# Immunization of Male Bonnet Monkeys (M. radiata) with a Recombinant FSH Receptor Preparation Affects Testicular Function and Fertility

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# IMMUNIZATION OF MALE BONNET MONKEYS (*M.radiata*) WITH A RECOMBINANT FSH RECEPTOR PREPARATION AFFECTS TESTICULAR FUNCTION AND FERTILITY

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## ABSTRACT

Immunization of proven fertile adult male monkeys (n=3) with a recombinant FSH receptor protein preparation (oFSHR-P) (representing amino acids 1-134 of the extracellular domain of the receptor Mr~15KDa) resulted in production of receptor blocking antibodies. The ability of the antibody to bind a particulate FSH receptor preparation and receptors in intact granulosa cells was markedly (by 30-80%) inhibited by FSH. Serum T levels and LH receptor function following immunization remained unchanged. The immunized monkeys showed a 50% reduction ($p<0.001$) in transformation of spermatogonia(2C) to primary spermatocytes (4C) as determined by flow cytometry and the 4C:2C ratio showed a correlative change (R 0.81, $p<0.0007$) with reduction in fertility index (sperm counts X motility score). Breeding studies indicated that monkeys became infertile between 242-368 days of immunization when the fertility index was in the range of 123±76 to 354±42 (compared to a value of 1602±384 on day 0). As the effects observed are near identical to that seen following immunization with FSH it is suggestive that oFSHR-P can substitute for FSH in the development of a contraceptive vaccine.

## INTRODUCTION

Neutralization of endogenous FSH with a specific antibody (ab) in the adult male bonnet monkey results in disruption of spermatogenesis leading to production of poor quality of sperms, oligospermia and infertility (1). In this method it is essential to maintain at all times high levels of FSH ab to ensure total neutralization of continuous outpouring of pituitary FSH. Following complete sequencing of FSH receptor and synthesis of portions of the extracellular domain by the recombinant route (2,3), use of receptor ab to obtain an FSH deprived effect appeared more attractive. As the Sertoli cells are limited in number and are not replenished in the adult it appears a low level of ab should be sufficient to block Sertoli cell function for long durations. The effect of actively immunizing adult male bonnet monkeys with a FSH-receptor protein fragment 1-134 (oFSHR-P) synthesized by recombinant route (3) is described here. In particular the bioefficacy of the ab to block FSH action, the effect of immunization on testicular germ cell transformation, sperm quality and counts and fertility status are described.

## MATERIALS AND METHODS

***The antigen:*** The oFSHR-P was a recombinant product engineered for production in *E.coli* based on the cDNA sequence of a naturally occurring alternately spliced FSH receptor variant in sheep testis (4). The cDNA clone called 151A1 was engineered for insertion into the Qiagen expression vector pQE31 with a His6 tag to enable purification. Construction and purification was done according to procedure described elsewhere (3). N-terminal sequence was verified by a automated sequencer. The final product of 134 amino acids (Mr~15kDa) following RP-HPLC purification was stored lyophilized at 4 C until use. This was free of host proteins as verified by electrophoresis and western blotting.

***Animals and Animal Experimentation:*** Proven fertile male bonnet monkeys (*M.radiata*) 9-10 yrs of age weighing 7-8 kg each were used for immunization. Procedures used for animal husbandry, housing, feeding etc. have been described earlier (1). Standards/guidelines set out for primate experimentation by the Ethics Committee of this Institute were followed.

***General Methodology:*** Procedures employed for obtaining periodic blood sampling, testicular volume, testicular biopsies, electroejaculation and determining sperm counts and motility score have been described earlier (1,5). Fertility testing of the immunized males was done by caging them with proven fertile cycling female monkeys between days 9-13 of cycle. Procedures employed for determining ovulatory nature of the cycle, establishment of pregnancy, analysis of serum for testosterone (T) and progesterone (in the females) levels by appropriate RIA have been described earlier (1,6).

