of adult male monkeys with characterized antiserum to oFSH leads to a drastic reduction in number of spermatozoa and onset of infertility (Murty *et al.*, 1979a; Sheela Rani *et al.*, 1978), but the fertility of these monkeys could be restored by stopping antiserum injections. Wickings & Neischlag (1980) and Raj *et al.* (1980) confirmed this observation by showing that immunization of monkeys with oFSH disrupts spermatogenesis and results in oligospermia without causing changes in serum testosterone concentration; however, no fertility tests were conducted by either of these investigators. Srinath *et al.* (1983) suggested that continuous active immunization of rhesus monkeys with oFSH (for more than 3 years) resulted in the monkeys starting to produce increased numbers of spermatozoa in less than 20% of the ejaculates suggesting that the animals may eventually be able to override the effect of FSH immunization. However, as only a few monkeys were used in this study and rhesus monkeys are seasonal breeders (Sehgal *et al.*, 1986), it is not clear whether the increment in the immunized monkey seminal sperm counts reported at about the time of seasonal recrudescence (July–August) is due to factors other than FSH regulating continuous production of spermatozoa. We therefore tested the long-term efficacy of oFSH as a contraceptive vaccine by undertaking studies in a male non-human primate model that does not exhibit overt seasonality in production of spermatozoa. The bonnet monkey (*Macaca radiata*) has been observed to continue to produce spermatozoa throughout the year (Murty *et al.*, 1979b). As the current study was undertaken as a prelude to clinical trials, every precaution was taken to use methods acceptable for eventual human use. We therefore used aluminium hydroxide gel as the vaccine carrier since it is an acceptable adjuvant for use in humans rather than Freund's complete adjuvant vaccine carrier.

Materials and Methods

Vaccine

The antigen used was oFSH isolated in the laboratory from frozen sheep pituitaries, according to the procedure of Sairam (1979) modified to suit laboratory conditions. The procedure included a passage through an LH antibody–Sepharose affinity column to remove traces of LH contamination (<0·05%). The final product was checked for homogeneity by a variety of physicochemical criteria, such as gel electrophoresis, high-performance liquid chromatography profile, amino-terminal analysis and radioreceptor:radioimmuno activity ratio.

Ovine FSH (1 ml of 10 mg ml^{-1}) solution in 0·9% sodium chloride was mixed with 19 ml of aluminium hydroxide (2%) gel (Alhydrogel: Superfos, Denmark) suspension, mixed thoroughly, left to stand at room temperature for 6 h and overnight at 4°C before use for immunization. All vaccinations were made using oFSH adsorbed on this gel except for the first group of monkeys (nos 375, 377, 391 and 498) which received oFSH adsorbed on laboratory prepared instead of commercial grade aluminium hydroxide gel only during the first year. The concentration of oFSH used, procedure adopted for obtaining adsorption as well as injection schedules were the same with both of the gel preparations.

Animals

Twenty-five healthy adult males, 8–10 years old, of proven fertility, were screened before initiation of the study for number of spermatozoa in seminal ejaculates obtained by electroejaculation as well as occurrence of nocturnal testosterone surges (Mukku *et al.*, 1981). Ten of the animals that showed satisfactory patterns and exhibited good health were recruited for the study. Parallel nonimmunized age-matched controls were monitored to obtain baseline data on serum testosterone, sperm quantity and quality and fertility index. The procedure followed for husbandry of these monkeys has been provided earlier (Moudgal *et al.*, 1985; Ravindranath & Moudgal, 1987).

Immunization protocol and general methods

The immunization protocol consisted of administering 1·0, 0·3, 0·1 and 0·1 mg of immunogen (oFSH adsorbed on Alhydrogel – 0·5 mg FSH ml^{-1} of gel suspension) to individual monkeys subcutaneously on days 1, 8, 16 and 24. The entire immunization study period of 1720–2088 days was divided into three phases. During phase I, from day 1 to day 550 when the first fertility testing was completed, animals received subcutaneously, every 90–100 days, 100 oFSH µg^{-1} adsorbed on alhydrogel as a booster injection; periodic blood sampling (from the femoral vein of unanaesthetized monkeys using vaccutainer tubes) to assess antibody titre, testosterone and LH concentrations in serum and penile

electrostimulation to study sperm quantity and quality were carried out; sampling ceased 15–20 days before the start of mating studies. In phase 2, from approximately days 550–1300 of immunization when animals were rested, no boosters were given and animals were checked for fertility once numbers and quality of spermatozoa returned to near normal. In phase 3, from about day 1300 to days 1720–2088, each monkey was rechallenged with 100 µg oFSH every 90–100 days and the effect on antibody titre and sperm production was monitored.

