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Development of male contraceptive vaccine—a perspective

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This paper reviews the recent advances that have occurred in the area of development of a male contraceptive vaccine. The vaccine candidates considered for review are hormone/hormone receptor-based proteins including luteinizing hormone-releasing hormone (LHRH)/LH, follicle stimulating hormone (FSH), as well as LH and FSH receptor proteins. The review also highlights the advances in our basic understanding of gonadotrophin action which have led to development of these vaccines. Focus is mainly on studies in the non-human primate which may be directly relevant to projected studies in the human. The data indicate that the vaccines are well tolerated by the primate (including the human based on limited data) and do not give rise to any known toxic symptoms or immediate health hazards. The response to the immunogen has been uniform and it may be possible to increase antibody titres as well as prolong the immune response by adding acceptable immune stimulators to the adjuvant cocktail and developing better immunization schedules or immunogen delivery systems. Contraceptive vaccines for the male are a feasible proposition and attention should now be focussed on evaluating carefully the bioefficacy of antibodies raised to recombinant ovine FSHβ or FSH receptor protein fragments in both human and non-human primates. The advantage of the FSH/FSH receptor over the LHRH/LH-based vaccine lies in the fact that the former does not require an exogenous testosterone supplement to maintain accessory gland function, libido etc. The LHRH/LH-based vaccine results in azoospermia, while the FSH vaccine causes the production of low numbers of poor quality spermatozoa which are incapable of impregnating cycling females.

Key words: azoospermia/contraception/gonadotrophins/oligozoospermia/testicular dysfunction

Introduction

Development of a viable male contraceptive agent of universal appeal has been a difficult task. For a method to succeed, it must be safe and must ensure that: (i) production of good quality spermatozoa is totally blocked (azoospermia) or affected to a highly significant extent (oligozoospermia accompanied by impairment in quality, in particular fertilizing ability) hence causing infertility in >90% of the volunteers tested; (ii) androgen-dependent accessory sex gland function and libido should not be impaired; and finally (iii) the process should be reversible after cessation of drug treatment.

Testicular testosterone has been shown to regulate spermatogenesis in primates, primarily by controlling meiosis, the step responsible for the production of spermatids (Aravindan *et al.*, 1993; Suresh *et al.*, 1995; Suresh and Moudgal, 1995). In the light of this, it is not surprising that azoospermia is invariably achieved by arresting the synthesis of testicular testosterone. Testosterone synthesis can be blocked by inhibiting pituitary luteinizing hormone (LH) secretion using either

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steroid-based feedback regulators (Cummings and Bremner, 1994) or gonadotrophin-releasing hormone (GnRH) agonists (Ravindranath *et al.*, 1992) and antagonists (Bagatell *et al.*, 1993) or an appropriate luteinizing hormone-releasing hormone (LHRH) vaccine (Ladd *et al.*, 1988). Alternately peripheral LH action can be blocked using antibodies to either LH (Jeyakumar *et al.*, 1995; Suresh *et al.*, 1995) or to LH receptors (Jeyakumar and Moudgal, 1996). In contrast to this, our studies in monkeys have shown that a blockade of follicle stimulating hormone (FSH) action by using an antibody to FSH or to FSH receptor leads to marked inhibition in the transformation of spermatogonia to primary spermatocytes as well as impairment in the spermiogenic process (Aravindan *et al.*, 1993; Suresh *et al.*, 1995; Moudgal *et al.*, 1997a). Though such a blockade resulted only in oligozoospermia, the quality of the spermatozoa produced was affected to a significant extent, leading to the establishment of infertility. Immunization with antibodies to either FSH or FSH receptor protein did not lead to a change in endogenous testosterone production.

In this review we aim to discuss the relative merits/demerits of using either LH or FSH-based vaccines for male contraception, with particular emphasis on the future prospects of developing such a product for use in man. Discussion on male gamete-based antigens, such as lactic dehydrogenase-X (LDH-X), sperm specific protein-10 (SP-10) and riboflavin carrier protein (RCP), are not included in the present review as they are contemplated mainly for use in women.

LHRH-based vaccines

Several attempts have been made in the past to use as a vaccine LHRH conjugated to carrier proteins, including bovine serum albumin (BSA), tetanus toxoid (TT), diphtheria toxoid (DT) and Keyhole limpet haemocyanin (kLH) (Fraser and Gunn, 1973; Shastri *et al.*, 1981; Silversides *et al.*, 1988). These studies highlighted primarily two variables. As well as the use of different carrier proteins, varying the amino acid residue of the LHRH to which the carrier protein is attached has been found to be important in determining the bioefficacy of the antibody produced. Secondly, replacement of Freund's complete adjuvant by permissible adjuvants such as TPS (a mixture of Tween 80, squalene Pluronic L121 in saline; Ladd *et al.*, 1988) and alum (Giri *et al.*, 1991) to produce bioeffective antibodies has been explored. The carrier proteins of choice are TT and DT. Ladd *et al.* (1990) observed that LHRH conjugated through its *N*-terminal (Glnl–LHRH) to TT provided a better antibody titre and greater bioefficacy compared with the use of GnRH conjugated to TT through its 10th amino acid residue (Ladd *et al.*, 1988). The Indian group developed a LHRH vaccine by substituting Gly at position 6 with D-Lys and using a spacer (6 amino hexanoic acid) to link it to DT (Gupta *et al.*, 1993). Although the LHRH vaccine has been tested for its efficacy in blocking LH and FSH secretion in both the female and male of several species of animals, its ability to block testicular as well as prostatic function has attracted particular attention. Both vaccines have been highly effective in bringing about a drastic reduction in testosterone production leading to a significant diminution in testicular and prostate weight (Giri *et al.*, 1991; Ladd *et al.*, 1994). Although the efficacy of such a vaccine in the male dog has been reported (Ladd *et al.*, 1994), no structured study has been carefully carried out in the male non-human primate to evaluate the contraceptive efficacy of such a vaccine. Using standard toxicological protocols, no acute or chronic side-effects resulting from the use of these two vaccines have been demonstrated (Pal and Talwar, 1995). Although the use of these LHRH conjugates as contraceptive vaccines for the human male (based on animal studies) has been mooted, they are currently being tested only for control of prostatic cancer (Pal and Talwar, 1995). In patients with prostate cancer and benign prostatic hypertrophy, three injections of the modified LHRH vaccine conjugated to DT (developed by the Indian group) adsorbed on alum followed by two further doses at 6 weekly intervals apparently led to production of anti-LHRH antibodies. This resulted in a reduction of FSH, LH and testosterone concentrations as well as, in some cases, to shrinkage of prostatic size (Pal and Talwar, 1995). From the available data with the current vaccine formulation it appears that reasonably frequent boosting with the LHRH-conjugate is required to maintain high antibody titres. Since the carrier protein(s) used for conjugation are potent immunogens, their antibody would remain in circulation in sufficient concentrations (compared with the rapidly used-up LHRH antibody) for a relatively long time. Hence, repeat immunization over a protracted period with the LHRH conjugate may not elicit the required response of maintaining high titres of LHRH antibody for prolonged periods. To overcome this problem of hyper-immunization to the chosen carrier, Talwar's group preferred to immunize women initially with a combination of human chorionic gonadotrophin (HCG)β TT + HCGβ DT followed by a booster at any one time with either of the conjugates alone (Gaur *et al.*, 1990). It is unclear whether this strategy would succeed in the human male.