***Immunization schedule and antibody titration:*** The injection schedule was as follows: on day 1, 200µg/monkey in Freund's complete adjuvant; on days 25 & 50, 150 and 100µg/monkey respectively in incomplete adjuvant and on days 150 & 300, 100 µg/monkey in saline. All injections were given s.c. Antibody titration was made by using an ELISA procedure (7). Microtiter plates coated with 100 ng oFSHR-P/ well in 0.1M $NaHCO_3$ pH 9.6 overnight at 4 C were washed thrice with phosphate buffered saline containing Tween-20 (PBST) excess sites being blocked with 250 µl of 5% skim milk powder. After washing thrice more with PBST the wells with different dilutions of antiserum (a/s) were incubated for 2 h at 25 C. Following copious washing with PBST, 200 µl of antibody (1:50 diluted) to monkey IgG conjugated to horse radish peroxidase was added to each of the wells and incubated for 1 h at 25 C. The colour developed following addition of orthophenyldiamine (40 µg) +$H_2O_2$ (0.03%) in citric acid buffer (pH 5.5) and incubation for 10 min was

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quantitated by measuring A at 490 nm in an ELISA reader.

***Biological Characterization of the oFSHR-P antibody:*** Ability of the a/s (100µl/tube) to inhibit binding of $^{125}$I hFSH to a particulate sheep testicular FSH receptor was assessed by preincubating a known quantity of the receptor preparation with IgG isolated from 100µl of normal monkey serum (NMS) or a/s for 120 min adding thereafter 1.5-2.0x$10^5$ cpm of $^{125}$I hFSH and continuing incubation for additional 60 min at 25 C. Following centrifugation and washing radioactivity in the pellet was quantitated in a clinigamma counter (LKB). For calculating % inhibition, binding of $^{125}$IhFSH in the absence of serum was considered as 100%.

Ability of the antisera (IgG fraction) to bind FSH receptors of intact granulosa cells (isolated from PMSG primed immature rats) was assessed using flowcytometry. Granulosa cells (GC; 1 mill/ml) were fixed in 3% buffered paraformaldehyde for 30 min at 4 C, washed thrice using 1 ml PBS containing glycine (GB-PBS; 10mM glycine and 1% BSA) and incubated with 50 µg IgG equivalent of respective a/s or NMS (for non-specific) for 60 mins at RT. Another aliquot of the GC was pre-incubated (for 20 mins at 25 C) with 1 µg oFSH to check specificity of the antibody binding to FSH receptors. Cells were washed thrice using 1 ml GB-PBS and incubated with anti-human IgG coupled with phycoerythrin (PE) (Sigma Chemical Co., St. Louis, USA) for 30 min at 25 C. Following washing thrice and resuspending in GB-PBS, the fluorescence of PE stained cells was measured using FACScan flow cytometer (Becton Dickinson, San Jose, USA) equipped with 15 mw argon-ion laser at 488nm. The red signals were collected at 564-606 (BP 585/42).

Monocellular suspensions of testicular germ cells (ethanol fixed) prepared from testicular biopsies taken on day 0, 66 and 141 of immunization were stained with ethidium bromide (EB, Sigma) and fluorescence intensity was analysed in a flow cytometer. Details of the procedures followed for flowcytometry (FCM) have been described elsewhere (5,6,8). Based on DNA content four main populations viz., elongated spermatids (HC), round spermatids (1C), spermatogonia and other non-germ cells (2C) and primary spermatocytes and $G_2$-spermatogonia (4C) could be visualized. As over 70% of the total germ cells analyzed are haploid cells (HC and 1C) (8) the overall changes recorded in the relative percentages of 2C and 4C populations in the flow cytogram could appear marginal. Consequently, to get a better insight into spermatogonial proliferation and transformation to primary spermatocytes the haploids were electronically gated out and 10,000 cells of only 2C and 4C cells were acquired and analyzed using LYSIS II Software (Becton Dickinson, San Jose, USA).

## RESULTS

***Antibody titration:*** The response of all three monkeys to the immunogen was verY similar, the ab titer remaining fairly constant (range between 0.3-0.4 A units) over the entire study period. Boosters did not significantly enhance the antibody titre (Fig. 1).

***Characterization of the antibody:*** While immunization had no effect on body weight, the testicular volume was reduced by 40% over day 0 values. Minor alterations in serum T levels seen in immunized monkeys were statistically (ANOVA) indistinguishable from controls (Fig. 2). The ab neither bound directly $^{125}$IFSH/LH nor inhibit binding of $^{125}$ILH to its receptor (data not shown). The a/s (IgG fraction) of all three monkeys inhibited by 29.2 ± 2.8% (observations n=7) binding of $^{125}$I hFSH to a particulate testicular receptor. An equivalent amount of NMS IgG inhibited $^{125}$IFSH binding to the receptor by < 5%. Using FCM analysis it was observed that the ab of all 3 monkeys bound to granulosa cell receptors and this was inhibited by 37-80% following preincubation of cells with FSH (Fig.3). Non-specific cell types like spleenocytes (rat) and testicular germ cells (both of rat and monkey) did not bind to oFSHR-P antibody (data not shown).