Hormone assays

Testosterone radioimmunoassay was carried out according to a standardized procedure (Mukku *et al.*, 1981) using a rabbit antiserum (T-IV) to testosterone–bovine serum albumin conjugate. The antiserum exhibited cross-reactivity with epitestosterone (0·1%), androstenedione (0·3%), androsterone (0·3%) and other C-18, -19, and -20 steroids (<0·01%). The assay sensitivity for testosterone was 0·01 ng per tube; the inter- and intra-assay coefficients of variation were 10·3 and 5·6%, respectively. Serum LH was measured using an LH radioreceptor assay standardized in the laboratory. The assay has a range of 0·6–10 ng hLH ml^{-1}, monkey LH (monkey pituitary extract as well as monkey serum) showing a parallel displacement (N. Selvaraj & N. R. Moudgal, unpublished). The inter- and intra-assay coefficients of variation of this assay were <10%. Radioimmunoassay procedures used for estimation of oestrogen, progesterone and monkey chorionic gonadotrophin were described in detail by Ravindranath & Moudgal (1987).

Antibody titration and determination of crossreactivity

The serum from the immunized monkeys was titrated for antibody concentration using two procedures. The first procedure consisted of determining the dilution at which 25% binding of ^{125}I-labelled ovine and human FSH occurs; 100 µl of different dilutions of the sera were incubated with about 50 000 c.p.m. of ^{125}I-labelled FSH (ovine or human) at room temperature for 20 h. The bound antigen–antibody complex was separated from the free by the addition of excess amount of goat antibody to monkey IgG and the radioactivity in the pellet monitored using an LKB auto-gamma spectrometer. Besides the above binding studies, the crossreactivity of the antisera with (a) ovine α and β subunits, (b) human FSH (hFSH) β subunit and human LH and (c) ovine LH was also determined. Iodination of gonadotrophic hormones and their subunits was according to procedures well standardized in the laboratory, iodogen being used as an oxidizing agent; the specific activity of the iodinated preparations ranged between 40 000 and 45 000 c.p.m. per ng of the hormone or subunit.

The bioneutralizing activity of the antisera was assessed by isolating granulosa cells from the ovary of 27–28-day-old female rats stimulated with pregnant mares' serum gonadotrophin 48 h earlier and incubating (50 000 cells per tube) with 50 ng of hFSH in the presence of 2·5 and 5·0 µl of normal monkey serum or test antiserum for 4 h at 37°C. The total volume of incubation per tube was 500 µl. The progesterone produced was monitored by an appropriate radioimmunoassay (Ravindranath *et al.*, 1989). The ability of different batches of antisera to inhibit the response to hFSH was calculated and the results expressed as percentage inhibition. Each assay included a standard laboratory preparation of hyperimmune FSH antiserum (a/s) used earlier to assess FSH bioneutralizating activity (Ravindranath *et al.*, 1989).

Counting and assessing quality of spermatozoa

Monkeys were tranquillized with ketamine hydrochloride (3 mg kg^{-1} body weight). Semen samples and the coagulum collected into dry 5 ml test tubes using a penile electrostimulator according to the method of Mastroianni & Manson (1963) were kept for 20 min in a water bath at 37°C for liquefaction. It was noticed that the animals become habituated to repeat ejaculatory procedure and ejaculate with very little electrostimulation (<20 mamps of 1–3 s duration per monkey). The volume of the ejaculate was noted and the number of spermatozoa in the ejaculate was counted using a haemocytometer.

The viability of spermatozoa (200 spermatozoa per sample) was assessed by the method of Swanson & Bearden (1951) using eosin and nigrosin to stain dead spermatozoa. The gross motility of the spermatozoa in the ejaculate was scored by subjecting a drop of semen after liquefaction to examination under a light microscope (Carl Zeiss, Germany), according to the method of Mann (1981).

The spermatozoa–cervical mucous interaction test originally described by Kremer (1965) was modified in the laboratory to measure sperm penetration of gel. The method involved the use of hen egg white instead of cervical mucus as the gel. The white of the egg was stirred for 20 min using a magnetic stirrer, centrifuged for 5 min at 12 000 r.p.m. (Eppendorf Microfuge, Germany), the supernatant degassed using suction and stored at 4°C as aliquots. On the day of assay, the gel was aspirated into rectangular capillary microslides (Vitro Dynamics, Inc., Rockaway, NJ, USA) and one end sealed by dipping lightly into molten paraffin wax. The open end was inserted into a rectangular opening made on the side of a reservoir fashioned from a test tube (12 × 100 mm^2) bottom in such a way that the microslides remained horizontal. Sperm suspension (0·5 ml; 10^6 spermatozoa ml^{-1}) in minimum essential medium (MEM) (Himedia, Bombay) was pipetted into the reservoir and the entire apparatus was incubated in a humidified chamber at 37°C. After 30 min, the slides were carefully removed and placed on an etched flat slide and the number of spermatozoa per field width was counted for the entire breadth of the microslide (at × 10 magnification) at 0·25, 0·5,

1.0, 2.0, 3.0, 4.0 and 5.0 cm along its length. Numbers of spermatozoa (mean $\pm$ SEM) determined from ejaculates of different monkeys at specific distances inside the micro-tube were plotted against distances moved to obtain comparison between groups.

