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Alterations in Sperm Characteristics of Follicle-Stimulating Hormone (FSH)-Immunized Men Are Similar to Those of FSH-Deprived Infertile Male Bonnet Monkeys

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ABSTRACT: The quality of sperm ejaculated by bonnet monkeys and normal, healthy proven fertile volunteer men, both actively immunized with ovine follicle-stimulating hormone (oFSH), was examined at different times of study for chromatin packaging and acrosomal glycoprotein concentration by flow cytometry. Susceptibility of sperm nuclear DNA to dithiothreitol (DTT)-induced decondensation, as measured by ethidium bromide binding, was markedly high compared with values at day 0 in men and monkeys during periods when FSH antibody titer was high. Sperm chromatin structure assay yields αt values, which is another index of chromatin packaging. Higher αt values, signifying poor packaging, occurred in both species following immunization with heterologous pituitary FSH. The binding of fluorosceinated pisum sativum agglutinin (PSA-FITC) to acrosome of sperm of monkeys and men was significantly low, compared with values at day 0 (control) during periods when cross-reactive FSH antibody titer was high and endogenous FSH was not detectable. Blockade of FSH function in monkeys by active immunization with a recombinant oFSH receptor protein corresponding to a naturally occurring messenger RNA (mRNA) also resulted in production of sperm with similar defects in chromatin packaging and reduced acrosomal glycoprotein concentration. Thus, it appears that in monkeys and men, lack of FSH signaling results in production of sperm that exhibit defective chromatin packaging and reduction in acrosomal glycoprotein content. These characteristics are similar to that exhibited by sperm of some class of infertile men. Interestingly, these alterations in sperm quality occur well ahead of decreased sperm counts in the ejaculate.

Key words: Chromatin packaging, FSH deprivation, sperm maturation, infertility.

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Spermatogenesis in mammals is a highly complex process and is largely regulated by the 2 pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). While the role of LH via its direct control of testicular testosterone secretion remains undisputed, the function of FSH in the male may vary according to species. The manner in which FSH regulates spermatogenesis in rodents appears to be different from primates, and hence, this has become a topic of many debates (Zirkin et al, 1994; Moudgal and Sairam, 1998). A variety of methods have been employed to block FSH signaling in order to study the regulatory role of FSH on rodent spermatogenesis. There is a large body of evidence in favor and against FSH's role in regulating spermatogenesis (Dym et al, 1979; Vaishnav and Moudgal, 1994; Sinha Hikkim and Swerdloff, 1995; Shetty et al, 1996). More recent gene disruption studies show that FSHβ gene knockout male mice, despite being fertile, show reduction in testicular size, sperm counts, and sperm motility (Kumar et al, 1997). However, FSH receptor deficient male mice not only show reduced testes size, sperm counts, and sperm motility, but also exhibit low testosterone levels. These mutant males also have reduced fertility (Dierich et al, 1998; Sairam et al, unpublished material). However, these reports on knockout models do not discuss the possible compensatory mechanisms that may be triggered in the absence of FSH signaling in the genetically manipulated condition, and extrapolation of such results across the species can be perilous (Moudgal and Sairam, 1998).

In other species, such as hamsters (Lerchl et al, 1993) and sheep (Kilgour et al, 1993), completion of spermatogenesis is dependent on FSH stimulation of testicular function. Loss of FSH action in sheep leads to decreases in daily production of B2 spermatogonia, leptotene, and

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pachytene spermatocytes (Kilgour et al, 1993). Studies reported over the past several years in primates are leading toward a clear consensus on the need for FSH in maintaining normal spermatogenesis in monkeys (Murty et al, 1979; Wickings et al, 1980; Moudgal, 1981; Raj et al, 1982; Van Alpen et al, 1988; Weinbauer et al, 1991; Moudgal et al, 1992) and in men (Matsumoto et al, 1986; Gromoll et al, 1996). In the adult male monkey, FSH deprivation induced by immunization with heterologous purified hormone results in impairment of testicular function leading to reduction in total sperm output (Murty et al, 1979). Similarly, blockade of FSH receptor function in male monkeys by an appropriate receptor antibody formation also has been shown to cause testicular dysfunction and infertility (Moudgal et al, 1997a). Recently, Tapanainen et al (1997) analyzed a family of men in Finland homozygous for an inactivating mutation of FSH receptor gene. It was observed that these men had a variable degree of spermatogenic failure, and they exhibited oligospermia with poor sperm characteristics. Two out of the 5 men examined, however, were apparently fertile and had testicular size, FSH levels, and LH levels that were in the normal range. However, there may not be total inactivation of the FSH receptor by such point mutations. From analysis of the inhibin levels of these men, it appears that there could be some residual receptor activity to maintain Sertoli cell function. Hence, that situation does not necessarily represent a condition of complete loss in FSH signaling. In contrast to this, recent reports of inactivating mutation of the FSH beta subunit gene indicate that lack of FSH action is perhaps responsible for the observed hypogonadism and absence of sperm in the ejaculate of affected individuals (Lindstedt et al, 1998, Phillip et al, 1998).

In previous studies in fertile adult male monkeys, we have shown that continuous bioneutralization of endogenous FSH by either passive or active immunization approaches leads to oligospermia and infertility (Murty et al, 1979; Moudgal et al, 1992). In addition to significant reduction in sperm counts (>70%), the poor quality of the ejaculated sperm exhibited by FSH-immunized monkeys contributed to infertility in these males. Analyses of a variety of parameters such as viability, gross motility, gel penetrability, and acrosin and hyaluronidase activity (Moudgal et al, 1992) supported the changes observed in the quality of spermatogenesis. In addition, it also has been independently demonstrated that spermatozoa from FSH-immunized monkeys fail to attach to zona-intact monkey eggs (Raj et al, 1991) and zona-free hamster eggs (Srivastava and Das, 1992). Further, we also have observed that FSH deprivation in monkeys affects sperm chromatin status as determined by the ability of sperm to undergo nuclear decondensation following exposure to a reducing agent like dithiothreitol (DTT; Aravindan et al, 1997, and unpublished data). Thus, compromising spermatogenesis and its quality at the same time might become an attainable option or consideration for male contraception. In the light of these interesting leads it was considered of interest to apply sensitive-flow cytometric methods (Spano and Evenson, 1993) to more thoroughly investigate the chromatin packaging quality of sperm obtained from monkeys and men deprived of endogenous FSH support by the same immunologic procedure. In the present study, we have compared the chromatin packaging and acrosomal functionality of sperm of normal, as well as specifically FSH-deprived, monkeys and men. At the same time, we have verified whether blocking FSH receptor at the testicular level can produce similar effects. A preliminary report of this work was presented at a recent meeting (Krishnamurthy et al, unpublished material).