***Effect of immunization on testicular germ cell transformation:*** Scanning of testicular germ cells prepared from biopsies taken on days 0, 66 and 141 of immunization by FCM showed that there was a relative percentage increase (23%) in 2C (primarily spermatogonial cells) and a decrease (38%) in 4C (spermatocytes) population. The overall transformation of 2C to 1C was reduced by 38 % (1C:2C ratio control 4.73±0.5 vs oFSHR-P 2.91±0.18; $p<0.03$). Confirmation of the changes observed in the relative proportionality of 2C and 4C cells was obtained by analyzing 10,000 cells of the above cell types. The 4C population as well as the germ cell transformation (4C:2C) ratio showed a significant ($p<0.0001$) reduction over the untreated control (Table 1). Essentially a similar effect was observed when animals were deprived of FSH support by actively immunizing with oFSH (Table.1).

***Effect of immunization on Fertility index and Fertility status:*** An idea of fertility status of immunized monkeys was obtained by monitoring the sperm density, motility score and viability of the ejaculated sperms obtained periodically during the immunization period. The % non-viable cells (stained with propidium iodide) as determined by FCM in the seminal ejaculates of control and immunized monkeys were 10.7±2.1 and 37.98±8% ($p<0.02$) respectively. All three monkeys showed marked reduction in the fertility index (product of sperm counts mill/eja X motility score) following immunization (Fig.4). In actual breeding studies carried out with proven fertile cycling females between 242-415 days of immunization no pregnancy occurred following 11 ovulatory cycle exposures (confirmed by determining serum progesterone levels between days 18-22 of cycle (Table2). However, monkey #3064 was able to impregnate a cycling female during a 4th mating episode. Around the time of mating (day 413 of immunization) this male had a fertility index of 1500 (sperm counts 375 million/ejaculate X motility score of 4).

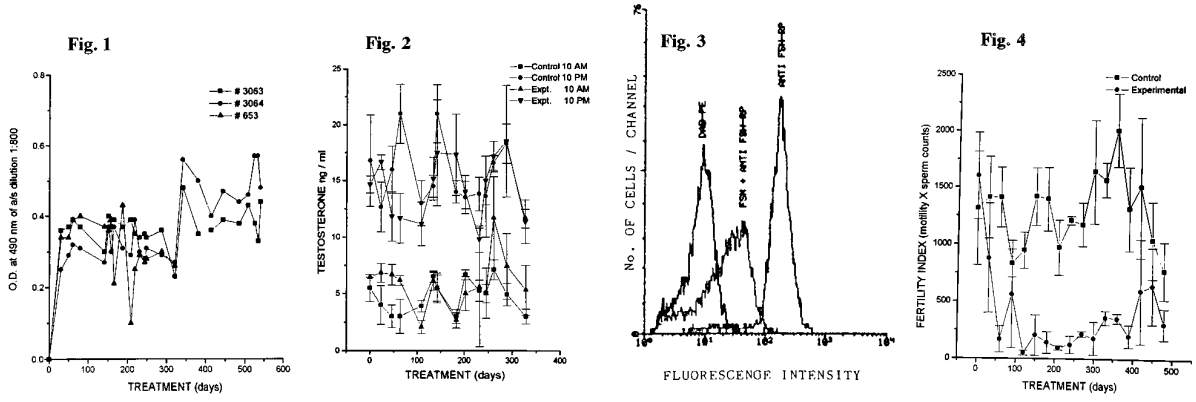


**Fig. 1.**Immune-responsiveness of three adult male bonnet monkeys to oFSHR-P. Antibody titer, expressed as A units, was determined by ELISA.

**Fig. 2.** Serum testosterone level of control and oFSHR-P immunized monkeys (ng/ml, mean ± SEM) n each group = 3.

**Fig. 3.** A representative flow cytogram illustrating the ability of oFSHR-P antibody to bind in vitro rat granulosa cells in the absence and presence of excess oFSH. Human anti IgG tagged to phycoerythrin was used to quantitate the fluorescence in a FACScan flow cytometer. DAb-PE : Nonspecific control.