Measurement of activity of acrosin and hyaluronidase in washed spermatozoa

Spermatozoa were washed three times with normal saline and acrosin activity was extracted according to the method of Goodpasture *et al.* (1980). Spermatozoa were suspended in 1 mmol HCl l^{-1} containing 50 mmol benzamidine l^{-1} (Sigma Chemical Co., MO, USA) and 10% glycerol (10^8 spermatozoa ml^{-1}), and extracted overnight at 4°C. After centrifugation, the supernatants were dialysed against 1 mmol HCl l^{-1} to remove benzamidine and stored at 4°C until assayed. Acrosin activity was determined by measuring the rate of hydrolysis of benzoyl arginyl ethyl ester (BAEE-Sigma). The aliquots of extracts were first incubated with 50 mmol Tris–HCl l^{-1}, pH 8.1 at 37°C for 1 h to convert proacrosin to acrosin. The acrosin reaction was initiated by addition of $CaCl_2$ (final concentration 50 mmol l^{-1}) and 0.5 mmol BAEE l^{-1}. Initial studies showed that addition of $CaCl_2$ during the activation step inhibited acrosin activity. Rate of hydrolysis of BAEE was monitored by determining the increase in absorbance at 253 nm at room temperature against 0.5 mM BAEE and was found to be linear up to 30 min. Molar absorption coefficient of 1150 was used to determine the hydrolysis of BAEE. The activity is expressed as IU (1 IU being umoles of BAEE hydrolysed min^{-1} 10^{-7} spermatozoa).

For determination of sperm hyaluronidase activity, washed spermatozoa were suspended in 50 mmol acetate buffer l^{-1}, pH 4.0 containing 0.1% Triton-X100 (10^6 spermatozoa ml^{-1}) and sonicated for 1 min. The extract was centrifuged in an Eppendorf microfuge at 14 000 g for 5 min and the supernatant was stored at -20°C until assayed. Aliquots of the supernatant were incubated with hyaluronic acid (Sigma) at a final concentration of 8 mg ml^{-1} in 50 mmol acetate buffer l^{-1}, pH 4.0 for 1 h at 37°C. The reaction was terminated by addition of 0.8 mmol potassium tetraborate l^{-1} and heating in a boiling water bath for 5 min, cooled and p-dimethyl aminobenzaldehyde (PDAMB-Merck, NJ, USA) added and the colour developed was read at 540 nm.

Assessment of fertility

The fertility of the monkeys was assessed by undertaking standardized mating studies as described earlier (Murty *et al.*, 1979a). Each of the males was caged at a time with a fertile regularly cycling female whose ovulatory nature was assessed by monitoring serum for oestrogen and progesterone (Ravindranath & Moudgal, 1987). Thus, each male was exposed to a total of five females, cohabitation being confined in each case to days 9–14 of the cycle. Establishment of pregnancy in the female was confirmed by monitoring serum for increment in oestrogen and progesterone and monkey chorionic gonadotrophin (mCG) concentrations from day 25 onwards (Ravindranath & Moudgal, 1987). The females that did not become pregnant after exposure to immunized males were subsequently cohabited with the breeder males in the colony to ensure their fertility status.

Results

Immunogenic response and antibody titration

Immunization was initiated in the first group of four monkeys (numbers 375, 377, 391 and 498) in August 1984 and in the second group of six monkeys (numbers 405, 422, 428, 482, 483 and 725) in August 1985. The vaccine formulation used in the two groups differed during the first year. Whereas the first group received the first six injections of oFSH (on days 1, 9, 18, 36, 100 and 200) adsorbed on laboratory prepared aluminium hydroxide gel (vaccine A), the second group used throughout FSH adsorbed on commercial aluminium hydroxide gel (vaccine B). The dose of oFSH used in both vaccine formulations was the same. Vaccine formulation A relative to B produced a poor immunogenic response as monitored by ^{125}I-labelled hFSH binding during the first 300 days (Fig. 1). The group I monkeys, however, upon receiving a booster dose of vaccine B on day 320 showed a spurt in their antibody titre (by 10–40 times Fig. 1a, b) and from then on no differences were observed between the two groups with respect to their antibody (Fig. 1c, d) and other physiological responses. The use of vaccine A in group I monkeys was discontinued from day 320.