Materials and Methods

Materials

Dulbecco phosphate-buffered saline solution (PBSS) was obtained from GIBCO Laboratories (Grand Island, NY). DTT, ethidium bromide (EB), fluorosceinated pisum sativum agglutinin (PSA-FITC) and Nonidet P-40 were obtained from Sigma Chemical Co (St Louis, Mo). Pepsin was obtained from Serraferin Biochemia (Heidelberg, Germany). Acridine Orange (Polysciences, Warrington, Penn) was a kindly gifted by Professor D. P. Evenson, South Dakota State University, SD. FACScan flow cytometer was obtained from Becton Dickinson (San Jose, Calif).

Monkey Study

The immunization procedure and protocols followed are described elsewhere (Moudgal et al, 1992). Briefly, adult male bonnet monkeys were actively immunized with 1-, 0.3-, 0.1-, and 0.1-mg ovine FSH (oFSH; adsorbed on alhydrogel suspension given SC) on days 1, 8, 16, and 24. The animals received booster immunizations once every 90 to 100 days with 0.1 mg of purified oFSH for more than 5 years. Periodically, blood samples were collected to assess antibody titer and testosterone and LH concentrations in the serum. Sperm samples were collected by electrostimulation, washed twice in PBSS, fixed in chilled 70% ethanol, and stored at −20°C. Similarly, sperm from a group of 3 adult male bonnet monkeys that were rendered infertile because of immunization for ~250 days with a recombinant ovine FSH receptor protein fragment (oFSHR-P) as described previously (Moudgal et al, 1997a) were also used for analysis. Sperm samples from unimmunized normal fertile monkeys collected at the same time as experimental samples served as corresponding controls. Control samples also were stored at −20°C in 70% ethanol until further analysis. The sperm samples were obtained from a group of control (n = 3–4) and oFSH-(n = 3–4) or oFSHR-P–(n = 3) immunized adult male bonnet monkeys. Testicular germ cells were obtained by needle biopsy from control and FSH-immunized monkeys according to procedure outlined earlier (Aravindan et al, 1993).

Human Study

Sperm samples collected from 5 volunteer, 34- to 41-year-old, normal, fertile, healthy men who underwent active immunization with oFSH as per protocol described earlier (Moudgal et al, 1997b) were used in the study. Briefly, each of the volunteers received subcutaneous injections of 1 mL Alugel (Alhydrogel, Superfos, Denmark) suspension containing 1 mg of oFSH on day 1, and on day 20, a further 1 mL of suspension containing 0.3 mg of oFSH was administered. Booster doses of 0.1 mg of oFSH were administered on days 40 and 70 of immunization. The human study was approved by the ethical committee of the Ramaiah Medical Teaching Hospital, Bangalore, India, where the clinical study was conducted and each of the volunteers provided individually signed consent letters. After a 3-day abstinence, the sperm samples were collected by masturbation between 9 AM and 11 PM on days 0, 30, 50, 70, and 110 or 140 of immunization. Because day-110 sample of ejaculate could not be obtained from 1 volunteer, we have used the posttreatment day-140 sample for all analyses. The volunteer men served as their own controls, and the sperm samples obtained during a pretreatment control phase and posttreatment recovery phase were compared with those provided during the treatment immunization phase (Moudgal et al, 1997b). The sperm samples analyzed in the human study were drawn from pretreatment (day 0), treatment (days 30, 50 and 70), and posttreatment (days 110 and 140) phases of immunization.

EB Binding to Sperm DNA Following DTT Treatment

The procedure for obtaining sperm nuclear decondensation by exposure to various concentrations of DTT has been described earlier (Aravindan et al, 1995, 1997). Briefly, ethanol-fixed sperm of individual human volunteers and monkeys were washed twice in PBSS, and the pellet was incubated with 0.2 mL of 0.5% pepsin in PBSS (pH 2) for 10 minutes at 37°C. Samples were washed and treated with 0.01% papain for 5 minutes at 37°C. In the case of EB-binding studies, each of the samples were washed, divided into 5 aliquots, and treated with 0, 2.5, 5, 10, and 20 mmol/L DTT (human volunteer) and 0, 2.5, 5, 10, and 50 mmol/L DTT (monkey) respectively for 20 minutes at 37°C (papain and DTT solutions were prepared in Tris-HCl buffer, pH 7.2). Following incubation, the samples were washed, and the sperm pellet was resuspended in 0.5 mL of EB (EB-staining solution; 25 μg EB/mL, 40 μg RNAase/mL, and 0.3% Nonidet P-40). The EB-stained samples were analyzed with a FACScan flow cytometer. The EB-stained sperm were excited at 488 nm, red fluorescence signals on a linear scale were collected at 564 to 606 nm, and fluorescence measurement in peak mode was processed with LYSIS II software.

Testicular germ cells obtained by needle biopsy from control and FSH-immunized monkeys (Aravindan et al, 1993) also were tested for DTT-induced decondensation of elongated spermatid population. Germ cells were exposed to 0 and 5 mmol/L DTT for 20 minutes at 37°C prior to staining with EB, and acquisition and analysis of fluorescence was done as described above.

Sperm Chromatin Structure Assay

As sperm chromatin structure assay (SCSA) has the advantage of distinguishing debris from sperm signals (Evenson and Jost, 1994), we analyzed the sperm ejaculated from FSH-immunized monkeys and men. The dye acridine orange (AO) intercalates into double-stranded DNA, producing a green fluorescence signal whereas its binding to single-stranded DNA or RNA causes a red fluorescence. The SCSA provides an assessment of the degree of DNA denaturation, which the sperm nucleus undergoes upon exposure to stringent acid treatment. The extent of denaturation can be determined by the metachromatic shift from green to red fluorescence of the AO-stained sperm. The AO-staining procedure used was essentially similar to that described by Evenson and Jost (1994). About 0.2×10^6 ethanol-fixed sperm were washed twice in TNE buffer (0.01 mol/L Tris, 0.15 mol/L NaCl, and 1 mmol/L EDTA, pH 7.4), and the pellet was resuspended in 200 μL TNE and treated with 0.4 mL of acid/triton X-100 solution (0.15 mol/L NaCl, 0.1 % Triton X-100, and 0.08 N HCl in double-distilled water). After 30 seconds, 1.2 mL of AO-staining solution (0.15 mol/L NaCl, 0.1 mol/L citric acid, 0.2 mol/L Na_2HPO_4, [pH 6] and 6 μg/mL AO) was added, and the stained samples were analyzed with a FACScan flow cytometer equipped with a 15-mW argon-ion laser. The AO-stained samples were excited at 488 nm, and the green fluorescence was collected at 515 to 545 nm, and the red fluorescence was collected at 564 to 606 nm and at 605 to 645 nm. Green and red fluorescence were processed in peak mode of signal with LYSIS II software. The αt values were calculated according the formula described by Evenson and Jost (1994). The extent of denaturation was quantitatively denoted by the term αt, a value that can range from 0 to 1. This is a ratio of red fluorescence to total (green and red) fluorescence (Evenson and Jost 1994). The higher shift from green to red signifies greater DNA denaturablity and loss of fertility. The αt values have shown to be useful in assessing the fertility rating of bulls (Ballachey et al, 1987, 1988).

