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Enhanced Susceptibility of Follicle-Stimulating-Hormone-Deprived Infertile Bonnet Monkey (*Macaca radiata*) Spermatozoa to Dithiothreitol-Induced DNA Decondensation *In Situ*

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ABSTRACT: Immunoneutralization of endogenous follicle-stimulating hormone (FSH) of adult male monkeys leads to oligospermia and infertility despite unchanged testosterone levels. The inability of these monkeys to impregnate despite repeated exposures to cycling females appeared to be due to abnormal alterations in the kinetics of germ cell transformations and deficient spermiogenesis. Here we investigated the stability of sperm chromatin in oFSH-immunized monkeys as a marker for spermiogenesis. The susceptibility of spermatozoa to *in vitro* decondensation induced by dithiothreitol (DTT, 0.05–50 mM) was studied by measuring the nuclear fluorescence of DTT-treated, ethidium bromide (EB)-stained sperm using flow cytometry. Changes in sperm morphology and binding of thiol-specific ^{14}C-iodoacetamide (^{14}C-IA) were also monitored under the same conditions. Sperm from the immunized monkeys decondensed at a lower concentration of DTT, bound more EB, and decondensed more extensively than those from control animals. The difference was apparent in sperm from all regions of the epididymis. Immunized monkey sperm also bound significantly more ^{14}C-IA at all concentrations of DTT. Overall, the effective concentration of DTT required to elicit 50% of maximal decondensation (ED_{50}) of epididymal and ejaculated sperm was significantly lower for the immunized monkeys than even the caput sperm of controls. These results suggest that FSH deprivation in monkeys results in production of sperm with limited potential for disulfide formation and reduced chromatin stability.

Key words: Flow cytometry, chromatin, primate, compaction, epididymis.

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Our earlier studies of adult male bonnet monkeys, using both a passive immunization approach with a precharacterized follicle-stimulating hormone (FSH) antibody (Sheela Rani et al, 1978; Murty et al, 1979) and an active immunization approach with an oFSH vaccine (Moudgal et al, 1992; Aravindan et al, 1993), have clearly shown that FSH deprivation leads to oligospermia and infertility and that this is not accompanied by any change in serum testosterone (*T*) levels. That oligospermia obtained following FSH deprivation is indeed compatible with infertility was shown by demonstrating that the quality of spermatozoa ejaculated, i.e., viability, motility, gel-penetrability, acrosin and hyaluronidase activities, and ability to bind/fertilize homologous monkey as well as zona denuded hamster oocytes, were significantly reduced (Moudgal et al, 1992; Sharma and Das, 1992; Aravindan et al, 1993). The poor quality of these spermatozoa, together with observations that FSH immunization alters the kinetics of testicular germ cell transformations during spermiogenesis (Aravindan et al, 1993), suggested that FSH deprivation has, in some currently undetermined manner, adversely affected the production of mature sperm that are capable of fertilization.

During mammalian spermiogenesis, highly basic protamines that are rich in arginine and cysteine replace the somatic histones in the spermatid nuclear chromatin and the numerous free sulfhydryls present within are progressively oxidized to form inter- as well as intraprotamine disulfide bridges as a function of spermiogenic development and epididymal maturation (Bellve and O'Brien, 1983; Leiva et al, 1994; Rousseaux and Rousseaux-Prevost, 1995). These changes impart a high degree of stability to the mammalian sperm nuclei, shielding them from exogenous influences (Evenson et al, 1993) and rendering their DNA transcriptionally inert (Bellve, 1979; Poccia, 1986; Perreault et al, 1988b) as well as structurally compact (Meistrich, 1989). Elongate spermatids, due to their compact chromatin, bind significantly less nucleic-acid-specific fluorochromes than round spermatids despite possessing the same amount of DNA (Clausen et

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al, 1978, 1979). Thus, when analyzed flow-cytometrically on the basis of emitted fluorescence intensity and apparent DNA content, these two populations of cells resolve into two separate peaks, facilitating their relative quantitation (Clausen et al, 1978, 1979; Clausen and Abyholm, 1980; Aravindan et al, 1990, 1993; Suresh et al, 1992). Treatment of elongate spermatids with agents that reduce chromatin disulfides has been shown to negate these differences in fluorescence-binding properties between these two populations of spermatids causing the merger of the two flow-cytometric peaks into one (Zante et al, 1977). Ejaculated sperm, having undergone not only the nuclear protein transitions and chromatin condensation steps of spermiogenesis, but also the subsequent epididymal maturation processes, understandably exhibit even greater reluctance to bind DNA-specific dyes (Hettwer et al, 1986; Evenson and Darzynkiewicz, 1990). Disulfide reduction *in vitro* and consequent decondensation of sperm nuclear chromatin are known to result in increases in nuclear size and fluorochrome bindability (Zante et al, 1977; Aravindan et al, 1995). The readiness to decondense on exposure to reducing agents *in vitro* is known to be a function of the nature or quantum of protamines and disulfide bridges present within the chromatin (Calvin and Bedford, 1971; Saowaros and Panyim, 1979; Sanchez-Vazquez et al, 1996). The current study was designed to determine whether FSH deprivation affects sperm chromatin stability by analyzing the nuclear chromatin status of epididymal and ejaculated spermatozoa from control and FSH-immunized monkeys. As a prelude to undertaking these investigations, the methodologies were thoroughly standardized using epididymal sperm from untreated adult rats (Aravindan et al, 1995).