LH and LH receptor-based vaccines

The use of heterologous gonadotrophin [(e.g. ovine (o) LH or FSH instead of human (h) LH, human HCG or human

FSH] as a candidate vaccine in the human is desirable as no carrier will be necessary in such cases to elicit an immune response. Because of the significant homology that exists between gonadotrophins of ovine and human origin (Ryan *et al.*, 1987), the cross-reactive antibody generated has been shown to be capable of neutralizing the bioactivity of circulating endogenous hormone (Moudgal and Sheela Rani, 1983; Moudgal *et al.*, 1988).

Attempts were therefore made to determine the efficacy of vaccines based on oLH and LH receptor protein (isolated from sheep testis) to block testicular function in the monkey (Suresh *et al.*, 1995) and rabbit (Jeyakumar *et al.*, 1995; Jeyakumar and Moudgal, 1996). The studies with oLH vaccine essentially showed that the antibodies generated were capable of binding both primate (monkey and human) and rabbit LH, resulting in a marked reduction (~90%) in serum testosterone concentrations after 8–16 weeks of immunization. Flow cytometric quantification of the germ cell population showed that, by 15–18 weeks of immunization, the spermatid population (round and elongating/elongated) had been reduced by >90%. The primary spermatocyte population was also reduced by >50% after 15 weeks of immunization. The resultant azoospermia is, we believe, primarily due to 'arrest' in meiosis which has been shown using different model systems to be under the control of testicular testosterone (Aravindan *et al.*, 1993; Suresh *et al.*, 1995; Suresh and Moudgal, 1995).

Ovine LH relative to oFSH appears to be a poor immunogen in both the monkey and rabbit and even with the use of Freund's complete adjuvant, the cross-reactive antibody (for both human and rabbit LH) titre obtained is generally low. The antibody, however, is bioeffective as indicated by a significant reduction in serum testosterone concentrations. Immunized animals exhibited a marked reduction in testicular weight. Particularly in the monkey, LH immunization resulted in a significant reduction in body weight, accompanied by noticeable muscle wastage and alopecia. This did not seem to be related to testosterone concentrations, as the drastic reduction in serum testosterone brought about by steroidal feed-back regulators (Suresh and Moudgal, 1995) or GnRH agonist (Ravindranath *et al.*, 1992) did not cause similar body weight loss or alopecia. These detrimental effects appeared only subsequent to oLH immunization and led us to abandon further studies on the evaluation of oLH as a potential contraceptive vaccine for the male. Immunization with oFSH did not cause similar changes.

Attempts to use the LH receptor, both the holo receptor protein and a synthetic nested peptide covering amino acid residues 21–41 of the extracellular domain (kindly donated by Dr R.Nagaraj, Center for Cellular and Molecular Biology, Hyderabad, India), instead of LH to obtain bioeffective antibodies in the rabbit have hitherto not met with complete success. While we were able to generate antibodies to both types of receptor preparation in the male rabbit, this was accompanied by a marked increase (3–6-fold) in serum testosterone concentrations instead of the expected reduction. This was particularly evident during the first 80–100 days of immunization. Detailed characterization of the antisera generated in rabbits to the holo LH receptor indicated that two types of receptor antibodies were produced, an initial response leading to production of antibodies able to bind the receptor and evoke a positive hormonal response and a secondary response leading to production of antibodies which were antagonistic. The presence of receptor antibodies with antagonistic activity became apparent when the immunized rabbits failed to respond to an exogenous bolus LH injection. The occurrence of two types of antibodies was also demonstrated using an in-vitro mouse Leydig cell system. Because of the apparent hormone-mimicking activity of the first type of receptor antibody, serum testosterone concentrations in the immunized rabbits were never reduced compared with normal values and, as a consequence, no change in testicular germ cell pattern (used as an index of spermatogenesis) was observed (Jeyakumar and Moudgal, 1996). The end effect observed following immunization with LH receptor was therefore different from that obtained with LH immunization.