Degradation of Sperm DNA

Ethanol-fixed human sperm (at different phases of treatment) were centrifuged and washed 3 times with PBSS and once with the lysis buffer (10 mmol/L Tris HCl, pH 8.0; 400 mmol/L NaCl, and 200 mmol/L EDTA). The cell suspension (1×10^6/mL lysis buffer) was treated with 10 μL proteinase K (20 mg/mL stock) and 100 μL of 10% sodium dodecyl sulfate (SDS) and incubated at 42°C for 24 hours. Nucleic acids in the supernatant of this digest were precipitated by adding an equal volume of isopropanol and storing it at −70°C for 6 to 12 hours. The DNA was washed with 70% ethanol, air-dried, and redissolved in Tris HCl-EDTA (TE) buffer for digestion with ribonuclease (0.1 mg/mL) for 3 hours. Following a phenol-chloroform extraction step, the DNA was again precipitated with ethanol, washed, and air dried. The final pellet was dissolved in TE buffer and quantitated by ultraviolet absorbency at 260 nm. Ten micrograms of each sample was subjected to electrophoresis on a 2% agarose gel at 2V/cm. The gel was stained with EB for visualization of the migration pattern. Monkey sperm samples were not available for this analysis.

Lectin-Binding Property of Sperm

Ethanol-permeabilized monkey and human sperm have been used to detect acrosomal content by staining with fluorescenated

lectin (Cross et al, 1986). About 0.2×10^6 ethanol-fixed sperm were washed 3 times in PBSS, and the pellet was suspended in 100 μL of 25 μg/mL PSA-FITC for 10 minutes at room temperature in the dark. The period of exposure provided optimal staining for quantification by flow cytometry, the nonspecific binding of PSA-FITC being negligible. The sperm were washed 2 times in PBSS to remove excess PSA-FITC and then resuspended in 0.5 mL PBSS. The sperm samples obtained from preimmunization, treatment (immunization), and postimmunization phases of human volunteers were stained and analyzed at the same time. Similarly, sperm from control and oFSH- and oFSHR-P–immunized monkeys also were processed and analyzed at the same time. The green fluorescence of the PSA-FITC–labeled sperm samples were recorded by a FACScan flow cytometer equipped with argon-ion laser. The sperm samples were excited at 488 nm, the green signals of FITC on log scale were collected at 515 to 545 nm, and fluorescence measurement at peak mode processed on LYSIS II software.

Statistical Analysis

Routinely statistical analysis was performed using Student's *t* test, but wherever necessary, analysis of variance (ANOVA) test also has been used to determine significance at $P < .05$.

Results

The treatment phase of the human study corresponded to the period where no free-endogenous FSH was measurable by a 2-step radioreceptor assay, and the human FSH (hFSH) cross-reactive antibody titer was maximal (binding capacity: 346 ± 185 ng hFSH/mL). While no hFSH cross-reactive antibody was titratable during the pretreatment control phase, in the posttreatment phase, antibody titers had fallen to very low levels (binding capacity: 10.5 ± 5.8 ng hFSH/mL), and free FSH had become detectable (Moudgal et al, 1997b). In the monkey study, the period when sperm samples were taken for analysis corresponded with the occurrence of high antibody titers (binding capacity, 9.1 ± 5.4 μg hFSH/mL) in the serum of oFSH-immunized monkeys (Moudgal et al, 1992). In the case of oFSHR-P–immunized monkeys, sperm samples were taken at periods when the serum (IgG fraction) inhibited the binding of FSH to a test FSH receptor preparation in vitro by 30% to 80% (Moudgal et al, 1997a). Considering that the period of immunization in the case of the human male covered only 1 sperm cycle (70 days), the overall decrease in sperm counts ranged from 33% to 64% (Moudgal et al, 1997b). In the case of the monkey study, the sperm samples obtained were following immunization with oFSH or oFSHR-P for more than 3 to 4 sperm cycles (>135 days), and as such, the sperm counts were reduced by more than 75% and 88%, respectively over the control (Moudgal et al, 1992, 1997a). This also was associated with a decrease in motility.

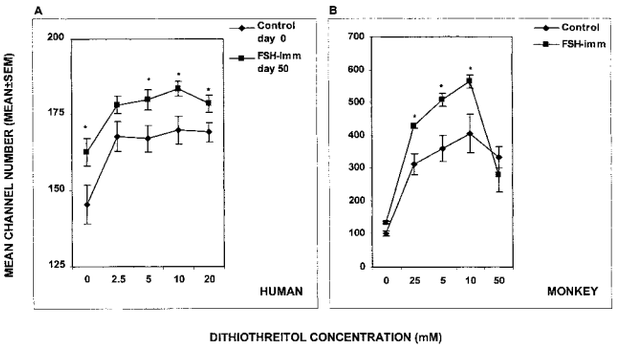

Figure 1. Effect of follicle-stimulating hormone (FSH) deprivation on ethidium bromide (EB) binding to dithiothreitol (DTT)-treated sperm voided by man and monkey. See text for experimental details. Asterisk indicates significantly different ($P < .05$).

Sperm Nuclear Decondensation

The EB-binding pattern of human sperm collected during treatment phase exhibited significantly ($P < .04$) high fluorescence compared with sperm of day 0, even in the absence of DTT (Figure 1A). The sample from day 50 of immunization was chosen for evaluation of this parameter, because the antibody titer was maximal during this period. Though the fluorescence intensity of FSH-immunized monkey sperm was higher than its respective control at 0 concentration of DTT, the increase was not statistically significant. The mean channel of fluorescence intensity in the case of human and monkey sperm increased with exposure to increasing concentrations of DTT (Figure 1A and B). Sperm samples from oFSH-immunized men and monkeys had a uniform increase in susceptibility to DTT-induced decondensation over their corresponding controls. Maximal and significant difference became apparent in human samples between 5 to 10 mmol/L DTT ($P < .02$) and in monkey samples between 2.5 to 5.0 mmol/L DTT ($P < .01$). The decrease in the fluorescence intensity of monkey sperm at 50 mmol/L concentration of DTT (Figure 1B) may be caused by a loss of DNA at higher concentrations of DTT. The increase in EB binding correlates to the increase in DTT concentration (between 0 and 10 mmol/L DTT), and the *r* values for control versus immunized groups were 0.85 vs 0.93 for the monkey, respectively, and 0.74 vs 0.84 (day 50) for man, respectively. These results concur with our earlier observations of increased susceptibility to DTT-induced decondensation of ejaculated and epididymal sperm from oFSH immunized monkeys (Aravindan et al, 1997).