Materials and Methods

Animals and General Methods

Eight- to 10-year-old adult male monkeys recruited for this study were healthy and proven fertile, with normal diurnal serum testosterone rhythms and sperm counts in seminal ejaculates (Mukku et al, 1981). The animal management and husbandry practices followed in the colony have been described earlier (Moudgal et al, 1985; Ravindranath and Moudgal, 1987). The procedures employed for immunization with oFSH vaccine, antibody titration, and characterization for cross-reactivity, bioneutralization ability and effects on serum testosterone levels, kinetics of testicular germ cell transformations, quality and quantity of spermatozoa ejaculated, and fertility status have been published elsewhere in detail (Aravindan et al, 1990, 1993; Moudgal et al, 1992).

Semen Collection and Processing

Semen was collected, along with coagulum, into dry 5-ml test tubes using a penile electrostimulator according to procedures described earlier (Mastroianni and Manson, 1963) and liquefied at 37°C for 20 minutes in a water bath. The dithiothreitol (DTT) dose-dependent decondensation of ejaculated sperm was performed using sperm obtained from three consecutive seminal ejaculates—spaced 15 days between ejaculations—from three control (nine ejaculates) and five oFSH-immunized (15 ejaculates) monkeys. Each of the ejaculated sperm samples was treated as a separate sample and not pooled. In addition to the dose-dependency experiments, a large number of semen samples obtained from six control monkeys (48 independent ejaculates) and five immunized oligospermic monkeys (70 individual ejaculates) were subjected to decondensation at a fixed concentration of DTT (10 mM) in order to ascertain consistency of effects within a large sample pool. Ejaculated spermatozoa from control and immunized monkeys were separated from semen by centrifugation (800 × *g*, 8 minutes) followed by two consecutive washes (800 × *g*, 5 minutes) with Dulbecco's phosphate-buffered saline (DPBS, pH 7.2; Himedia, Bombay, India). The final pellet containing the spermatozoa was finally reconstituted in 0.5 ml of DPBS, fixed in chilled 90% ethanol, and stored at 4°C. For objectivity, all flow-cytometric analyses on these sperm samples were performed on the same day under identical conditions. The results obtained were averaged and statistically analyzed as described below.

Preparation of Epididymal Sperm

Epididymes from 5 control and 10 oFSH-immunized monkeys that were euthanatized for an independent investigation on possible long-term immunotoxicological effects related to oFSH immunization (Sehgal et al, 1991) were collected immediately following euthanasia, and the caput (CAP), corpus (COR), and cauda (CAU) regions were separated (Meistrich et al, 1976) and transferred immediately into separate sterile petri dishes containing DPBS in ice for recovery of epididymal sperm. Each of the epididymal regions was thoroughly minced in separate petri dishes using sterile surgical blades in 5.0 ml of DPBS, and the supernatants from each sample were collected separately. The minced tissue was again washed with an additional 5.0 ml DPBS to maximize sperm recovery, and the respective supernatants from each epididymal region of each monkey were individually pooled, filtered through 50-μm nylon mesh (Swiss Silk, Zurich, Switzerland), and centrifuged at 800 × *g* for 5 minutes, and the resultant pellet was washed twice with DPBS (5 ml each), reconstituted in 0.5 ml DPBS, counted, and finally fixed in 90% chilled ethanol for storage at 4°C until analysis. Preliminary experiments on decondensation patterns of epididymal sperm from both groups within individual epididymal region revealed no differences between the two testes of each monkey.

Decondensation of Sperm Nuclei

The procedures used for the *in vitro* decondensation of spermatozoa from the ejaculate as well as from each of the regions of the epididymes are identical to those used in our earlier study on rat sperm (Aravindan et al, 1995) except for the doses of DTT used. About 2.5 × 10^6 sperm from the previously counted ejaculated and epididymal sperm samples in ethanol were aliquoted and separately centrifuged (800 × *g*, 5 minutes), and the resultant pellets were rehydrated in 1 ml of DPBS for 20 minutes at room temperature, vortexed for 30 seconds to dissociate