Using the nested peptide of the LH receptor (21–41 amino acids of the receptor protein) as the immunogen also failed to abolish in the male rabbit the initial hormone agonistic (LH-like) activity. However, after further immunization, the serum testosterone concentrations tended to fall significantly below normal and examination of testicular biopsies showed a partial to total blockade in testicular germ cell transformation (H.N.Krishnamurthy, S.Surekha, H.Krishnamurthy, M.Jeyakumar, R.Nagaraj and N.R.Moudgal, unpublished data). This is encouraging and suggests that it may still be possible to obtain antibodies which are purely antagonistic using either other nested peptides of the LH receptor or by chemically modifying the 21–41 nested peptide already being used as the immunogen.

FSH and FSH receptor-based vaccines

The impetus for research in this area was primarily provided by the classic observation made in 1978–79 that monkeys immunized with oFSH showed testicular dysfunction, despite not showing any change in serum

testosterone concentrations (Sheela Rani *et al.*, 1978) and consequently were rendered infertile (Murty *et al.*, 1979). That testicular function/spermatogenesis of the monkey was arrested following FSH deprivation was latter confirmed by several groups of workers (Wickings *et al.*, 1980; Raj *et al.*, 1980; Moudgal, 1981; Srinath *et al.*, 1983; Srivatsave and Das, 1992; Aravindan *et al.*, 1993; Suresh *et al.*, 1995). Even in the human male, Matsumoto *et al.* (1986) have been able to show that selective deprivation of FSH leads to significant reduction in sperm output and that quantitative restoration of this to a normal value is achieved only after exogenous FSH administration.

The observations of Gromoll *et al.* (1996) concerning the sustained spermatogenesis in a hypophysectomized man with an activating mutation of the FSH receptor are interesting. As well as concluding that the activating mutation of the FSH receptor could autonomously sustain spermatogenesis, they suggested that FSH could maintain sperm production in man permanently, even in the absence of adequate concentrations of intratesticular testosterone. They concluded that the crucial role of testosterone in spermatogenesis is to prepare the ground for FSH, facilitating its action through a mechanism involving the FSH receptors.

Studies in the monkey, however, have provided positive evidence for the role of testosterone in the specific regulation of meiosis and spermatid production (Suresh *et al.*, 1995; Suresh and Moudgal, 1995). FSH appears to have no role in this step. In contrast to the study referred to above concerning the hypophysectomized man with activating mutation of FSH receptor, Tapanainen *et al.* (1997) analysed a group of five homozygous males with an apparently inactivating mutation of the FSH receptor gene (566c ---T). They concluded that FSH may not play an essential role in spermatogenesis of normal hurnan males. However, careful analysis of their data indicates that two out of the five were apparently fertile and exhibited normal testicular volume and normal FSH and LH values. The other three had a 51–73% reduction in testicular volume and a two-fold increase in FSH and LH concentrations. None of the group exhibited normal sperm parameters. Most were acutely oligozoospermic and any spermatozoa present were not norrnal, a situation very similar to that occurring when monkeys are deprived of FSH by immunoneutralization. A recent clinical report on the occurrence of ovarian dysgenesis in a group of women exhibiting receptor mutation 566 --- T concluded that residual receptor activity was present since the women were shown to have follicles (Aittomaki *et al.*, 1996). In the light of this, the conclusion of Tapanainen *et al.* (1997) that FSH is not needed for maintenance of spermatogenesis and fertility in man is open to question. Supportive evidence for a role for FSH in maintaining spermatogenesis and fertility is provided by results obtained from immunizing male monkeys with a recombinant FSH receptor protein preparation (discussed in detail below). There appears to be species variation with regard to the need for FSH to maintain quantitative spermatogenesis during adulthood. Whereas a clear FSH requirement has been shown in the adult male hamster (Lerchl *et al.*, 1993) and ram (Kilgour *et al.*, 1994), spermatogenesis seems to occur normally in the absence of FSH in the adult but not the immature rat (Dym *et al.*, 1979; Vaishnav and Moudgal, 1994) and adult mice (Singh *et al.*, 1995; Rajendra Kumar *et al.*, 1997).

Most of the work carried out has used ovine instead of human FSH as the immunogen The rationale for this is: (i) at the time these studies were started (20 years ago), a plentiful supply of highly purified ovine instead of human FSH was available; (ii) oFSH, in addition to being a heterologous protein, was a good immunogen and as such a high titre of antibodies could be obtained using only alum, an adjuvant cleared for human use as the adsorbent; (iii) the ability of antibodies raised to oFSH to cross-react and bioneutralize FSH of other species including that of the primate (both monkey and human) was clearly established (Rao and Moudgal, 1970; Moudgal and Sheela Rani, 1983; Moudgal *et al.*, 1988); and (iv) by using ovine instead of human FSH, the chances of producing antibodies capable of cross-reacting with human LH and thyroid stimulating hormone (TSH) (due to similarities in their α-subunits) was avoided.

Although the ability of antibodies raised to both oFSH (Moudgal, 1981; Moudgal *et al.*, 1992) and to its β subunit (Raj *et al.*, 1980) to bind and bioneutralize primate FSH has been clearly shown, hitherto intact oFSH continues to be used as the immunogen, since the affinity of binding of its antibody to FSH is higher by one order of magnitude than that generated to the FSHβ subunit (Moudgal *et al.*, 1989). However, we currently recommend use of oFSHβ as the immunogen for the following reasons: (i) it is easier to produce oFSHβ instead of the annealed holo FSH molecule by the recombinant route, which is the preferred method of producing the immunogen in large amounts; (ii) use of the sodium pthalate derivative of lipopolysaccharide (SPLPS) as an adjuvant extender for oFSHβ vaccine significantly facilitated an increase in the titre of cross-reactive/bioneutralizing antibodies; (iii) chances of reinitiating testicular spermatogenesis following administration of extra physiological amounts of the hormone as a booster (particularly when the animal is deprived of FSH support for a long time) does not arise, as FSHβ (unlike intact FSH) is not biologically active; and (iv) by using oFSHβ instead of intact oFSH, the immunogen load is reduced and the production of non-specific antibodies to epitopes in the α-subunit of the hormone is minimized.