Analysis of testicular germ cells of control and oFSH-immunized monkeys for chromatin status, with DTT-induced decondensability as a parameter, once again revealed that lack of FSH renders the spermatid population highly susceptible to DTT treatment. The frequency his-

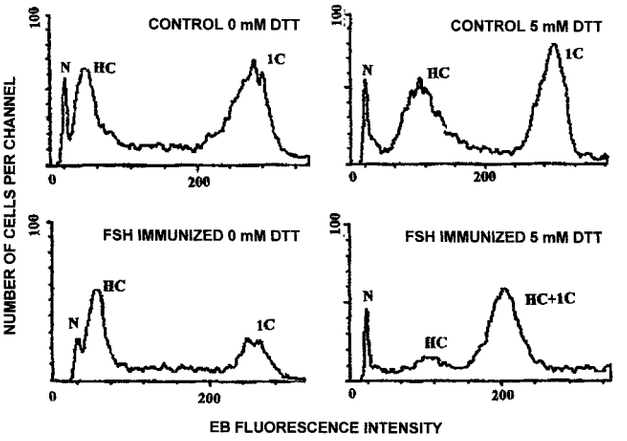

Figure 2. Representative flow cytograms showing EB-DNA–binding pattern to the elongating/elongated and round spermatid population of testis of control **(top 2 panels)** and ovine FSH (oFSH) immunized **(bottom 2 panels)** monkeys. The testicular germ cell population was exposed to 0 and 5 mmol/L DTT for 20 minutes before staining. N indicates nonspecific signals; H, hypostainability caused by compaction; HC, elongated spermatids; and 1C, round spermatids. For clarity, only the zoomed-in peaks of HC and 1C are presented.

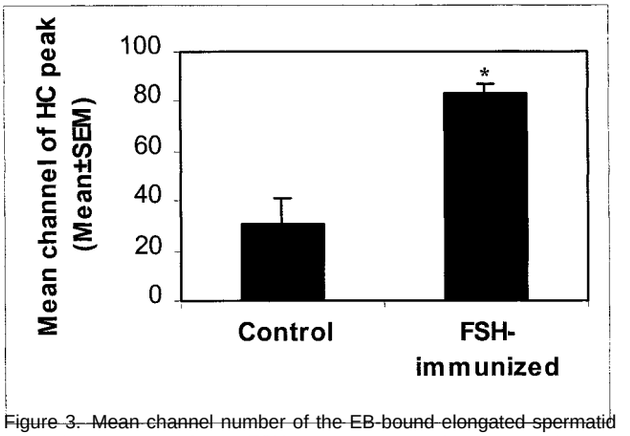

Figure 3. Mean channel number of the EB-bound elongated spermatid peak of control (n = 4) and FSH-immunized (n = 4) monkeys after exposing the germ cells to 5 mmol/L DTT. HC indicates elongated spermatid peak; asterisk, significantly different.

tograms obtained for the testicular germ cells treated with or without DTT are presented (Figure 2). The elongated spermatids (HC) and round spermatids (1C) peaks of control and FSH immunized monkeys were essentially identical in absence of DTT (Figure 2). The mean channel shift in EB fluorescence of HC population following exposure to 5 mmol/L DTT, however, was significantly different for the 2 groups. Deprivation of FSH resulted in a 2.7-fold ($P < .01$) increase in the mean channel shift compared with corresponding controls (Figure 3). The 1C population of control monkey testis did not show significant change in its disposition following DTT treatment as expected. In the FSH-immunized monkeys, however, the 1C population also appeared to be susceptible to the reducing action of DTT. The apparent 1C peak that appears displaced to the left could be representing both the true 1C and the HC that have undergone decondensation. Such a shift to the left in the 1C peak would indicate a decrease in EB stainability, which could be caused by loss of DNA following treatment with DTT (Figure 2). Thus, the spermatid population in the FSH-deprived monkeys is more susceptible.

Sperm Chromatin Structure Assay

From the AO-binding pattern of sperm ejaculated by oFSH-immunized volunteer men and monkeys (Figure 4) it is clear that on a per-cell basis, the red fluorescence increases, and the green fluorescence decreases with the progress of immunization. The human data, in addition to showing that the effect seen is highly reproducible, also makes it evident that with the fall of hormone antibody titer (by day 110 or 140) the relative fluorescence intensity pattern shows a reverse trend. While in 2 volunteers the maximal shift could be seen as early as day 30, in the other 3 volunteers this effect was evident by day 50. The αt values of the sperm produced by human male volunteers significantly ($P < .0001$) increased from a pretreatment control value of 0.31 to 0.54 during treatment phase. This increase was restored to normalcy (0.32) during the posttreatment phase (by day 110 or 140 of immunization, Table 1). The sperm of oFSH-immunized monkeys also exhibited significantly ($P < .0001$) high αt values compared with that of control monkey sperm (Table 1). The sperm of monkeys rendered infertile because of immunization with the recombinant oFSHR-P (Moudgal et al, 1997b) also showed significantly ($P < .0001$) high αt values compared with controls (Table 1), indicating that similar effects could be achieved by different methods of inducing loss of FSH signaling.

Degradation of Sperm DNA

The gel migration pattern of ejaculated sperm DNA samples from each of the 5 FSH-immunized volunteers are shown in Figure 5. In this experiment, migration of nucleic acid into the gel is an indication of action of endogenous endonuclease or other effects resulting in cleavage. In 4 of the 5 volunteers, the preimmunization sperm DNA at day 0 either did not migrate or showed slight mobility, indicating its high molecular weight (compare 10 and 12 with 1 and 5). Only the volunteer No. 9 sample exhibited a smear pattern on day 0. This could be caused by some technical problem during sample preparation. This interpretation is reasonable because the same sample analyzed for other parameters like SCSA (Figure 4) was no different compared with day 0 samples of the other 4 volunteers. Interestingly, 50 days after FSH immunization (ie, when antibody titers were maximum; Moudgal et al, 1997b) the migration of each DNA sample was altered