clumps, and counted again. From each of the sample tubes, 2×10^6 sperm were further aliquoted (volume made up to 1 ml using DPBS) and centrifuged again ($800 \times g$, 5 minutes), and the sperm pellet was reconstituted in 0.5 ml of 0.5% pepsin (Serrafein Biochemica, Heidelberg, Germany) in DPBS (pH 2.0) and incubated at 37°C for 1 hour. At the end of pepsin treatment, the cells were washed with 20 mM Tris-HCl (Himedia, Bombay; pH 7.2; $800 \times g$, 5 minutes) once and reconstituted in 0.5 ml solution of papain (0.01% in Tris-HCl, pH 7.2) and incubated for 5 min at 37°C. The working reagent of papain was freshly prepared by first making a 1% solution of adsorbed papain in 1 ml of 20 mM Tris-HCl, vortexing for 1 minute, and filtering through a single layer of tissue paper. The filtrate was then appropriately diluted with Tris-HCl to obtain the 0.01% working reagent of papain. At the end of incubation with papain, 300 µl of Tris-HCl was added, and the cell suspension was divided into eight equal aliquots of 100 µl each (volume made up to 500 µl in each tube) and centrifuged. The resulting cell pellet, containing 2.5×10^5 sperm in each tube, was reconstituted in 0, 0.05, 0.1, 0.5, 1, 5, 10, or 50 mM solution of DTT (0.5 ml, Sigma Chemical Co., St. Louis, Missouri) in Tris-HCl (20 mM, pH 7.2) and incubated at 37°C for 1 hour in the dark. Preliminary experiments using a variety of decondensation agents including sodium dodecyl sulfate indicated that monkey sperm chromatin decondensed maximally and most consistently with a combination of papain and DTT.

Flow Cytometry and Microscopy of Sperm Following Decondensation

Although the changes in forward scatter (FSc; cell size) and side scatter (SSc; cell "granularity") were also measured as indicators of induced nuclear decondensation and remained consistent with our earlier report (Aravindan et al, 1995), the data are deemed not within the scope of this report and hence are not presented. The flow cytometry of ethidium bromide (EB)-stained sperm decondensed with DTT was conducted as described in detail earlier (Aravindan et al, 1995). Briefly, following DTT treatment, the decondensed sperm pellet ($800 \times g$, 5 minutes) was directly reconstituted in 0.5 ml of a staining solution (25 µg EB/ml, 40 µg RNase/ml, 0.3% Nonidet P-40; Sigma Chemical Company, St. Louis, Missouri) and incubated for 20 minutes in the dark at 4°C. Changes in nuclear size and morphology of the EB-stained sperm were studied at the end of incubation using a Carl Zeiss fluorescence microscope. The intensity of fluorescence emission at 567–607 nm (FL2 threshold) wavelength was quantitated using a FACScan flow cytometer (Becton Dickinson, San Jose, California) equipped with an argon ion laser exciting at a fixed 488 nm. The data were acquired in list mode using the Consort-30 data acquisition program. For each sample, 10,000 cells were acquired and analyzed individually. The intensities of EB fluorescence emitted by both undecondensed and fully decondensed (possessing DNA equivalent to round spermatids) sperm were standardized using both human peripheral blood leukocytes and monkey total testicular germ cells to identify flow-cytometric peak positions (Aravindan et al, 1990, 1995). The channel number representing maximal staining intensity of normal monkey sperm in the absence of DTT was kept constant at about 55 by manipulating the photomultiplier voltages of the flow cytometer in order to maintain consistency throughout these studies and to facilitate comparisons. All samples belonging to each experiment were analyzed on the same day in a single-instrument run that was repeated at least three times subsequently. The channel of maximum cell concentration (CMC), representing the fluorescence intensity exhibited by the maximum number of cells within each sample, was arrived at by placing digital markers at the channel of maximum histogram peak height. The heterogeneity of responses of sperm within each sample to any given concentration of DTT was represented by cells exhibiting differential fluorescence intensities and was studied by quantitating them within two equidistant zones along the x-axis of the flow cytograms, i.e., within channels 1–128 and 129–256. The effective concentration of DTT required to elicit 50% of maximal decondensation (ED_{50}) was calculated for each of the parameters studied from respective dose–response curves.

^{14}C-Iodoacetamide Uptake by Sperm Nuclei

The ability of the radiolabeled sulphydryl-blocking agent ^{14}C-Iodoacetamide (^{14}C-IA) to successfully bind free sulphydryls liberated by DTT-induced reduction of disulfides within nucleoproteins of sperm nuclei was studied with a view to establishing its relationship with observed changes in EB uptake and morphology. The procedure followed for the ^{14}C-IA uptake studies was identical to that published earlier (Aravindan et al, 1995) except that the concentrations of DTT used in the current studies were 0.01, 0.05, 0.1, 0.2, 0.5, and 2.5 mM. At least 12 separate experiments were performed with identical results, except for differences in baseline values, and results from representative experiments are presented. Although concentrations of DTT above 2.5 mM (the maximum concentration used in ^{14}C-IA-binding studies) would be expected to potentially reduce greater amounts of disulfides, we observed that the net specific ^{14}C-IA bound to plateau at this concentration, since residual DTT tended to directly reduce the radiolabeled IA (Aravindan et al, 1995). Excessive repeat washings of cells following DTT treatment were not favored, other than the two successive washings with 90% ethanol and Tris-HCl (pH 7.2) employed in the current study, in order to optimize cell recovery and to minimize differential cell loss. For each experiment, the specificity of the DTT effect was studied by monitoring the ability of 500 µM unlabeled IA to competitively bind free thiols and displace specific ^{14}C-IA binding.

Statistics

The significance of differences among different sperm populations for all parameters at any given concentration of DTT treatment was calculated using Student's *t*-test.