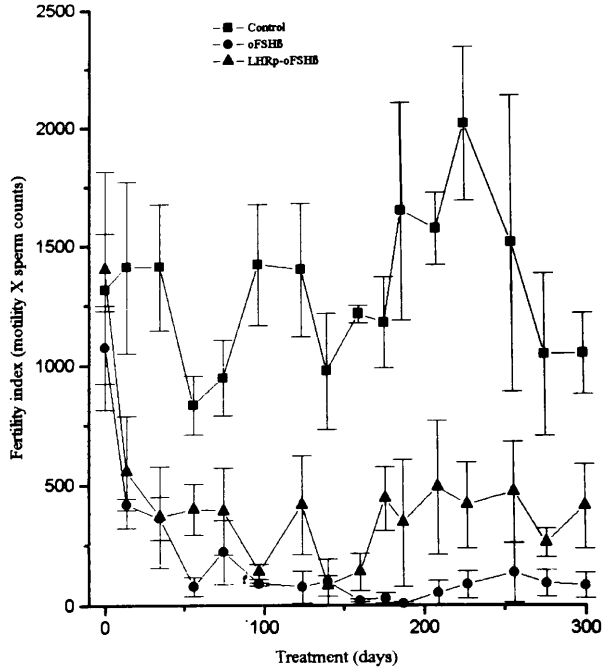

Figure 1. Effect of immunization with ovine follicle stimulating hormone (FSH)β on fertility index. Fertility index = sperm count ($\times 10^6$/ejaculation) × motility score. Control and oFSHβ groups, n =3 each; n = 5for LHRp–oFSHβ. For other details see text.

Table I. Immunogenicity of ovine follicle stimulating hormone (oFSH) and its derivatives determined in rats

Group	Immunogen[a]	human FSH binding ED_{50} values[b] (mean ± SEM)
1	oFSH	720 ± 50
2	oFSH-DT	836 ± 239
3	oFSH + SPLPS	850 ± 165
4	oFSHβ	770 ± 118
5	oFSHβ-DT	1760 ± 571
6	oFSHβ + SPLPS	1912 ± 449

[a]Normally immunogen is given adsorbed on alum, diphtheria toxoid (DT), or sodium pthalyl derivative of lipo polysaccharide (SPLPS). Injections were given on day 1 (50 μg), day 20 (25 μg), day 40 (15 μg in saline). Autopsy on day 100. In each group, n = 5. SPLPS (50 μg/rat) was administered on day 1 only.
[b]Dilution of antiserum at which 50% of maximal binding was observed. Binding determined using enzyme-linked immunosorbent assay on sera collected at autopsy.

oFSH vaccine, as purified and formulated in the authors' laboratory, has been tested for immunogenecity in over 50 monkeys in three independent research centres. The proportion of non-responders was <3%. Although oFSH is a good immunogen, the increase in cross-reactive antibody titre following a booster injection does not last more than 90–100 days (Moudgal *et al.*, 1992; Aravindan *et al.*, 1993). This is primarily because the antibody is rapidly used up to bind and bioneutralize the continuous production of FSH from the pituitary of the immunized animal. Both in the monkey and the human, as long as substantial amounts of 'free' cross-reactive antibody titre are measurable, circulating concentrations of endogenous FSH cannot be detected using a two-step radioreceptor assay (Moudgal and Aravindan, 1993; Moudgal *et al.*, 1997b).

Studies conducted hitherto both in monkey and man have mainly used aluminium hydroxide gel (Superfos, Denmark) as the adjuvant. An attempt was made to increase the immunogenicity of oFSH by administering it either as a conjugate of DT or with SPLPS as a generalized adjuvant extender. The pilot study conducted in adult rats essentially showed that SPLPS was able to boost cross-reactive antibody titres, particularly when oFSHβ was used as the immunogen (Table I). Interestingly, the use of SPLPS along with the first injection of the immunogen, even in monkeys, greatly facilitated an increase in the relevant antibody titres (Table II). Although conjugation of FSHβ to a carrier protein or hapten peptide using carbodiamide activation also increased antibody titres, as assessed by direct ligand binding studies, it had a detrimental effect upon the bioefficacy of the antibody generated. Monkeys immunized with oFSHβ alone showed a consistent and marked reduction in sperm quality and concentration compared with those immunized with a conjugate of oFSHβ and a synthetic LHRP nested peptide (21–41) (Figure 1). These results correlate well with the effect on spermatogonial transformation (2C) to elongated spermatids (HC) shown using DNA flow cytometry (HC:2C ratio; control 2.63 ± 0.27 versus FSHβ alone 0.59 ± 0.23 versus LHRP–FSHβ 1.13 ± 0.24, P <0.01).

Although Nieschlag (1985) found that immunization of rhesus monkeys with oFSH led to severe impairment in spermatogenesis over a 1–2 year period, he did not recommend this approach for male contraception. The reasons for this were two-fold: firstly, consistent azoospermia could not be achieved following FSH immunization, and secondly, repeat boosting at short time intervals over a 4.5 year period led to a qualitative, but not quantitative, resurgence in spermatogenic activity (Srinath *et al.*, 1983). No fertility testing was undertaken by this group. Two other groups independently tested the efficacy of the vaccine and observed that immunized cyanomologous (Raj *et al.*, 1991) and Bonnet (Srivastav and Das, 1992) monkeys became infertile despite the presence of oligozoospermia. The reason why repeat immunizations with oFSH over several years lead to a degree of recovery in spermatogenic activity is not clear. It may be immunological in nature and related to changes in the type of antibodies (non-bioneutralizing) produced with the

passage of time, or it is possible that the biologically active FSH given as a booster (in 50–200 μg) at times when the antibody titre is low could, in conjuction with the reappearance of free endogenous FSH, reinitiate the spermatogenic process. Our experience over the years has shown that this problem can largely be overcome by administering (biologically inactive) oFSHβ instead of oFSH as a booster.