The affinity of antibodies produced by the ten monkeys calculated using Scatchard analysis of binding data ranged from $0.13 - 2.0 \times 10^{10}$ mmol l^{-1}. Although the affinity did not change either with the progress of immunization or with the source of the aluminium hydroxide gel, the capacity of the antisera to bind FSH showed wide variation (mean $\pm$ SD 9.1 ± 5.4 µg FSH ml^{-1}). The ^{125}I-labelled hormone binding assay also showed that the antibody generated to oFSH bound the α and
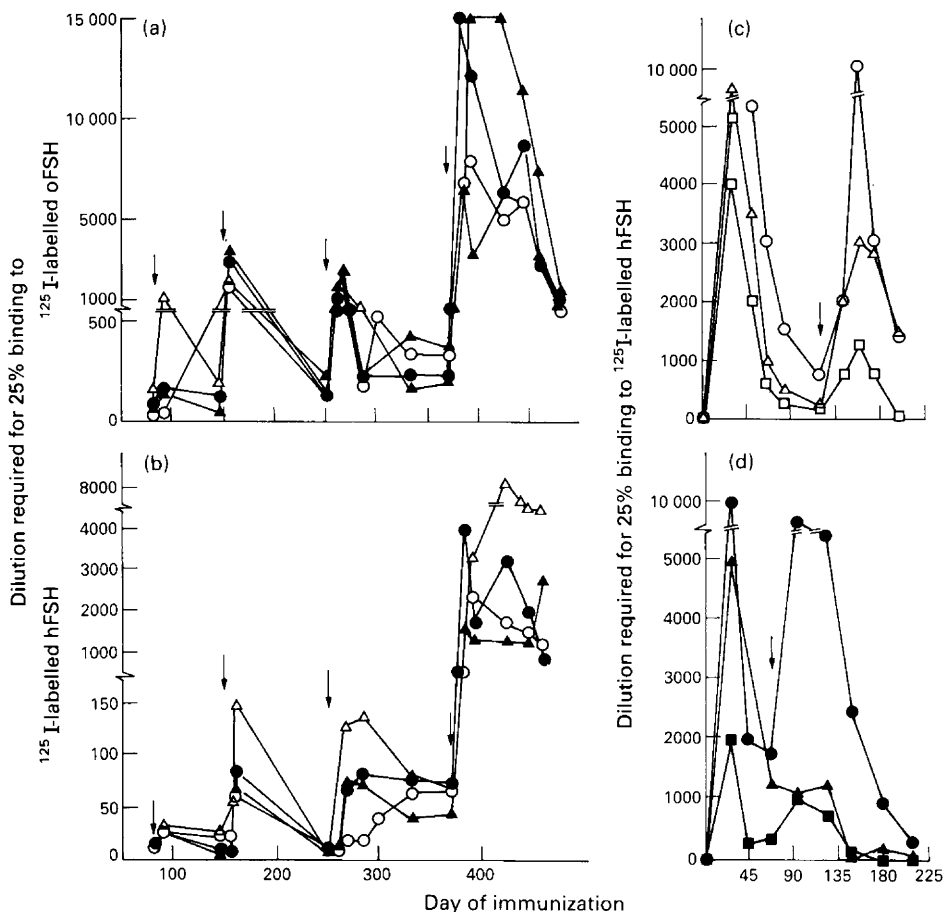
Fig. 1. Immune responsiveness of individual bonnet monkeys to ovine follicle-stimulating hormone (oFSH) vaccine. Binding of ^{125}I-labelled (a) ovine and (b) human FSH (hFSH) to sera of monkeys in group I (monkey nos 375 (○); 377 (●); 391 (△) and 498 (▲). The first three injections (↓) of oFSH were given adsorbed on laboratory prepared aluminium hydroxide gel (vaccine A) whereas the fourth injection (on day 320 and beyond) of oFSH was given adsorbed on commercial Alhydrogel (vaccine B). (c) and (d) represent human FSH antibody titres before and after a single booster injection (↓) of Group II monkeys immunized throughout with oFSH adsorbed on commercial Alhydrogel (vaccine B) (monkey nos 422 (□); 428 (○); 483 (△); 405 (▲); 482 (●) and 725 (■).

β subunits of ^{125}I-labelled oFSH in addition to ^{125}I-labelled oFSH (Table 1). The fact that addition of excess cold oFSH to the antisera abolished binding to ^{125}I-labelled oLH (data not shown) suggested that the crossreactivity is due to epitopes in the common α subunit of the two hormones. All antisera showed binding to ^{125}I-labelled hFSH but not to ^{125}I-labelled hFSH β subunit or ^{125}I-labelled hLH (Table 1). The efficacy of the antibody to bioneutralize primate FSH (hFSH used as an example) was tested using the *in vitro* rat ovarian granulosa cell assay system. The capacity of the antibody generated calculated using this assay was $5.0 \pm 0.35\,\mu g$ hFSH ml^{-1}.

Determination of the specificity of the immunization procedure

Serum testosterone of immunized monkeys (basal and nocturnal surge concentration) remained unchanged throughout the test period (control: 14.9 ± 0.7, experimental: 15.2 ± 0.5 ng ml^{-1},

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Table 1. Differential binding of ^{125}I-labelled hormones to ovine follicle-stimulating hormone antisera raised in bonnet monkeys

Monkey no.	^{125}I-labelled ligand bound (%)				
	oFSH	oFSHβ	oLH	oLHα	hFSH
375	62·5	21·6	14·7	17·9	57·7
377	57·8	9·0	25·3	22·1	73·4
391	80·3	8·2	42·1	51·0	93·5
405	70·6	32·8	35·3	44·5	85·6
422	79·4	49·9	57·3	54·6	62·4
428	35·0	—	5·3	11·5	33·0
482	31·5	—	4·8	24·9	53·8
483	38·2	2·4	20·6	33·0	26·2
725	32·4	37·9	13·8	32·5	4·2