Results

DTT-Induced Changes in the Morphology of Control and Immunized Monkey Spermatozoa

The decondensation patterns signified by changes in nuclear size and morphology of control (left panel) and oFSH-immunized-monkey-ejaculated sperm nuclei (right

panel) treated with varying concentrations of DTT and visualized at the same magnification (×400) by fluorescence microscopy of EB-bound DNA are presented in Figure 1. Even though differences in cell size were not apparent at DTT concentrations of 0.05–0.1 mM (Fig. 1A,B), and only marginal difference in nuclear size was apparent at 1 mM DTT (Fig. 1C,D), significant differences between control and immunized monkey sperm in cell size and in the amount of EB bound were clearly appreciable at all DTT concentrations between 5 (Fig. 1E,F) and 50 mM (Fig. 1G,H). At 50 mM DTT, the immunized monkey nuclear chromatin appeared to disassemble and externalize beyond its nuclear boundaries (Fig. 1H), while the control monkey sperm nuclei had maintained their integrity (Fig. 1G), having neither decondensed nor bound EB in equivalent extents.

Flow Cytometry of Decondensed Ejaculated Spermatozoa

The representative DNA flow-cytometric profiles of normal and oFSH-immunized-monkey-ejaculated sperm following exposure to varying concentrations of DTT are presented in Figure 2. Spermatozoa from both control and immunized monkeys exhibited very low fluorescence in the absence of DTT treatment. However, the progressive dose-dependent increase in EB bindability following treatment with 0.05, 0.1, 0.5, 1, 5, 10, and 50 mM DTT visualized by a shift in the histogram patterns was markedly higher for the oFSH-immunized monkey sperm than for those of controls (Fig. 2B). Increased EB bindability was detected in control monkey sperm only beyond 1 mM DTT, and the extent of increase was consistently lower than that for the immunized monkey sperm at any given concentration of DTT. The height of the histograms tended to decrease with increase in DTT due to increased heterogeneity of responses of cells, resulting in greater distribution in a horizontal plane. The channel of maximum cell concentration (CMC), representing the extent to which the majority of sperm from each semen sample had decondensed (Fig. 3), indicated that normal monkey sperm tended to decondense only at concentrations beyond 1 mM DTT, and the uptake of EB progressively increased with increasing DTT without achieving complete decondensation even at 50 mM DTT. Immunized monkey sperm, on the other hand, exhibited significant decondensation and greater CMC values ($P < 0.001$; Fig. 3) at DTT concentrations as low as 0.05 mM, exhibiting significantly greater EB bindability at all concentrations studied ($P < 0.001$), compared to corresponding controls. The flow-cytometric results were consistent with the morphological observations.

Comparison of Epididymal and Ejaculated Sperm Decondensation Patterns

In order to determine if the observed patterns of differential decondensation of normal and oFSH-immunized monkey sperm are indeed related to the extent of condensation of their nuclear chromatin, spermatozoa collected from the caput (CAP), corpus (COR), and cauda (CAU) epididymal regions from 5 normal and 10 immunized monkeys were subjected to *in vitro* decondensation and flow cytometry. The flow cytograms from the three regions from each of the monkeys were overlaid, and the results were compared between groups (Fig. 4). The three epididymal sperm populations of controls (Fig. 4, left panel) and immunized monkeys (Fig. 4, right panel) exhibited progressive decondensation and enhanced EB uptake as a function of increasing DTT concentration. For any given concentration of DTT, the CAP sperm exhibited the highest EB uptake and the CAU sperm the lowest, the COR sperm being positioned in between the other two, indicating that decrease in EB binding is a function of chromatin compaction. In preliminary experiments, testicular elongate spermatids closely followed this pattern of decondensation exhibiting the greatest susceptibility to DTT among all these cell populations. Especially beyond 0.1 mM DTT, CMC values of each of the three epididymal sperm populations from the immunized monkeys not only stayed significantly ($P < 0.001$) higher than those of the corresponding epididymal sperm population of control monkeys, but also tended to remain higher than even those of the caput sperm of controls ($P > 0.01$–0.001; Fig. 5).

Uptake of [^{14}C]-iodoacetamide

The pattern of uptake of radiolabeled thiol-specific iodoacetamide ([^{14}C]-IA) by control and oFSH-immunized-monkey-ejaculated sperm before and after exposure to different concentrations of DTT was studied in order to ascertain the relationship between EB bindability and disulfide content of chromatin, and the results are presented in Figure 6. The nonspecific binding of [^{14}C]-IA in the presence of excess unlabeled IA (500 μM) was very low (133 ± 21 cpm) and did not vary between control and immunized monkey sperm irrespective of concentration of DTT. In the absence of DTT, the net specific binding of [^{14}C]-IA was very low and remained almost indistinguishable from nonspecific binding for both control and immunized monkey sperm. At all concentrations of DTT, the sperm from immunized monkeys bound significantly greater amounts of [^{14}C]-IA than did those of controls ($P < 0.001$). The control monkey sperm bound very little [^{14}C]-IA, except at 2.5 mM DTT ($P < 0.01$). These results indicated that the chromatin of oFSH-immunized monkeys contained significantly higher amounts of free thiols at all concentrations of DTT used and the rate of formation of -SH- groups increased as the concentration of DTT was increased.