**Fig. 4.** Analysis of seminal ejaculates of monkeys immunized with oFSHR-P at different times of immunization for Fertility index. Fertility index = (mill/eja) X motility score (5 -> 0).

**TABLE 1 : Effect of immunization with oFSHR-P on transformation of spermatogonia to primary spermatocytes as analyzed by flow cytometry.**

| | | % of germ cells (Mean±SEM) | | |
|---|---|---|---|---|
| Group | Treatment (n) | Spermatogonia (2C) | Primary Spermatocytes (4C) | Ratio (4C:2C) |
| I | Untreated control (6) | 45.93 ± 0.80 | 46.62 ± 1.50 | 1.01 ± 0.04 |
| II | oFSH immunized + (5) | 58.52 ± 4.40[a] | 24.20 ± 2.60[c] | 0.44 ± 0.06[c] |
| III | oFSHR-P immunized(3) | 59.02 ± 2.80[b] | 29.00 ± 1.90[c] | 0.50 ± 0.04[c] |

In the case of gr III data from samples of two biopsies taken at 2 different times were analyzed to ensure reproducibility of results. A total of 10,000 germ cells of non haploid category were analyzed to obtain% distribution values.

+ Results of analysis of testicular biopsy samples taken from long-term oFSH immunized monkeys as per protocol described earlier (1) is included as positive control. P Value a<0.01; b<0.0008 and c<0.0001

**TABLE 2: Results of a breeding study* carried out with oFSHR-P immunized monkeys**

| Monkey # | Period of immunization days | Fertility index during breeding period | No. pregnant /No. ovulating females exposed |
|---|---|---|---|
| 653 | 242-298 | 47-465 | 0/4 |
| 3063 | 290-368 | 12-325 | 0/4 |
| 3064 | 342-415 | 300-1500 | 1+/4 |

* Mating with proven fertile cycling females was confined to days 9-13 of the cycle. Ovulatory cycle was confirmed by monitoring serum progesterone levels between days 18-22 of cycle. Pregnancy establishment was determined by measuring serum progesterone/CG beyond day 24 of the cycle.

+Pregnancy occurred during 4th ovulatory cycle exposure at which point the sperm counts were 375 mill/eja and motility score of 4 (Fertility index 1500).

## DISCUSSION

The primary objective of the present study was two fold. Firstly to determine if the recombinant oFSHR-P preparation of 134 residues as coded by a natural cDNA elicits a satisfactory immune response in the male bonnet monkey and secondly to see if the antibody so produced is able to block FSH action *in vivo* and *in vitro* by virtue of its ability to bind FSH receptors. It is evident from the results presented that all the immunized monkeys produced a near equivalent antibody response and the ab titers could be sustained for over 300 days. It appears that a single booster per year instead of multiple as given in the current study would be adequate to maintain high antibody titers. The prolonged immune response seen with oFSHR-P in monkeys is quite unlike the short lived immune response to oFSH in monkeys requiring booster once in 100 days (1). Antibodies to a rat FSH-receptor fragment 5-125 (RF1) has been observed to inhibit binding of both $^{125}$I labelled FSH and LH to rat testis receptor (2). The current study, however, has not provided any evidence for monkey ab to oFSHR-P to block LH function. Antibodies to

oFSHR-P unlike those of LH receptor have not been observed to exhibit upon binding to receptor, hormone mimicking activity (9).

In the immature rat, spermatogenesis, particularly production of primary spermatocytes, is inhibited following injecton of a/s to either oFSH (10) or a rat FSH receptor (11). Two types of evidences have been presented here to prove that the monkey ab to oFSHR-P binds to FSH receptor and block FSH action. Besides showing that the oFSHR-P ab markedly inhibits binding of FSH to particulate receptor preparation and intact granulosa cells *in vitro*, we have demonstrated that active immunization with oFSHR-P leads to significant ($p<0.0001$) reduction in transformation of spermatogonia to primary spermatocytes. It is essential to note here that the effects on germ cells distribution pattern seen here cannot be ascribed to either the use of non-specific agents like Freund's complete adjuvant (12) or blockade of LH function (6). Since the overall effects observed with oFSHR-P immunization are mostly identical to that obtained with oFSH immunization (5,6 and the current study), it can be concluded that a specific "FSH deprivation effect" has been obtained by immunizing with oFSHR-P.