Table II. Immunological efficacy of ovine follicle stimulating hormone (FSH)β administered in different modes as tested in adult male bonnet monkeys

Immunogen	Day of immunization	Antibody titre[a] oFSHβ binding ng/ml	Percentage reduction in sperm counts occuring between days 150–296 of immunization
oFSHβ	91	<10	70–85
	169	283	
	190[a]	1289	
	355[a]	1256	
oFSHβ–$LHRP_{21–41}$	91	1013	33–42
	143	3061	
	169	652	
	190[a]	2174	
	355[a]	4275	
oFSHβ + SPLPS	40	4033	73–92**
	61	1967	
	277*	2684	
	297	1351	
oFSHβ–$LHRP_{21–41}$ + SPLPS	40	1959	48–90[b]
	61	1372	
	277[a]	4771	
	297	1062	

SPLPS = sodium pthalyl derivative of lipo polysaccharide; $LHRP_{21–41}$ = a synthetic polypeptide representing amino acids 21–41 of luteinizing hormone receptor protein.
Adult male bonnet monkeys were immunized on days 1, 30, 60 and 170 when SPLPS was not included and on days 1, 30 and 170 when SPLPS was included. SPLPS (1 mg/monkey) was given only during first immunization.
[a]Antibody titre was generally determined using sera sample collected 10–20 days post-booster.
[b]Significant reduction in sperm counts observed from day 100.

Table III. Assessment of quality of spermatozoa produced by monkeys immunized with follicle stimulating hormone (FSH)

Parameter	Effect observed	Reference
Counts/ejaculate	Reduced by 75% in 80% of ejaculates	Moudgal *et al.*, 1992
Viability	Reduced by 50%	Moudgal *et al.*, 1992
Gross motility	Reduced by 40–50%	Moudgal *et al.*, 1992
Gel penetrability	Reduced by 90% at 15mm	Moudgal *et al.*, 1992
Acrosin activity of washed spermatozoa	Reduced by 74%	Moudgal *et al.*, 1992
Hyaluronidase activity of washed spermatozoa	Reduced by 34%	Moudgal *et al.*, 1992
cAMP level of washed spermatozoa	Reduced by 42%	Raj *et al.*, 1991
ATPase activity of washed spermatozoa	Increased by 500%	Srivatsav and Das, 1992
Binding to homologous monkey oocytes	Markedly inhibited	Raj *et al.*, 1991
Penetration of zona-denuded hamster eggs	Blocked	Sharma and Das, 1992
Sperm chromatin structure assay[a]	Acridine Orange binding following acid denaturation: α testosterone values significantly high	Krishnamurthy *et al.*, 1997
Decondensation of chromatin*	Highly susceptible to DTT (dose related) induced decondensation	Aravindan *et al.*, 1991 Krishnamurthy *et al.*, 1997
Acrosomal content[a]	PSA–FITC binding significantly reduced	Krishnamurthy *et al.*, 1997

[a]Effect reproduced in spermatozoa from immunized human volunteers.
DTT = dithiothreitol; PSA–FITC = *Pisum sativum* agglutinin–fluorescein isothiocyanate.

Table IV. Effect on fertility status of immunization of male monkeys with ovine follicle stimulating hormone (FSH)

Immunogen	Centre	No. of immunized males used	No. pregnant/ no. ovulatory cycles exposed	References
FSH	IIS, Bangalore[a]	10	0/52	Moudgal *et al.*, 1992
	LSU, New Orleans[b]	9	0/54	Raj *et al.*, 1991
	NIHFW, New Delhi[c]	10	2/30	Srivatsava and Das, 1992
rFSH–RP	IIS, Bangalore[c]	3	1/12	Moudgal *et al.*, 1996

IIS = Indian Institute of Science; LSU = Louisiana State University Medical School; NIHFW = National Institute of Health and Family Welfare; rFSH–RP = recombinant FSH receptor protein.
Fertility was checked at time points when animals had become oligozoospermic by exposing to proven fertile females between days 9–14 of ovulatory cycle. In all three centres the fertility index of control monkeys was >80%.
[a]Fertility was checked between 100–500 days of immunization. Nine monkeys recovered fertility following stoppage of boosting. Time taken to recover fertility extended from 80–700 days.
[b]Fertility checked following 3 years of immunization. Three monkeys recovered fertility 6 months later following cessation of boosting.
[c]Fertility checked following 5 years of immunization. Two monkeys showed recovery of fertility when their antibody titre was low.
[d]At the 12th ovulatory cycle exposure one monkey successfully impregnated a female. At this time its antibody titre was low and sperm counts had recovered to near normality.

It should be possible to achieve selective FSH deprivation in the primate by generating antibodies to FSH receptor protein instead of to the circulating hormone. Recent attempts to achieve this using an FSH receptor protein fragment (1–134 amino acids) obtained by the recombinant route have been successful. The pilot study conducted in adult male Bonnet monkeys indicated that: (i) effective antibody titres of prolonged bioefficacy (>300 days) can be obtained after only two or three injections of the immunogen; (ii) the antibody binds to cell-associated receptors effectively and inhibits binding of FSH; (iii) the consequent FSH deprivation results in inhibition of testicular germ cell transformation, a situation identical to that seen in FSH-immunized monkeys; and (iv) the FSH receptor protein-immunized monkeys exhibited a significant reduction in their fertility index (sperm counts $\times$ 10^6/ejaculation $\times$ motility score) and proved in mating studies to be infertile (Moudgal *et al.*, 1997a). The specificity of the effect was underscored by the fact that, similar to the results from the FSH vaccine study, immunization with FSH receptor protein did not lead to any change in serum testosterone concentrations.