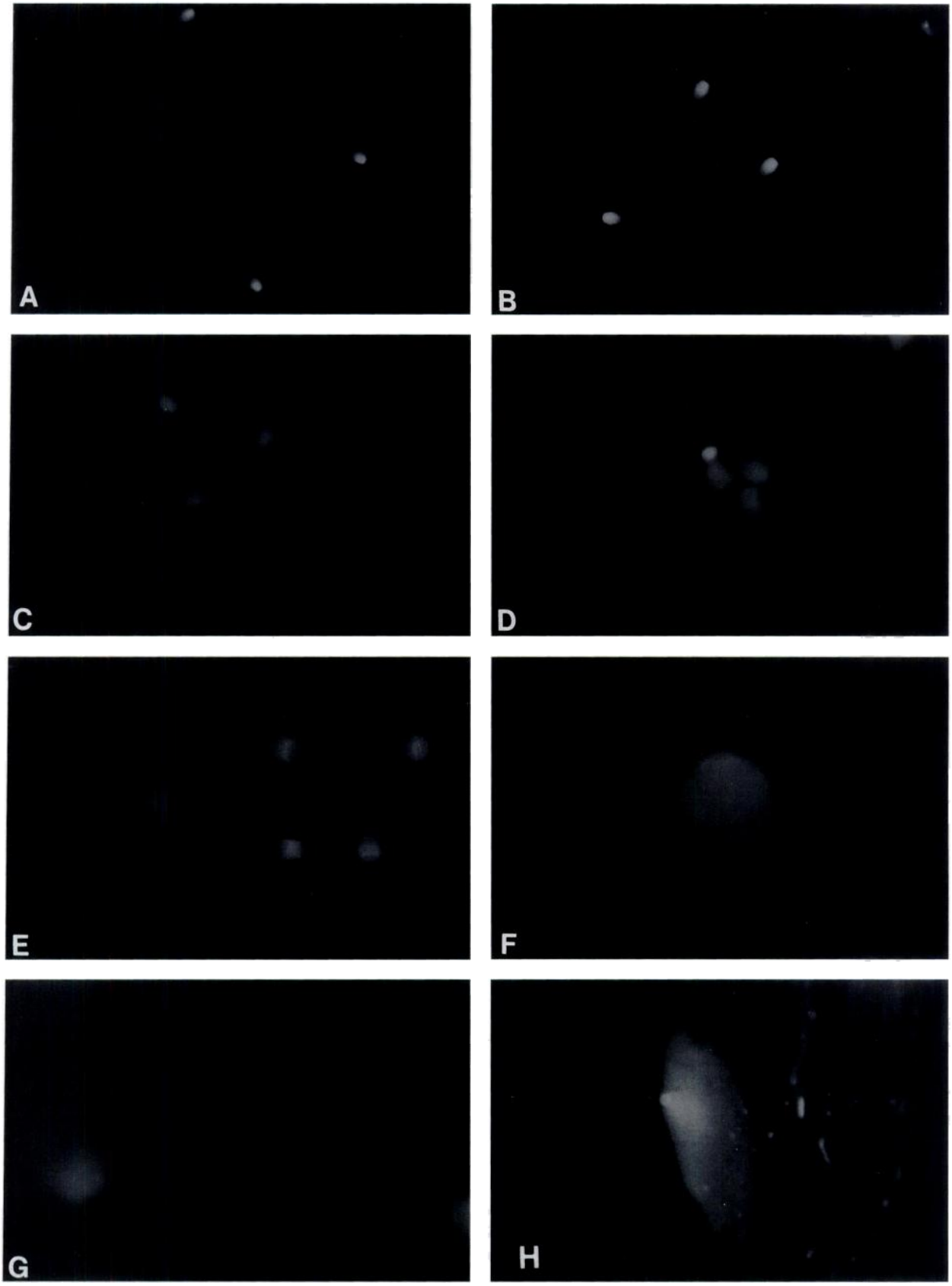

FIG. 1. Photomicrographs (×400) of control (left panel) and oFSH-immunized (right panel) monkey spermatozoa subjected to DNA decondensation *in situ* using 0.1- **(A, B)**, 1.0- **(C, D)**, 5.0- **(E, F)**, and 50-mM **(G, H)** concentrations of dithiothreitol (DTT) and stained with ethidium bromide (EB).