Analysis of seminal ejaculate showed the sperm count and quality (motility as well as viability) to be markedly affected following oFSHR-P immunization, an effect very similar to that obtained following immunization of monkeys with oFSH (1). A good correlation exists between the decrease in germ cell transformation (4C:2C ratio) and reduction in fertility index ($R = 0.81$, $p < 0.0007$) observed following immunization with either oFSHR-P or FSH indicating that the former event is a cause of the observed diminution in sperm production.

The fertility index provides a reasonable idea about the actual fertility status of the monkey. Monkeys became infertile (checked by breeding studies) when the fertility index was well below 500. At the time a single pregnancy was recorded (by monkey #3064 at 4th mating) the fertility index of this monkey was 1500 similar to that of fertile monkeys. Even in this issue the results of the oFSHR-P immunization are comparable to that noted earlier by us with oFSH immunization (1). In conclusion, the foregoing clearly shows that it is possible to obtain an "FSH deficient" status in adult male monkeys by immunizing with a recombinant oFSHR-P. Like in the case of FSH immunization, this does not lead to reduction in serum T but to blockade in fertility due to disruption in spermatogenic process leading to production of low number of poor quality sperms. Considering that the oFSHR-P antibody titers compared to that of FSH are relatively long lasting due perhaps to limited number of FSH receptors available in target cells it could become feasible to use oFSHR-P instead of FSH for development of a viable contraceptive vaccine.

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**REFERENCES**

1. **Moudgal NR, Ravindranath N, Murthy GS, Dighe RR, Aravindan GR, Martin F** 1992 Long term contraceptive efficacy of vaccine of ovine follicle stimulating hormone in male bonnet monkeys (*Macaca radiata*). J. Reprod. Fertil. 96: 91-102.

2. **Sharma SC, Catterall JF** 1995 Expression of extracellular domain peptides of the FSH receptors and their effect on receptor-ligand interactions *in vitro*. Mol. Cell. Endo. 115:87-93.

3. **Khan H, Jiang LG, Jayashree GN, Yarney TA, Sairam MR** 1997 Ligand recognition and immunological characteristics of recombinant ovine testicular follitropin receptor protein variant expressed in *E. coli*. (under preparation).

4. **Khan H, Yarney TA, Sairam MR** 1993 Cloning of alternately spliced mRNA transcripts coding for variants of ovine testicular follitropin receptor lacking the G-protein coupling domains. Biochem. Biophys. Res. Comm. 190: 888-894.

5. **Aravindan GR, Gopalakrishnan K, Ravindranath N, Moudgal NR** 1993 Effect of altering endogenous gonadotropin concentrations on the kinetics of testicular germ cell turnover in the bonnet monkey (*Macaca radiata*). J. Endocrinol. 137:485- 495.

6. **Suresh R, Medhamurthy R, Moudgal NR** 1996 Comparative studies on the effect of specific immunoneutralization of endogenous FSH or LH on testicular germ cell transformation in the adult bonnet monkey (*Macaca radiata*) Am. J. Reprod. Imm. 34: 35-43.

7. **Suter M** 1982 A modified ELISA technique for anti-hapten antibodies. J. Imm. Methods. 53:103-108.

8.**Suresh R, Aravindan GR, Moudgal NR** 1992 Quantitation of spermatogenesis by DNA flowcytometry: Comparative study among different species of mammals. J. Biosci., 7(4): 413-419.

9. **Jeyakumar M, Moudgal NR** 1996 Immunization of male rabbits with sheep luteal receptor to luteinizing hormone results in production of antibodies exhibiting hormone agonistic and antagonistic activities. J. Endocrinol. 150, 431-443.

10. **Vaishnav MY, Moudgal NR** 1994 Role of FSH in regulating testicular germ cell transformations in the rat: a study using DNA flow cytometry. Andrologia, 26, 111-117.

11. **Catterall JF, Sharma SC, Shetty J, Samaddar M, Gandre SS, Dighe RR** 1996 Characterization of the extracellular domain of the FSH receptor using anti-peptide and monoclonal antibodies. 10th International congress of Endocrinology, San Francisco, USA, June 12-15th, (Abstract No.OR8-7).

12. **Jeyakumar M, Suresh R, Krishnamurthy HN and Moudgal NR** 1995 Changes in testicular function following specific deprivation of LH in adult male rabbit. J. Endocrinol. 147, 111-120.