The studies with FSH/FSH receptor vaccine carried out in primates have suggested that infertility can be achieved in the absence of azoospermia. It is essential to realise that it is not possible to achieve consistent azoospermia with FSH/FSH receptor immunization, as a lack of FSH significantly affects spermatogonial proliferation and quantitative production of primary spermatocytes, but not meiosis, the step that regulates production of spermatids (Suresh *et al.*, 1995). Due to the marked reduction in the quantity of primary spermatocytes available for meiotic division, a significant diminution in the transformation of spermatogonia to spermatids is seen resulting in oligozoospermia but not azoospermia. There is, however, ample evidence to suggest that FSH deprivation also affects the spermiogenic process leading to production of poor quality or immature spermatozoa and this is responsible for demonstrable infertility (Aravindan *et al.*, 1991, 1997; Moudgal *et al.*, 1992; Moudgal and Aravindan, 1993). Although the level of oligozoospermia required for acceptable infertility is unknown, it is believed that hypogonadotrophic men can become fertile after gonadotrophin replacement, producing $<5 \times 10^6$ spermatozoa/ml (Cummings and Bremner, 1994). It is presumed that, even though spermatogenesis is reinitiated by gonadotrophin treatment, the spermatozoa that are produced are in fewer in number but of good quality. The oligozoospermia induced by FSH deprivation, however, appears to be distinctly different. In addition to a marked reduction in sperm output, their quality (as determined by a variety of criteria) has been shown to be significantly affected (Table III). That immunization with oFSH results in infertility was established not only by undertaking mating studies with proven fertile cycling females (Table IV) but also from in-vitro studies which showed that the spermatozoa are incapable of binding to either homologous monkey oocytes (Raj *et al.*, 1991) or to zona-denuded hamster oocytes (Sharma and Das, 1992). These last authors observed that, while 60% of the zona-denuded hamster oocytes incubated with spermatozoa from control monkeys exhibited pronuclear formation, this figure was reduced to 0% for spermatozoa obtained from immunized monkeys 4 weeks after an FSH booster injection. The

infertility achieved by this procedure has also been shown to be reversible in 90% of cases, but recovery depends upon the time taken for the bioeffective antibody to fall below detectable amounts.

Since the oFSH vaccine being tested in the non-human primate model was intended for eventual human trials, from the beginning every attempt was made to ensure that the oFSH vaccine being produced was able to pass a variety of safety tests. Firstly, oFSH was isolated from lyophilized sheep pituitaries imported from New Zealand, as sheep from that country are certified free from a variety of schedule 1 diseases, including foot-and-mouth, rinderpest, vesicular stomatitis, anthrax, swine fever, lumpy skin disease, contagious bovine pleuropneumonia and scrapie. Following physicochemical and biological characterization of the isolated protein, it was passaged through a series of quality control tests used by the Serum Institute of India Ltd, Poona, India, to routinely certify vaccines produced for human usage.

Thus far, experience with gonadotrophin vaccines suggests that their safety and toxicity has been relatively good. Although immunization of male monkeys with intact oLH resulted in problems, the β subunit of oLHβ has been extensively tested for safety and toxicity in female monkeys (Thau, 1988) and no toxic events were observed following long-term (5 year) immunization with this immunogen. Earlier studies using immunofluorescence techniques by Nieschlag's group with oFSH have shown that immunization with oFSH does not lead to the presence of immune complexes either in the circulation or precipitated in a variety of tissues (Srinath *et al.*, 1983). The oFSH vaccine used in our studies was also subjected as per the guidelines of the Toxicology Review Committee of the Indian Council of Medical Research to acute (10 day), subacute (90 day) and chronic (365 day) toxicity testing in rats (Dr Nirmal Sethi of Central Drug Research Institute, Lucknow) and monkeys (Dr Shobha Sehgal of Post-Graduate Institute of Medical Education and Research, Chandigarh). As well as assessment of routine toxicological parameters, the tissue specimens were carefully screened for the occurrence of immunopathology. It was concluded that oFSH vaccine at the doses tested (1× and 5× the intended human dose) was not toxic for rats and monkeys (personal communication and Sehgal *et al.*, 1991).

A pilot study carried out in five human male volunteers (oFSH immunizations given on days 0, 20, 40 and 70 of the study) indicated that the vaccine in its current formulation and dosage was well tolerated and did not cause any immediate adverse effects. No significant immunopathological effects were recorded. The hormonal concentrations, especially LH, TSH, testosterone, thytoxine (T4) and triiodothyronine (T3), remained unaltered. Though no volunteer showed any significant change in serum prolactin or cortisol concentrations, in a different study conducted by another collaborating centre, the concentrations of prolactin in two or three of the volunteers appeared elevated for a single day. As this could be a stress-related effect, prolactin concentrations (multiple samples taken on three consecutive days) of long-term FSH immunized monkeys were carefully monitored, but no significant change was noted. A recently-concluded, closely-monitored monkey study (unpublished) showed no correlation between serum prolactin concentrations and antibody titres. This perhaps is understandable as monkeys in captivity are husbanded under carefully controlled conditions, unlike human volunteers which are exposed to the stresses and strains of everyday life. All volunteers responded to the vaccine by producing cross-reactive antibodies of high affinity (0.3×10^9–1.6×10^9/M) and cross-reactivity with hFSH in the range 25–45%. During the period when free antibody titre was high (binding capacity 252.40 ± 72.5 ng hFSH/ml), no serum FSH was measurable (value in normal males 1–5 ng/ml). Because of the short duration of the clinical study, it appears that circulating FSH may be neutralized for a period of only 50–70 days (Moudgal *et al.*, 1997b). Sertoli cell function, as assessed by measuring the values of seminal plasma transferrin, was markedly affected following FSH deprivation in both the human and the monkey (Moudgal *et al.*, 1997b). Androgen binding protein (ABP) is a Sertoli cell protein whose production has been shown to be regulated in the rat by FSH (Bardin *et al.*, 1994). Such an event may also occur in primates. However, the non-availability of a sensitive and specific radioimmunoassay for this protein in the primate precludes such measurements. Considering that the FSH deprivation achieved in the human was for only one sperm cycle (~65 days), the effect on actual sperm counts in the ejaculate was only marginal (33–64%). However, using flow cytometric analytical criteria standardized for the monkey study (Aravindan *et al.*, 1991, 1997), it was observed that the spermatozoa of human male volunteers had a similar distinct downward trend in quality which was correlated with antibody titre. The parameters measured were: (i) susceptibility to acid denaturation followed by Acridine Orange (which assessed sperm chromatin structure assay by binding only to single-stranded DNA); (ii) chromatin decondensation as measured by binding of ethidium bromide following exposure to different concentration of dithiothreitol (DTT); and (iii) acrosomal content as measured by *Pisum sativin* agglutinin–fluorescein isothiocyanate (PSA–FITC) binding (Krishnamurthy *et al.*,