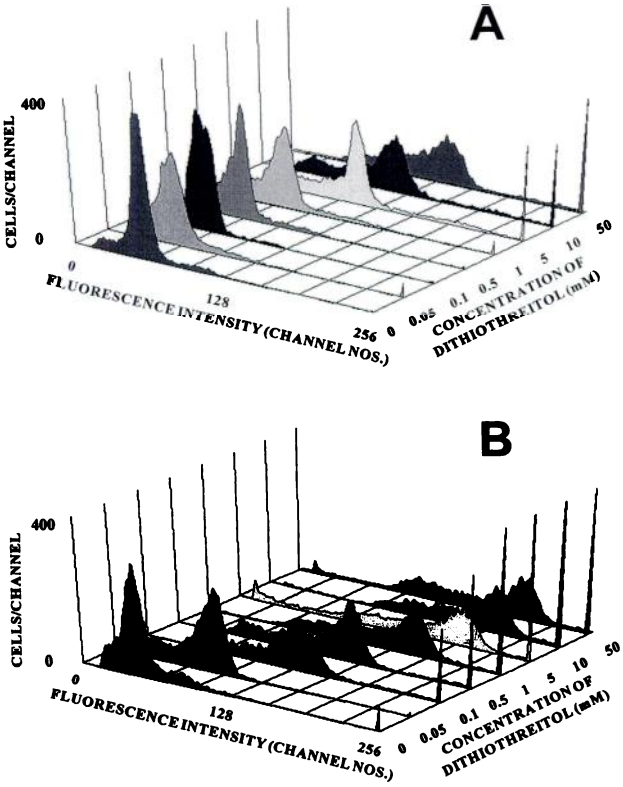

FIG. 2. Two-dimensional overlays of representative flowcytograms of (A) control and (B) oFSH-immunized-monkey-ejaculated spermatozoa subjected to different concentrations of DTT *in vitro*. Bindability of EB fluorescence as a function of DTT concentration was observed to be more pronounced for the sperm from the immunized monkeys than for those from the controls.

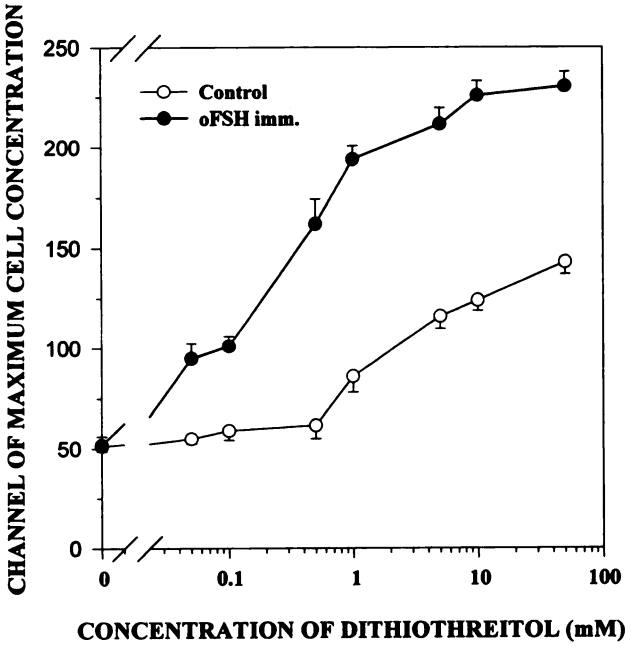

FIG. 3. The channel of maximum cell concentration (CMC) of control and oFSH-immunized monkey sperm following exposure to different concentrations of DTT were quantitated by identifying the x-axis channel fluorescence intensities of histogram peaks. For each group, in the absence of DTT, the CMC values were the lowest, and at 50 mM DTT concentration they were the highest. At any given concentration of DTT, the CMC values were significantly ($P < 0.001$) greater for the immunized monkey spermatozoa than for those of the controls.

Decondensation of Ejaculated Sperm at 10 mM DTT

In order to establish a concentration of DTT that is sensitive to differences in the extent of chromatin condensation between control and oFSH-immunized monkeys, a large number of ejaculated semen samples from both groups were subjected to decondensation at 10 mM DTT concentration, arrived at from the results of the dose-dependency experiments (Fig. 7). Also, due to the heterogeneity of response of ejaculated sperm to DTT evidenced by the broadening of the histogram peaks (Fig. 2), the numbers of sperm in channels 0–128 and 129–256 were quantitated by placing equidistant digital markers. The oFSH-immunized monkeys were clearly oligospermic, the average sperm counts in their ejaculates being 39 ± 19 (Fig. 7A). The CMC values (Fig. 7B) as well as the percentage of cell populations of the two groups falling within the two fluorescence intensity ranges of 0–128 and 129–256 (Fig. 7C) were significantly different between the two groups ($P < 0.001$). These results indicated that flow cytometric evaluation of *in situ* decondensation using 10 mM DTT is a reliable index of the state of the chromatin.

ED_{50} of Monkey Sperm Chromatin Susceptibility to DTT

The DTT concentration required to elicit 50% of the maximal effect was calculated for each of the parameters studied, and the results are presented in Table 1. The maximum effect observed for each of the parameters irrespective of treatment was considered to be 100% for calculating ED_{50}. The ED_{50} values for the oFSH immunized monkey sperm were found to be significantly different from those of controls for all parameters without exception ($P < 0.001$). The ED_{50} values also significantly differed among parameters within each treatment group ($P < 0.01$–0.001). However, within each treatment group and within each parameter tabulated, the ED_{50} values were consistently highest for the ejaculated sperm, followed sequentially by the CAU, COR, and CAP spermatozoa, clearly delineating the relative extent of compaction of their chromatin. The ED_{50} of DTT concentration required to facilitate ^{14}C-IA binding by the reduction of disulfides was dramatically lower for the immunized monkey sperm than for those of controls.