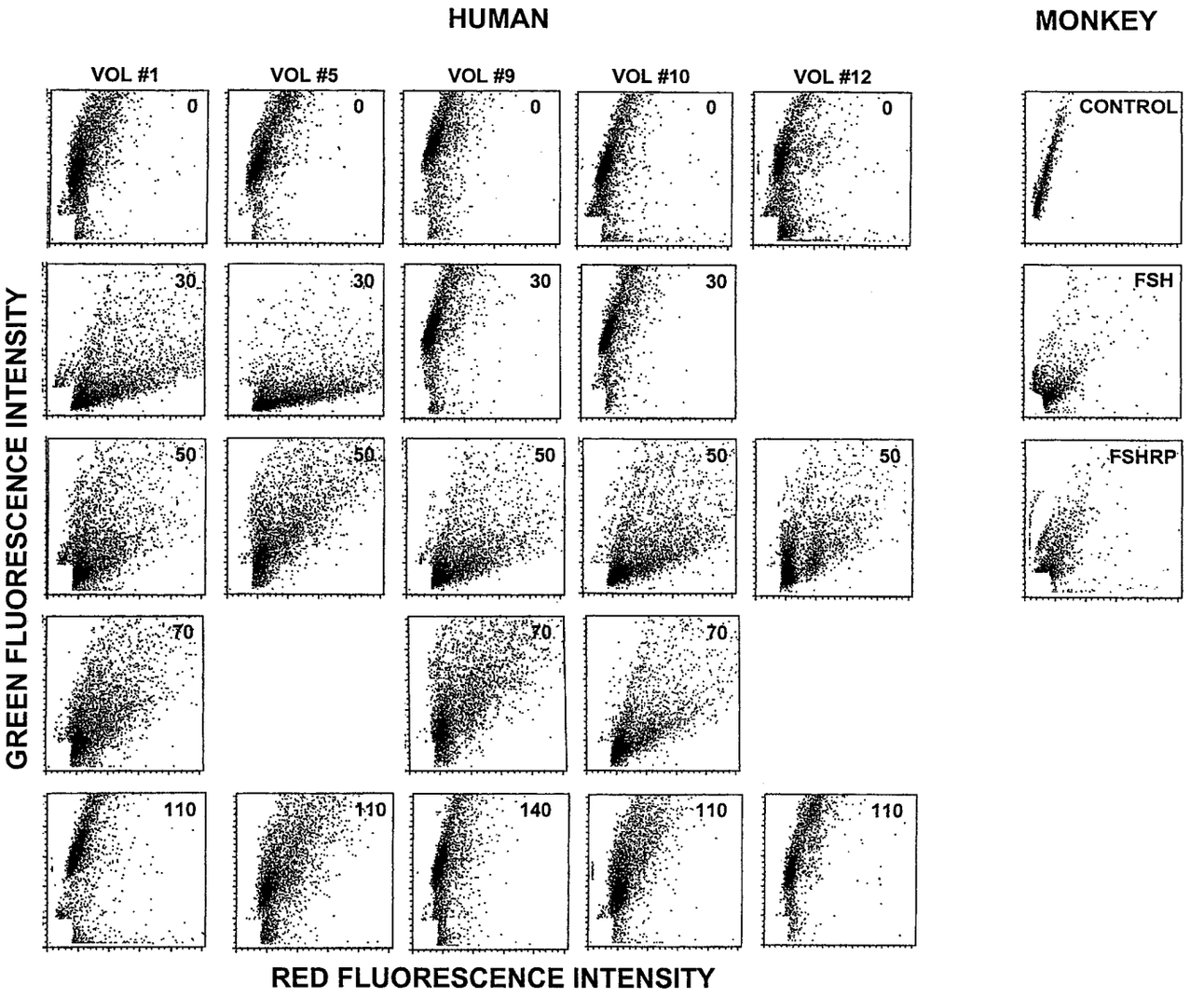

Figure 4. Dot plots showing the green (y-axis) and red (x-axis) fluorescence of acridine orange–bound sperm ejaculated by men and monkeys was determined by sperm chromatin structure assay. Dot plots from 5 individual human volunteers **(left panels)** on days 0, 30, 50, 70, and 110 or 140 (numbers provided in the right-hand top corner) of oFSH immunization. Representative dot plots showing green and red florescence of acridine orange–bound sperm voided by control, oFSH, and oFSH receptor protein fragment (oFSHR-P)-immunized monkeys **(right panel)**. In each case, 10 000 cells were analyzed following staining with acridine orange.

Table 1. *Sperm chromatin structure assay: αt values exhibited by the sperm voided by follicle-stimulating hormone–deprived man and monkey*

Human (n = 5)		Monkey	
Phase of Immunization*	αt Values† (mean ± SEM)	Immunogen	αt Values† (mean ± SEM)
Pretreatment	0.31 ± 0.001	Control (n = 7)	0.24 ± 0.001
Treatment	0.54 ± 0.002‡	oFSH (n = 4)	0.35 ± 0.003‡
Posttreatment	0.32 ± 0.002	oFSHR-P (n = 3)	0.41 ± 0.002‡

* Day 0 sample from each of the volunteers; αt values for control pretreatment phase. During treatment phase (days 30–70), 3 samples per volunteer were analyzed, and during the posttreatment/recovery phase (days 110 and 140) one sample per volunteer was analyzed.

† αt indicates mean channel of red fluorescence/mean channel of green fluorescence + mean channel of red fluorescence; oFSH, ovine follicle-stimulating hormone; and oFSHR-P, ovine follicle-stimulating hormone receptor protein fragment.

‡ $P < .0001$ pretreatment or control versus treatment.

substantially, showing a broad smear indicating breakage of the genetic material. Examination at day 110 or 140, when FSH-antibody titers had declined, showed a remarkable recovery of the patterns. During this phase, the reduced smearing effect indicated an increase in the stability of DNA, suggesting that the process induced by deprivation of FSH in circulation is completely reversible. Because there is no difference in the AO bindability of sperm ejaculated by volunteer No. 9 on the day 0 (Figure 4), we feel that the DNA degradation observed may be caused by technical problems while processing the sample.

Lectin-Binding Property

PSA-FITC binding to sperm quantitated by determining the mean channel of fluorescence intensity has been used here to assess the total acrosomal content of glycoprotein in sperm. Although the frequency histograms of PSA-FITC-bound sperm from control and immunized men and monkeys were showing overlaps caused by heterogenity, the fluorescence intensity (mean channel number) of human volunteer sperm collected during treatment phase of immunization showed a significant reduction (by 62%, $P < .001$) over corresponding pretreatment controls. The effect on acrosomal glycoprotein content had not returned to normalcy even by day 110 to 140 of immunization (reduced by 55%, $P < .001$). The sperm of monkeys immunized either with oFSH or oFSHR-P also showed a significant reduction (by 39%, $P < .001$ and 55% $P < .04$, respectively) in the binding of PSA-FITC over sperm of control monkeys (Figure 6; Table 2). It should be noted here that preincubation of control sperm with galactose reduced PSA-FITC binding to a marked extent. Galactose, which was used as a check of specificity, binds to the lectin conjugate and prevented subsequent recognition on cells (data not shown).