B_{max} for each of the ^{125}I-labelled hormones determined using specific antisera was considered as 100% and actual binding observed with different (100 µl of 1:100 diluted) antisera expressed as % of the B_{max}. Binding to ^{125}I-labelled hFSH β subunit and hLH was < 10% in all cases.
LH: luteinizing hormone; FSH: follicle-stimulating hormone; o: ovine; h: human.

mean ± SEM of 48 and 120 samples, respectively. More recently using a radioreceptor assay that can measure monkey serum LH, it has been observed that monkeys immunized with FSH do not show a marked change in the concentrations of LH in sera (mean ± SD hLH equivalent ng ml^{-1} – control: 17·6 ± 0·7, experimental: 12·9 ± 2·7) (N. Selvaraj & N. R. Moudgal, unpublished).

Effect of immunization on the quality and quantity of spermatozoa voided

Within 150 days of immunization, all of the ten monkeys showed a significant reduction in number of spermatozoa per ejaculate, mean ± SEM (day 0: 356 ± 76 versus day 150: 32 ± 23 $P < 0.01$). This state of oligospermia interspersed with occasional azoospermia was maintained by periodic boosting until the first set of fertility testing was completed. During the entire period of immunization, which extended over 46–58 months, 24–29 ejaculates of each monkey were examined for determination of number of spermatozoa. A >75% reduction in number of spermatozoa was observed in 80% of the ejaculates of seven monkeys and in 50% of ejaculates of three monkeys. It should be noted that the total period screened included 6–12 months when no booster was given and the animals were allowed to recover their normal testicular function. During this period the number of spermatozoa gradually returned to normal as indicated by examining three to six ejaculates per monkey.

The quality of spermatozoa voided was assessed by a variety of parameters using standardized procedures including viability (Fig. 2a), gross motility (Fig. 2b) and gel penetrability (Fig. 3). Since bonnet monkeys normally exhibit at least marginally higher sperm production during August–December of each year, the number of spermatozoa of both control and immunized monkeys over a 12 month period was examined (Fig. 2a). The total and viable number of spermatozoa of the controls were significantly ($P < 0.01$) different from the immunized monkeys throughout the year. Of the three parameters, the motility and the gel penetrability of the spermatozoa of immunized monkeys were significantly lower ($P < 0.01$) than in controls. A significant decrease in the activity of acrosin ($P < 0.001$) of washed spermatozoa was also observed in all the immunized monkeys (Table 2). The hyaluronidase activity of spermatozoa of the immunized monkeys showed only a marginal decrease which was not statistically significant.
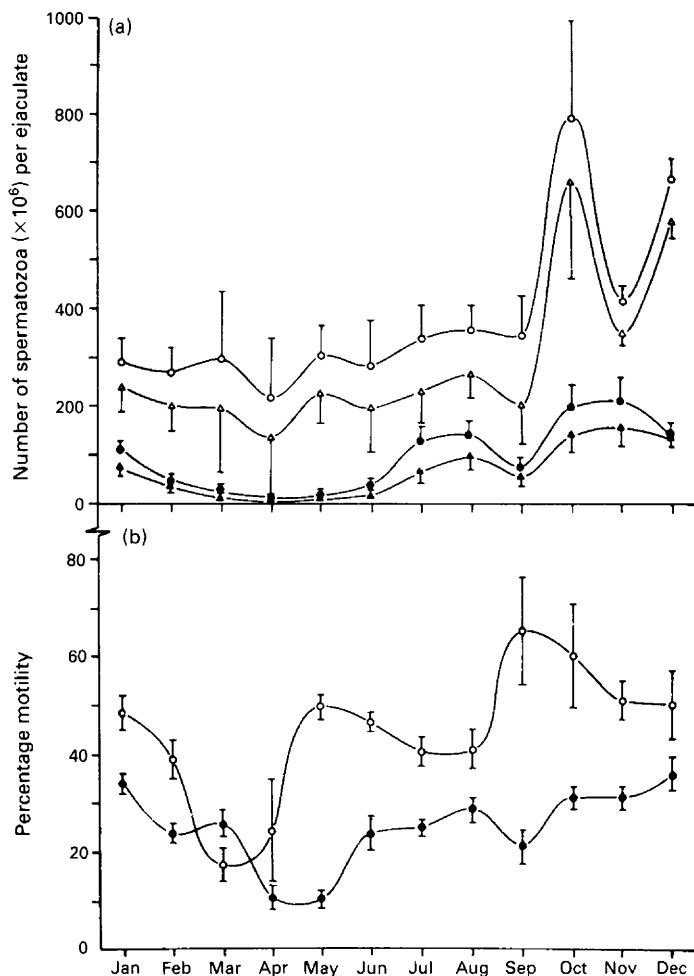
Fig. 2. (a) Numbers of total (○) and viable (△) spermatozoa of control and numbers of total (●) and viable (▲) spermatozoa of bonnet monkeys immunized with ovine follicle-stimulating hormone over 12 months. (b) Motility (%) of spermatozoa of control (○) and ovine follicle-stimulating hormone immunized (●) bonnet monkeys over 12 months. Data are means ± SEM; in each group *n* = 10.