Discussion

The objective of this study was to investigate abnormalities in the nuclear chromatin of sperm from FSH-de-

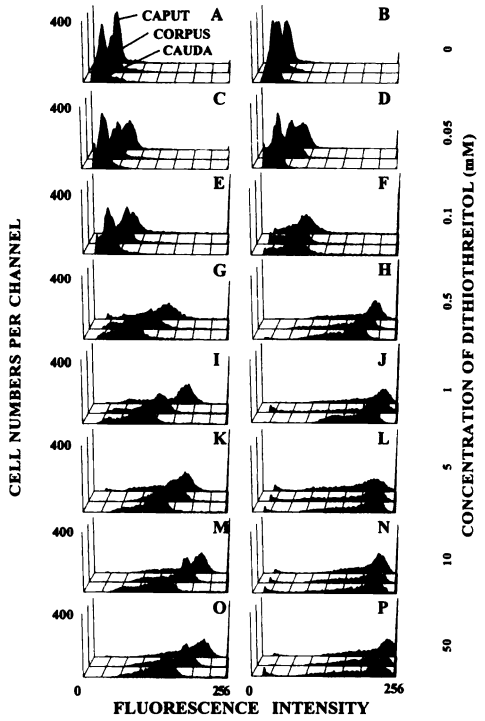

FIG. 4. Two-dimensional overlays of representative flow cytograms of control (left panel) and oFSH-immunized (right panel) monkey epididymal spermatozoa treated with varying concentrations of DTT. Within each group, the EB fluorescence uptake was consistently greatest for the CAP sperm and least for the CAU sperm, with those from COR positioned in between. With increasing DTT concentrations, increases in EB uptake were noted by gradual shifting of the histograms to the channels of greater fluorescence intensity. Beyond 0.5 mM DTT, the extent of shift in EB fluorescence uptake was consistently greater for the FSH-deprived group than for corresponding controls.

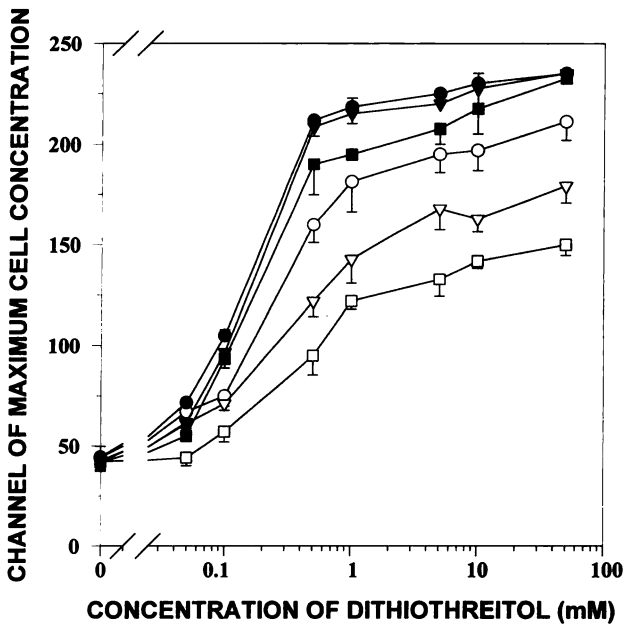

FIG. 5. The channel of maximum cell concentration (CMC) of each of the epididymal sperm populations of control and oFSH-immunized monkeys was quantitated as a function of DTT concentration. At any given concentration of DTT greater than 0.5 mM, the CAU (—□—), COR (—▽—) and CAP (—○—) sperm of controls were significantly different from each other ($P < 0.001$), with the CAP sperm exhibiting the highest CMC values, the CAU sperm exhibiting the lowest values, and the COR sperm staying in between the two. The CAU (—■—), COR (—▼—), and CAP (—●—) sperm of oFSH-immunized monkeys exhibited CMC values greater than even those of CAP sperm of controls at any given concentration of DTT and were found to be significantly higher than the respective values exhibited by control monkey spermatozoa.

prived monkeys as a marker for spermiogenesis. This has been achieved by showing by a variety of criteria that distinct differences exist in the thiol status of chromatin of sperm ejaculated by control and FSH-deprived monkeys. Most of the earlier investigations on chromatin stability of mammalian spermatozoa have employed monitoring of the time course of susceptibility of sperm to decondensation in the presence of disulfide-reducing agents, either alone (Lung, 1972; Marushige and Marushige, 1975a, 1978; Wagner et al, 1978; Incharoensakdi and Panyim, 1981), in combination with proteases (Zirkin et al, 1980), and salts (Rodman et al, 1982; Zirkin et al, 1982), or by microinjecting spermatozoa into oocytes (Perreault et al, 1984, 1987, 1988a; Yanagida et al, 1991). Maintaining a fixed time schedule but varying the concentration of DTT has enabled us to demonstrate that FSH-deprived monkey spermatozoa are significantly more vulnerable to DTT-induced decondensation than those of corresponding controls. Analyses of a large number of semen samples from normal and clearly infertile oligospermic immunized monkeys after decondensation specifically at 10 mM DTT, revealed significant differ-