1997). It may be of particular interest to point out here that high αt value derived from the sperm chromatin structure assay (Evenson and Jost, 1994) has been correlated with sub- or infertility observed in a number of species including the human. The spermatozoa of both monkeys and man exhibited these high αt values (in the monkey from a control of 0.03 ± 0.01 to 0.56 ± 0.08 and in man from a control of 0.19 ± 0.03 to 0.85 ± 0.04) following immunization with oFSH. The spermatozoa of recombinant FSH receptor-immunized monkeys also exhibited high αt values (0.80 ± 0.01), indicating that FSH deprivation achieved by immunizing with either FSH or FSH receptor leads to the same end result (Krishnamurthy *et al.*, 1997). Whether a change in sperm quality is responsible for the inability of the spermatozoa of FSH-immunized monkeys to fertilize oocytes *in vitro* is worth considering (Raj *et al.*, 1991; Sharma and Das, 1992). It is also interesting to observe here that Acosta *et al.* (1992) have noted that the fertilizing ability of spermatozoa associated with some types of human male infertility can be significantly improved by administering pure hFSH.

Table V. Identification of amino acid residues at the epitopic region of human follicle stimulating hormone (hFSH) by chemical modification

Modification	Amino acid residue modified	Activity profile (%) of modified derivatives using immobilized hFSH MAb[a]
TNBS	K	165–200
TNM	Y	27–30
CDI	D/E	14–16
CHYMOTRYPSIN	F, Y	80–100
TRYPSIN	K, R	100
RCM	S–S	<1

TNBS = trinitrobenzene sulphonic acid; TNM = tetranitro methane; CDI = carbamide; RCM = reduced carbodymethylated (reproduced with permission from Murthy and Srilatha, 1996).
[a]hFSH monoclonal antibody (MAb) used is MAb 68 K12 1D12 kindly donated by Dr Dias. This MAb is specific to FSHβ and inhibits binding of [125I] FSH to its receptor.

As the criteria of quality impairment (analysed by flow cytometry) in spermatozoa produced by human volunteers immunized with oFSH appear to be very similar to those of monkeys rendered infertile due to FSH immunization, it is tempting to suggest that we can also achieve infertility in the human male by continuing the FSH immunization schedule. Based on the monkey study, to achieve infertility the monkey/man must be deprived of endogenous FSH support for three or four sperm cycles. Thus, in the monkey, FSH must be neutralized on a continuing basis for ~120 days to achieve infertility, whereas in the human male this needs to be prolonged to ~180 days. This can only be verified by future human studies.

Two groups (Santa Coloma and Reichert, 1990; Santa Coloma *et al.*, 1990 and Meloen *et al.*, 1991; Hage-Van-Noort *et al.*, 1992) have recently attempted to identify, using a synthetic approach, linear peptide sequences in the hFSHβ subunit that can act as hormone agonists/ antagonists. The ability of these peptides to generate receptor-blocking antibodies *in vivo*, when administered either alone or together, remains to be demonstrated satisfactorily. More recently, Lal *et al*. (1997) reported, using an in-vitro rat granulosa assay system, that antibodies raised to 31–52, 66–75 but not to 86–95 amino acid residues of the hFSHβ subunit, do possess bio-neutralizing activity and block FSH stimulated progesterone production. Interestingly, the disulphide loop peptide of 31–52 directly blocked FSH stimulated progesterone production *in vitro*. Since the homology between ovine and human FSHβ is considerable (>90%, see Figure 2), we checked the ability of synthetic linear peptides corresponding to three epitopes of hFSHβ (kindly provided by Dr K.S.Iyer of Institute of Research in Reproduction, Bombay, India) to cross-react with the antibody generated to oFSH in the human volunteers. Using an enzyme-linked immunosorbent assay to detect cross-reactivity, we observed that all three epitopes were indeed recognized by the antisera raised in humans to oFSH (Figure 2). The importance of amino acid sequences 37–55 in the FSHβ subunit in generating FSH-specific antibody is also stressed by the recent work of Westhoff *et al.* (1996). More recently, using a novel innovative procedure, Murthy and Srilatha (1996) have reported the mapping of a hFSH β-specific epitope which may be involved in receptor binding. The necessity of conformational integrity for the reaction of hFSH with a receptor blocking FSHβ-specific monoclonal antibody (MAb) was shown by the failure of reduced carboxymethylated FSH to react with MAb (Table V). Furthermore, by examining the ability of chemically derivatized hFSHβ to interact with an hFSHβ-specific Mab which had been shown to block FSH-receptor interaction, they were able to conclude that a multiple disulphide-linked cysteine knot peptide (Figure 2 inset) was the hFSH-β-specific core peptide involved in receptor binding. Interestingly, this peptide encompasses two of the linear peptides used earlier by our group to detect the presence of specific antibodies in human volunteers. It would be of interest to determine the immunogenicity and bioefficacy of the antibody generated to a synthetic version of the disulphide-linked peptide representing the above epitope of the hFSHβ subunit.