Discussion

The primary objective of the current study was to examine if deprivation of endogenous FSH support in normal proven fertile men and monkeys produces similar effects on sperm quality. Among methods currently in use for assessing sperm quality, flow cytometry techniques were preferred, because they provide an objective single-cell analysis with relatively large sample size and more than 1 parameter of sperm function. Direct comparison between samples collected on different days of treatment was feasible because all samples that were used were fixed in ethanol and stored in cold conditions. The parameters we chose for our analysis, such as defective chromatin packaging and reduced acrosomal glycoprotein concentration, have been correlated to infertility in men and monkeys (Cross et al, 1986; Acosta et al, 1992; Engh et al, 1992; Kosower et al, 1992; Moudgal et al, 1992; Bartoov et al, 1994; Bianchi et al, 1996; Sakkas et al, 1996; Aravaindan et al, 1997, Baccetti et al, 1997; Bench et al, 1998; Kamishke et al, 1998).

Earlier studies in FSH-immunized monkeys had indicated that the observed deleterious changes in the quality of sperm precede actual reduction in sperm counts in the ejaculate and induction of infertility (Moudgal et al, 1992). The overall effect is perhaps caused by a marked decrease in the production of adequate numbers of qualitatively normal sperm. In monkeys, Aravindan et al, (1993, 1997) noted that FSH deprivation reduced spermatogonial proliferation and altered spermiogenesis. Ejaculated and epididymal sperm of FSH-immunized monkeys bound significantly more ^{14}C-labeled iodoacetamide compared with corresponding controls. The failure of the oFSH-immunized monkey sperm to achieve nuclear compaction that is even equivalent to caput sperm of normal monkeys, indicated not so much an impaired epididymal maturation, but rather a distinctly reduced potential for –S–S– bond formation. This is clearly suggestive of defective spermiogenesis (Aravindan et al, 1997).

In the light of the above mentioned studies, it was felt appropriate to undertake a flow cytometric analysis of sperm from human volunteers immunized with oFSH. The results, we felt, could reflect the effectiveness of the FSH immunization procedure used in the human beings. Immunization of human volunteers with oFSH led to the production of antibodies capable of bioneutralizing hFSH between days 30 and 80 of immunization. During this period, circulating free FSH was not detected. FSH deprivation led to a marked reduction in seminal plasma transferrin (in both men and monkeys), used as a marker of Sertoli cell function (Moudgal et al, 1997b). Coinciding with this are our current observations on changes in sperm characteristics. It should be noted here that these changes occurred before large reductions in sperm counts were observed. In contrast, the monkeys were immunized with oFSH for more than 3 to 4 sperm cycles before significant reduction in sperm counts was observed. Thus, it is reasonable to propose that reductions in fertility by decreasing sperm quality might be possible before affecting sperm counts.

Sperm maturation, which is initiated at the spermiogenic stage in the testis, continues during transit of the sperm through the epididymis. During epididymal transit, the free SH groups of protamines of maturing spermatozoa undergo oxidation, resulting in greater degree of condensation leading to stabilization of sperm nucleus (Calvin and Bedford, 1971). The susceptibility of the sperm DNA to acid denaturation in vitro has been correlated with infertility (Ballachey et al, 1986). Our observation that the sperm ejaculated by FSH-immunized volunteer