Effect of immunization on fertility

Immunization with oFSH vaccine at no time affected mating behaviour of monkeys and this is perhaps due to unaltered concentrations of serum testosterone. The experimental monkeys were tested for fertility at three different periods of immunization. Whereas one set of three monkeys was tested for their fertility (ability to impregnate proven fertile females) between 96–170 days of immunization, the second (*n* = 3) and third (*n* = 4) sets of monkeys were tested for their fertility between 138–220 and 320–508 days of immunization, respectively. No pregnancies could be recorded despite exposing the ten monkeys to a total of 52 proven fertile females (Table 3). Each male was exposed to a minimum of five females and the number of spermatozoa of the monkeys at about the fertility testing period ranged from 10 to 160×10^6 per ejaculate. All of the control group of nine untreated males proved their fertility when tested at the same time as experimental animals. They were exposed to a total of 26 females leading to 15 pregnancies. One exposure per female is usual for the colony (Ravindranath & Moudgal, 1987). The data were analysed by both
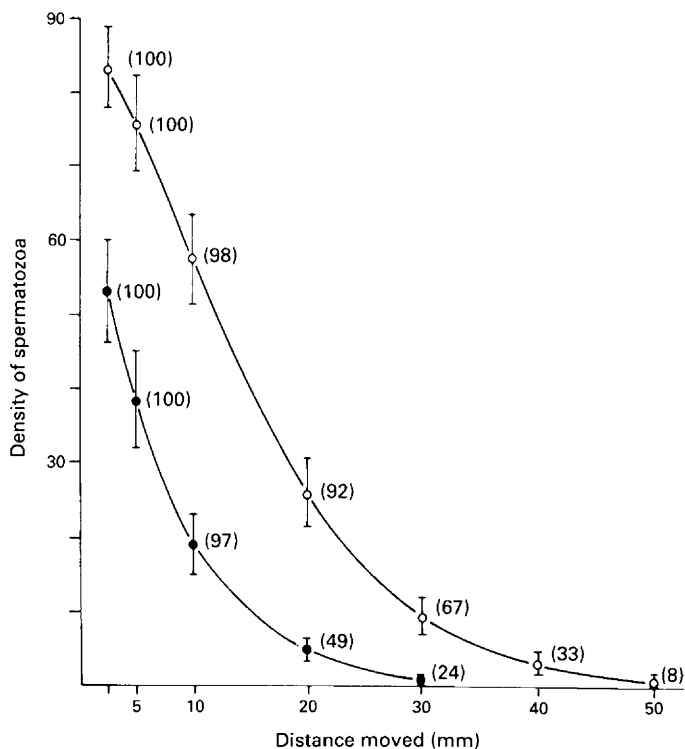
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Fig. 3. Gel penetrability (distance travelled in mm) and sperm density (number of spermatozoa) at specific distances (mean numbers ± SEM) of control (○; $n = 14$; 48 ejaculates) and immunized (●; $n = 10$; 35 ejaculates) bonnet monkeys as assessed by modified Kremer's test. Figures in parentheses indicate the percentage of ejaculates in which spermatozoa had moved to corresponding distances.

Table 2. Biochemical determinants of sperm quality in bonnet monkeys

	Controls ($n = 9$)	Immunized with oFSH ($n = 10$)	% change	P
Hyaluronidase (μg *N*-acetylgalactos-amine 10^{-8} spermatozoa h^{-1}	15.25 ± 3.04	10.12 ± 5.81	34	ns
Acrosin miu 10^{-7} spermatozoa	15.57 ± 2.72	3.68 ± 2.65	74	<0.001

Values are means ± SD.
miu: amount of enzyme required for hydrolysis of *n* moles benzoyl arginine ethylester min^{-1}. ns: not significant; oFSH: ovine follicle-stimulating hormone.
The spermatozoa from both control and immunized monkeys were washed three times with normal saline and then suspended in 1 mmol HCl l^{-1} containing 10% glycerol and 50 mmol benzamidine l^{-1} (10^8 spermatozoa ml^{-1}) for extraction of acrosin activity and 50 mmol acetate buffer l^{-1} (pH 4·0) containing 0·1% Triton X-100 for extraction of hyaluronidase activity.

χ^2 and Fisher test and showed that the breeding performance of the immunized monkeys was significantly different from the controls ($P < 0.01$).