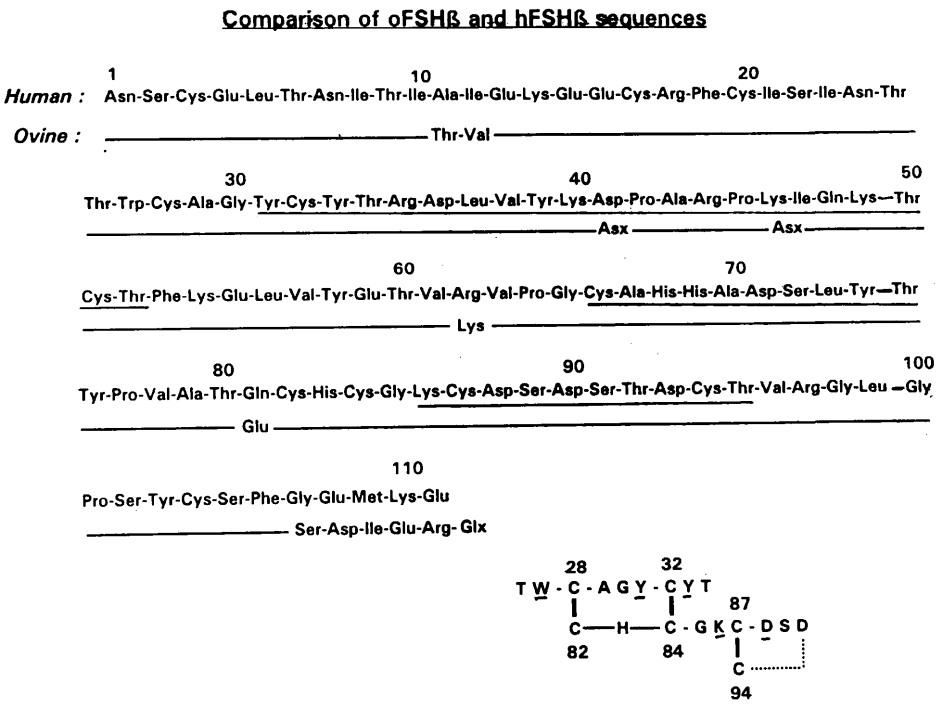

Figure 2. Comparison of ovine and human follicle stimulating hormone (FSH)β sequences. The sequence of three peptides of hFSHβ (31–52); (66–75) and (86–95) which showed cross-reactivity with the oFSH antisera generated by human volunteers is underlined. The inset shows the amino acid sequence of disulphide-linked polypeptide supposed to represent the domain that interacts with the receptor (after Murthy and Srilatha, 1996).

Conclusions and future considerations

The foregoing suggests that development of a viable hormonally-based contraceptive vaccine for the male is distinctly possible. If azoospermia is the desired goal, LHRH or a modified version of the LH receptor-nested peptide may be the candidate vaccine of choice. In such a case, exogenous testosterone supplementation will be required to maintain libido and accessory gland function. Research, however, needs to determine the proper choice of carrier protein, adjuvant and immunization protocol which would entail a booster only once in 6–12 months. Considering that receptors for LHRH have been noted in other tissues as well as the pituitary (including the gonad, prostate etc), long-term immunization with LHRH conjugate must be carefully assessed for any pathology in apparently non-specific tissues.

The available data on the efficacy of FSH/FSHβ and FSH receptor antibodies in blocking testicular function in the male non-human primate and thus leading to infertility, suggest that these may be the prospective vaccine candidates for the human male. The major advantage of this approach is that infertility can be achieved without affecting testosterone production or libido, and that fertility can be restored following cessation of antibody administration. Generally, the response to the heterologous immunogen (oFSH or oFSHβ subunit or FSH receptor protein) has been good (from studies conducted both in monkey and man) and, within the limits of the toxicology studies conducted, the oFSH vaccine appears to be relatively non-toxic and has not resulted in any untoward health hazards. Since the immunogen isolated from natural sources may not be acceptable for long-term human trials, it is recommended that future studies are carried out with the recombinant product. The recombinant FSH receptor protein may have an advantage over FSH or FSHβ as the contraceptive vaccine for the future, as receptor protein antibodies appear to provide long-term coverage. However, more basic work and toxicological screening needs to be carried out on the FSH receptor protein before it becomes acceptable. An apparent drawback of the FSH-based vaccine is that it does not lead to azoospermia, a desired norm for present day male contraceptives under trial. However, sufficient evidence is forthcoming from long-term studies in monkeys as well as short-term pilot studies in human volunteers to suggest that the reduction in the quality of spermatozoa, together with the oligozoospermia achieved, may be compatible with infertility. Additional human studies are required to verify the truth of this concept.

The vaccine approach for male contraception, as currently envisaged, entails the administration of two injections of the immunogen (20 days apart) as a primary immunization followed by boosters (either LHRH or FSH) once every 3–6 months. It is possible that with the use of better adjuvants, we may be able to extend the period of coverage of each booster by another 3–6 months. Based on our limited experience with the recombinant FSH receptor protein as immunogen in the monkey, we believe that high antibody titres can be maintianed in this case by administering only one or two booster injections per year. This is not surprising as the FSH receptor cells (Sertoli cells) in the adult primate are limited in number and the turnover of receptors may not be rapid, unlike the continuous production of LHRH and FSH by the hypothalamus and pituitary respectively. Considering that the currently recommended steroid hormone-based male contraceptive procedure requires the injection of testosterone with or without a progestin every 1–4 weeks, the apparent frequency in immunization schedule recommended may not be daunting.

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