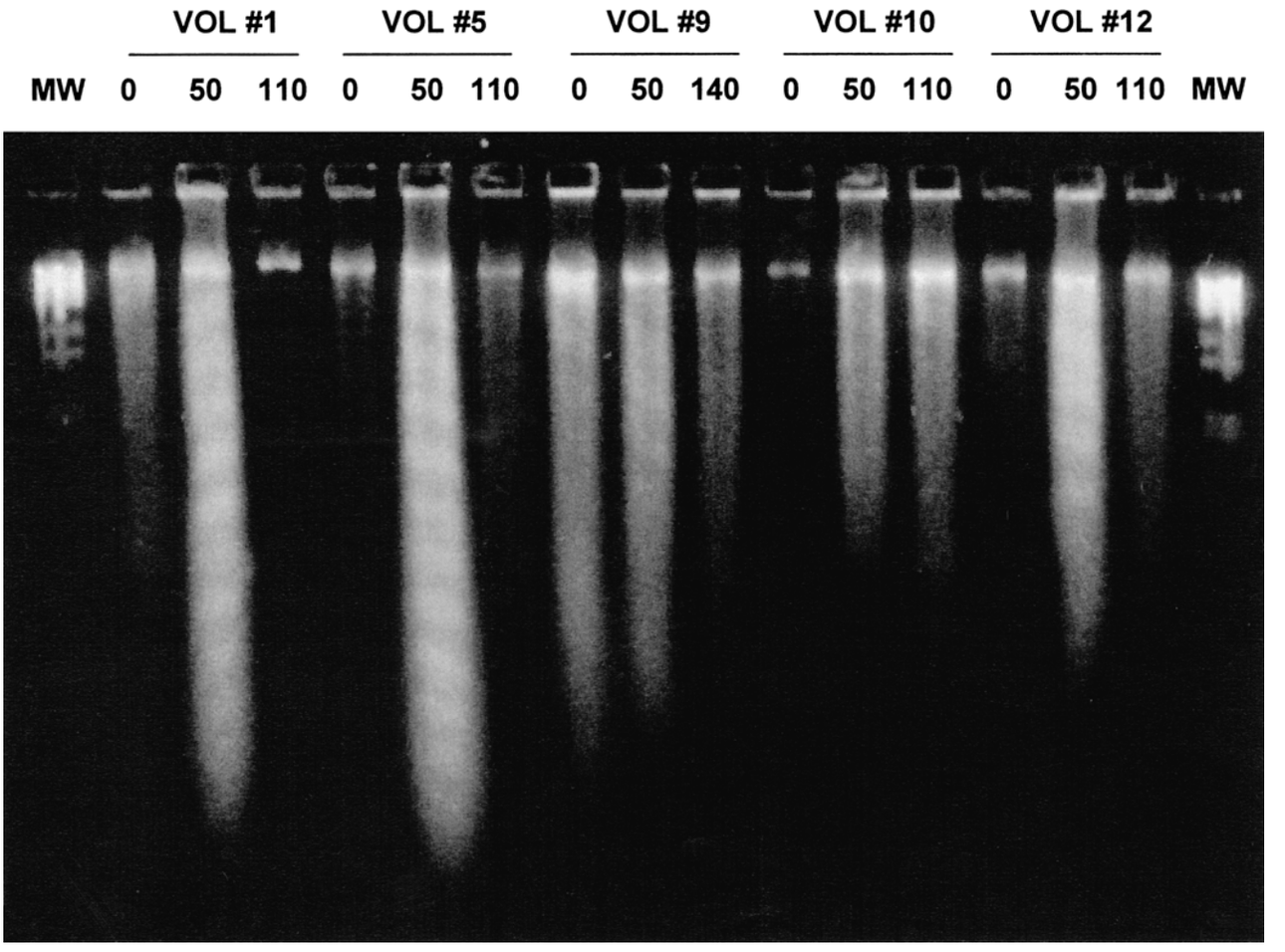

Figure 5. EB-stained gel showing the DNA degradation pattern of the sperm voided by volunteer men on days 0, 50, and 110 or 140 of immunization. Smear signified DNA instability. MW indicates DNA molecular weight marker.

men show increased susceptibility to DTT-induced decondensation compared with day 0 control sperm (Figure 1) is in line with this conclusion. These changes also are observed in monkeys (Figure 1; Arvindan et al, 1997). Other clinical observations also correlate infertility with improper DNA packaging. Thus, sperm from patients attending an infertility clinic show increased DNA stainability (Engh et al, 1992). Similarly, increase in guanine-cytosine–specific fluorochrome, chromomycine A_3 (CMA_3) binding reflects poor packaging quality of human sperm chromatin. According to Sakkas et al (1996), this also provides an indirect visualization of protamine deficiency and denaturation of DNA. Bianchi et al (1996), studying the staining of morphologic characteristics of normal human sperm with CMA_3, observed an inverse relationship between dye binding and in vitro fertilization. They also attributed the lowered fertilization rate to the absence or reduction in protamines and anomaly in chromatin packaging. We have demonstrated that this phenomenon can be experimentally induced in monkeys (Figure 2). Corroborative evidence for FSH deprivation affecting spermiogenic process in primates has been provided in the current study by analyzing the vulnerability of the HC population (Figure 3). Thus, it is evident that changes in packaging are already apparent at the testicular level in primates.

By use of the more sensitive bivariate SCSA procedure, both DNA stainability and the degree of DNA denaturation in the sperm can be studied by measuring the metachromatic shift from green to red fluorescence that occurs following AO binding (Evenson and Jost, 1994). The αt values calculated from the flow cytometric SCSA appears to show minimal variation within a single group over a given time. Thus, Evenson (1991) observed that the coefficient of variation is as low as 10% for a group of 45 volunteer men over 8 months. In our own experience we have observed that the αt of sperm from proven fertile men (in addition to the current study) is lower than 0.3. Generally, in a sperm chromatin structure assay, a 45° angle shift observed in the dot plot or scattergram is

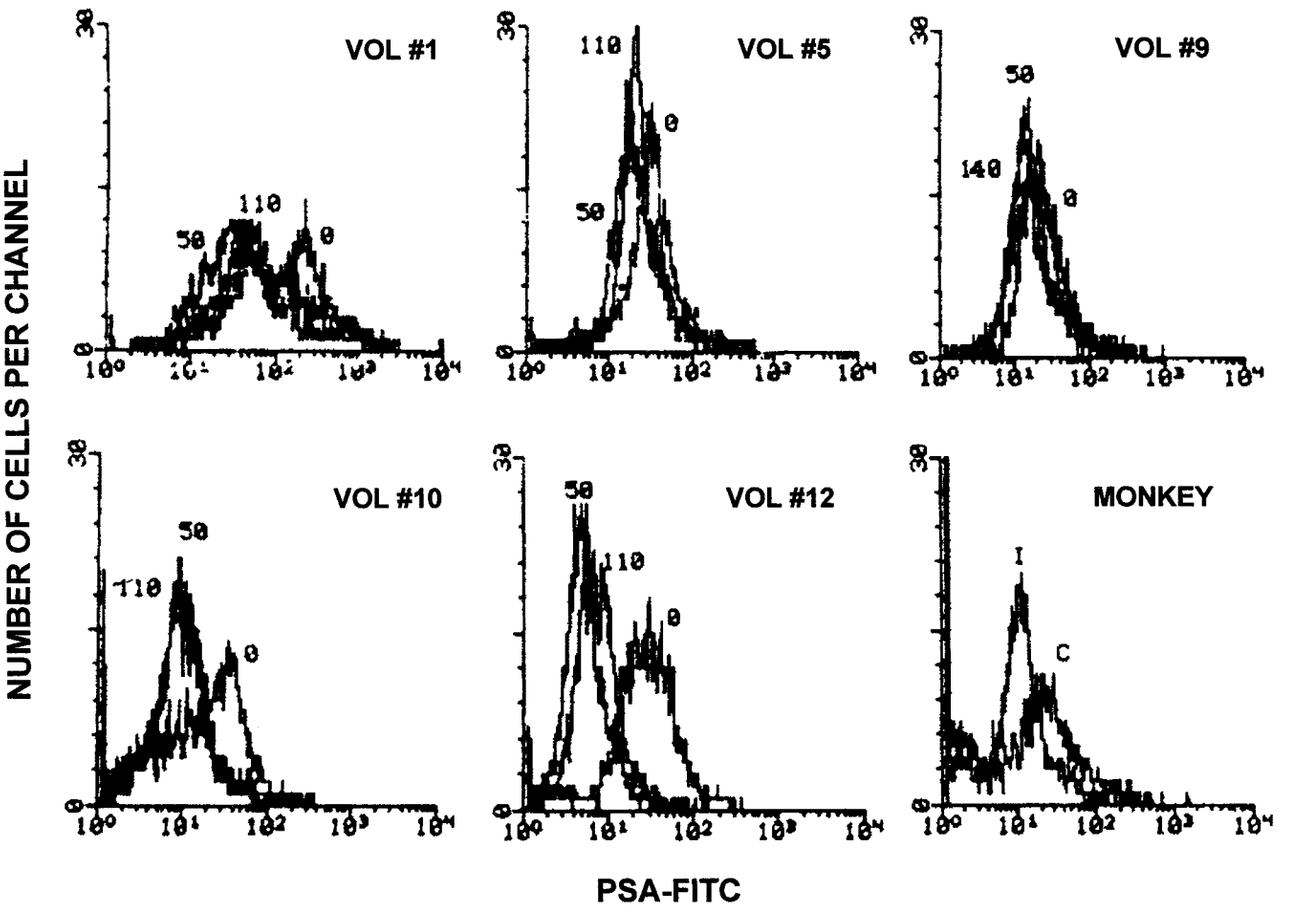

Figure 6. Fluorosceinated pisum sativum agglutinin (PSA-FITC)-binding pattern of sperm obtained from 5 individual volunteer men (volunteer numbers are provided in the upper right-hand corner) on days 0, 50, and 110 of immunization with oFSH. For comparison, representative flow cytograms of the PSA-FITC–binding pattern of sperm from control and oFSH-immunized monkeys also are shown. C indicates control; I, oFSH-immunized monkeys.