Although there was variation among monkeys, it generally took 100–120 days for the antibody titres to fall to low concentrations (10–15% binding when 100 μl of undiluted sera was incubated with ^{125}I-labelled hFSH) when no further boosters were given. The number of spermatozoa

Table 3. Fertility status of bonnet monkeys immunized with ovine follice-stimulating hormone

Monkey No.[a]	Active immunization phase (days 1–550)			Recovery phase (days 550–1400)	
	Period of fertility testing[b] (days)	Number of spermatozoa[c] (10^6 per ejaculate)	Fertility status[d] (number pregnant/ number of females mated)	Number of spermatozoa[c] (10^6 per ejaculate)	Days taken for restoration of fertility[e]
405 (3)	96–170	10–119	0/5	17–248	430
482 (1)	96–170	10	0/5	73–360	430
725 (1)	96–170	10–69	0/5	151–524	780
422 (1)	138–220	10	0/5	67–140	325
428 (2)	138–220	10	0/5	29–146	325
483 (1)	138–220	10	0/5	140–222	700
375 (1)	320–508	73–125	0/5	146–473	80
377 (2)	320–508	10–160	0/5	14–282	80
391 (1)	320–508	10	0/6	10–132	Infertile up to 892 days
498 (1)	320–508	10	0/6	12–213	700

[a]All monkeys used were proven fertile, numbers in parentheses refer to number of babies sired in the colony before immunization. [b]Refers to days of immunization when fertility was tested. [c]Number of spermatozoa just before initiation of mating studies. [d]Fertility testing was carried out by caging the male with a proven fertile female between days 9 and 14 of the ovulatory cycle. 0/n indicates infertile status of the male. [e]Refers to days after the last booster injection of ovine follicle-stimulating hormone when the animal was able to impregnate. The booster was administered between 500 and 550 days after immunization.

increased thereafter and reached normal values 100–150 days later for eight of the monkeys, whereas the remaining two (numbers 391 and 498) showed improvement in the number of spermatozoa only to levels well below normal. Fertility testing at this time or beyond revealed that nine out of ten of the immunized monkeys had recovered their ability to impregnate females (Table 3) but the resting period required for successful recovery and impregnation differed among monkeys. Whereas two monkeys recovered fertility by 80 days after final booster, four required 325–430 days and three of the remaining four monkeys took as long as 700–780 days to achieve first successful impregnation (Table 3). Even after 892 days of recovery monkey number 391 was found to be infertile despite showing reasonable numbers of spermatozoa in its ejaculate (about 100 millions per ejaculate).

Studies after the recovery period

When fertility was recovered, the monkeys were re-boosted by injecting 100 μg oFSH every 90–100 days. In response to the booster, the antibody titres once again increased promptly. This was reflected in an increase in both the ^{125}I-labelled hormone binding and bioneutralizing capacity of the antibody (Fig. 4). However, the magnitude of response showed considerable variation and the direct binding data did not always correspond to the capacity of the antibody to bioneutralize hFSH. This rise in antibody titre was followed by a rapid fall in the quality and quantity of spermatozoa voided (data not shown). The response to the boosting has been observed to be better if the antibody titres are allowed to fall very low before the administration of the vaccine.

Discussion

This study was undertaken to assess the long-term contraceptive efficacy and potential of oFSH vaccine when administered adsorbed on Alhydrogel, an adjuvant cleared for human use. The data,
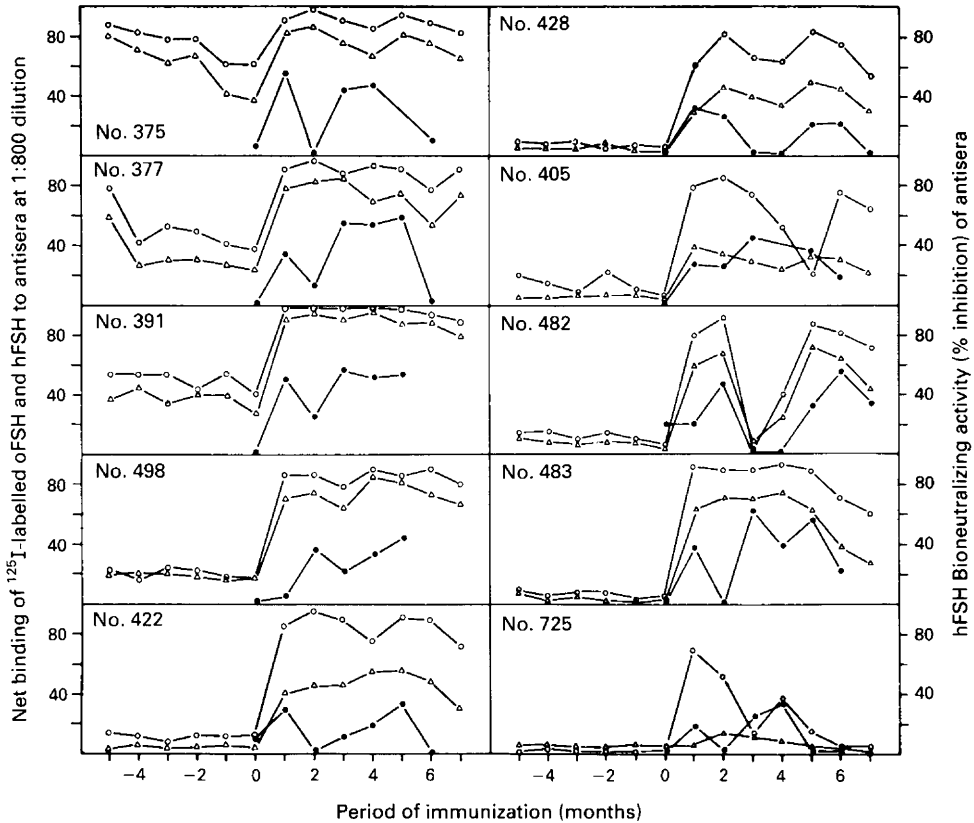
Fig. 4. Ability of ten bonnet monkeys (numbers shown in figure) to respond to boosters of ovine follicle-stimulating hormone (oFSH) after different periods of recovery. The titres of antibody that bind oFSH (○) and human FSH (hFSH) (△) versus the titre of bioneutralizing antibody (●) that blocks the ability of rat granulosa cell system to respond to added hFSH is shown. The booster injection of oFSH (100 μg per monkey) was given at time 0. The antibody titres before this (−4 to 0 months) represent values present during the recovery period.