caused by a relative increase in the red fluorescence and decrease in the green fluorescence (Evenson and Jost, 1994). In the present study, the percentage of AO-stainable sperm (that exhibiting high green and low red fluorescence) showed a drastic reduction with the progress of immunization reaching a nadir between days 50 and 70 of immunization. However, the percentage of AO-stainable sperm returned to normalcy following a drop in the antibody titer (by days 110–140 of immunization). Sperm samples from the FSH- as well as FSH-RP–immunized

Table 2. *PSA-FITC binding (acrosomal content of glycoproteins) to sperm of follicle stimulating hormone–deprived man and monkey**

Group	Group or Immunization Phase†	Mean Channel of Fluorescence Intensity, mean ± SEM	Change, %
Human (n = 5)	Pretreatment (5)	32.71 ± 2.08	
	Treatment (11)	12.37 ± 1.74 c‡	62
	Posttreatment (6)	15.00 ± 2.45 d	54
Monkey (n = 5)	Control	21.95 ± 0.01	
	oFSH immunized	13.41 ± 0.68 c	39
Monkey (n = 3)	Control	66.30 ± 13.75	
	oFSHR-P immunized	30.33 ± 6.11 a	54

* Results of each set of experiments carried out independently are shown. oFSH indicates ovine follicle-stimulating hormone; oFSHR-P, ovine follicle-stimulating hormone receptor protein fragment.

† The numbers in parentheses indicate the total number of samples obtained from 5 volunteers. *P* values provided are in comparison with respective controls.

‡ The letter a indicates $P < .05$; b, $P < .01$; c, $P < .001$; d, $P < .00001$.

monkeys also exhibited reduced AO stainability. Therefore, it appears from the current study that sperm ejaculated by FSH-immunized men and monkeys are highly susceptible to the acid-detergent– and DTT-induced denaturation of sperm chromatin. In the light of this, any significant increment in the αt values over the day 0 control becomes meaningful. The high αt values exhibited by the spermatozoa of FSH-deprived men and monkeys over their respective controls (Table 1) would then indicate that packaging of chromatin could be poor in both cases because of protamine deficiency or alteration and denatured status of DNA. The significant upward shift in the αt values following FSH immunization cannot be ascribed to reduced DNA stainability, because under such circumstances both red and green fluorescence should reduce proportionally, leading to no change in the ratio. By use of flow cytometry and application of quadrant analysis to determine AO binding pattern of ejaculated sperm, it has been observed that in adult male bonnet monkeys, the percentage of sperm exhibiting low green-high red fluorescence increased significantly ($P < .01$) from 28% of control monkeys to 37% of FSH-immunized monkeys (Aravindan and Moudgal, 1998).

The observation that blockade of FSH action by a specific FSH receptor antibody also leads to production of sperm having poor chromatin packaging (higher αt values) lends credence to our thinking that any method that results in blockade of FSH support in primates significantly affects sperm maturation. At present we cannot rule out the possibility that after exposure to denaturing agents like DTT (>50 mmol/L; Figure 1B) or acid, there is a loss in the DNA of sperm obtained from FSH-deprived monkeys and men. The tertiary and quaternary structure of protamine-complexed sperm DNA is likely to be important for the protection of genetic information and possibly for early genetic events of postfertilization (Ward and Coffy, 1991). Evenson et al (1993) comment that even though fertilization could occur with sperm having high αt value, postfertilization development is terminated prematurely, resulting in early embryonic death. The αt values also have been useful in assessing the fertility rating of bulls (Ballachey et al, 1987, 1988).

The gel electrophoretic pattern of sperm DNA of volunteer men at different times of FSH immunization also revealed definite degradative patterns (Figure 5). This is a clear indication of DNA instability, which might be attributed to improper condensation and chromatin packaging allowing action by DNA degradative processes. Once the immunization effects abated the DNA, as examined on day 110 or 140 (postimmunization phase), it reverted to its stable state in 4 out of 5 men. These data are in complete accord with results of SCSA.

Earlier studies have shown that round spermatids actively synthesize several glycoproteins (Clermont and Tang, 1985; Gerton and Milette, 1986) and some of these (eg, acrosin and hyaluronidase enzymes) are packed into the acrosome. Acrosomal enzymes are believed to have crucial roles in fertilization, and lack (partially/completely) of them could be a cause of infertility (Chulavatnatol, 1990). The PSA-FITC assay we have used is rapid and useful in detecting the loss of acrosomal contents. Use of ethanol-permeabilized sperm in this assay provides, despite disruption in sperm membrane, an index of glycoprotein concentration of acrosome and other cytoplasmic remnants (Cross et al, 1986). The significant decrease in the binding of PSA-FITC to the sperm of FSH-deprived volunteer men and monkeys (following immunization with oFSH and oFSHR-P, Table 2) compared with their respective controls would indicate that the acrosomal content of stainable glycoproteins is reduced in an FSH-deficient state. It is possible that reduction in PSA-FITC binding in FSH-immunized men and monkeys could be caused by the alterations in acrosomal glycosylation and glycoprotein content. This is pertinent because earlier data showed that acrosin and hyaluronidase activities of sperm of FSH-deprived monkeys are significantly reduced (Moudgal et al, 1992) and that the sperm of such animals are incapable of attaching to intact monkey eggs (Raj et al, 1991) and zona-free hamster eggs (Srivastava and Das, 1992).

Based on the severity of effect produced on individual parameters of sperm quality, like acrosomal content of glycoproteins and chromatin status/packaging in FSH-deprived men and monkeys, it would appear that the process of spermiogenesis in the latter is more sensitive to FSH deprivation. Whether men, like monkeys, could be rendered infertile by deprivation of endogenous FSH support for a prolonged period is a question that remains to be answered. Our phase I study (Moudgal et al 1997b) was not designed for this purpose.

In conclusion, the current study has shown, for the first time, that endogenous FSH deprivation in normal men could affect the chromatin packaging and acrosomal glycoprotein concentration of ejaculated sperm, in a manner very similar to that noticed in monkeys lacking FSH support. The specificity of the effect in men is underscored by the fact that it is reversible as restoration to normal FSH levels leads to regain of normal chromatin packaging.

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