besides clearly showing that oFSH does produce a satisfactory immunogenic response in all of the ten monkeys tested, underscore its ability to produce antibodies that can crossreact with hFSH but not hLH. Neither serum testosterone nor LH concentrations showed a significant change after immunization with oFSH confirming the specificity of the antibody response. Each booster injection brought about an increase in hFSH bioneutralizing antibody titre that lasted no more than 100 days necessitating repeat boosting.

The capacity of the antibody to bioneutralize primate FSH (mean 6.9 ± 0.18 μg hFSH ml^{-1}) was more than adequate to neutralize endogenous FSH concentrations of normal male monkeys (1–3 ng hFSH equivalent ml^{-1}). No 'free FSH' could be detected by radioimmunoassay in the sera of any of the monkeys following precipitation of antigen–antibody complex with polyethylene glycol.

From the crossreactivity profile of the oFSH antiserum (Table 1) it appears that antibodies produced to conformational epitope(s) common to intact ovine and hFSH rather than FSH β subunit are responsible for the bioneutralizing activity (Moudgal *et al.*, 1989).

This investigation demonstrated that chronic FSH deprivation results in infertility in all of the ten monkeys, and fertility was restored in 90% of the monkeys by stopping booster injections thus allowing FSH concentrations to return to normal. By carrying out fertility testing at different times

after immunization, it was observed that infertility begins as early as 96–170 days after treatment (Table 3). The demonstration that the experimental monkeys were fertile before the start of immunization and recovered their fertility after withdrawal of boosting underscores the specificity of the immunization effect. An important aspect of the present study is the observation that the recovered monkeys could once again be rendered oligospermic and infertile by oFSH booster injections. All monkeys have responded positively to the post-recovery oFSH boosters (Fig. 4) and the number of spermatozoa was reduced by 75% of the original ($300 \pm 75 \times 10^6$ per ejaculate).

This study shows that it is possible to render experimental animals infertile without necessarily obtaining azoospermia. Examination of the quality of the spermatozoa voided by these infertile monkeys showed that besides significant ($P < 0.01$) reductions in number of spermatozoa, viability, gross motility and gel penetrability of the voided spermatozoa were markedly affected. This may result in a reduction in the effective concentration of spermatozoa in the ejaculate that can positively interact with the oocyte. Fertilization *per se* depends upon the ability of the spermatozoa to bring about effective dispersion of the cumulus and gain entry into the oocyte. The two sperm acrosomal enzymes hyaluronidase and acrosin which are involved in the control of the above functions (Reddy *et al.*, 1980; Beyler and Zaneveld, 1982) have been shown in the present study to be markedly reduced (Table 2). Besides this, Raj *et al.* (1991) using eggs retrieved from bonnet monkeys at the periovulatory period and spermatozoa from FSH-immunized monkeys showed that the ability of these spermatozoa to attach to the egg itself is very poor compared with that noted with spermatozoa from control monkeys. These results thus support the contention that it is not just the total number of spermatozoa but the quality of spermatozoa that determines the fertilizing ability (Koukoulis *et al.*, 1989).

The immunogenicity of the oFSH vaccine used in the current study and the crossreactivity of the antisera generated with hFSH have been verified independently in an additional group of ten bonnet monkeys at National Institute of Health and Family Welfare, New Delhi (R. P. Das, pers. commun.) and nine cynomolgus macaques at the LSU Medical centre, New Orleans (H. G. M. Raj, pers. commun.). These investigators also observed induction of acute oligospermia and infertility in their monkeys after immunization with oFSH.

The current study has indicated that oFSH can be developed as a viable male contraceptive vaccine for use by man. An advantage of this procedure over other hormonally based male contraceptives is that it leaves normal libido and accessory gland function unimpaired. Successful application of this vaccine as a human male contraceptive, however, may depend on several additional factors. Refinement of methods to obtain sustained antibody response requiring boosting perhaps only once in 12 months, and use of an FSH derivative that retains its immunogenicity but has lost its bioactivity would help to achieve this goal.

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