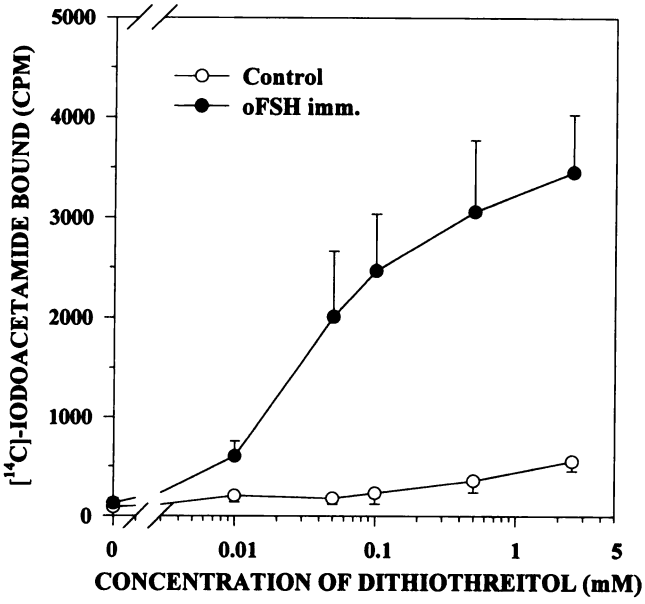

FIG. 6. Net specific binding of [^{14}C]-iodoacetamide to control (n = 4, ejaculates = 12) and oFSH-immunized (n = 4, ejaculates = 12) monkey sperm following treatment with varying concentrations of DTT.

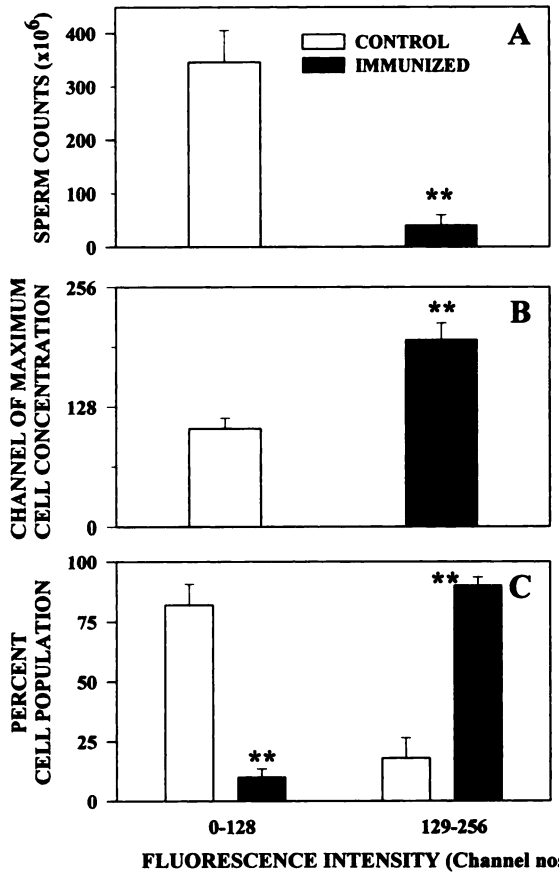

FIG. 7. Spermatozoa from a large number of semen samples from both control (n = 48) and oFSH-immunized (n = 70) monkeys exhibiting varying numbers of sperm in their ejaculates **(A)** were subjected to decondensation at 10-mM concentrations of DTT, and the channel of maximum cell concentration **(B)** as well as the percentages of cells accumulating varying degrees of fluorescence at channels 0–128 and 129–256 **(C)** were quantitated. ** indicates $P < 0.001$.

ences between the two groups, indicating that this concentration can serve as a dose that is both sensitive and practical for routine semen analyses. Susceptibilities of chromatin of sperm from the two groups of monkeys to acid-induced denaturation *in vitro* followed by binding of acridine orange have been strikingly similar (Aravindan, unpublished data).

The dramatically pronounced effect of disulfide reduction in the FSH-deprived monkey sperm, assayed using a variety of parameters, suggested that these sperm are significantly less stabilized by disulfide-induced chromatin compaction than are those of controls. Under the current experimental conditions, it appears that the amount of DTT-reducible disulfide groups in normal sperm is relatively small compared to those of FSH-deprived monkeys. If one were to assume that chromatin compaction normally leads to diminution in the availability of disulfide groups for reduction by DTT, this would suggest that FSH deprivation results in poor or defective disulfide-induced compaction. Since the potential for free sulfhydryl oxidation and nuclear compaction is determined by the nuclear protein transitions occurring during spermiogenesis (Seligman and Shalgi, 1991), and the ability of sperm chromatin to decondense and form free thiols in response to reducing agents is a function of the initial presence of chromatin-stabilizing factors, especially the inter- and intraprotamine disulfide linkages (Lung, 1972), the results of the current study suggests deficient spermiogenesis due to lack of FSH.

Results of studies on epididymal sperm of normal and immunized monkeys support our contention that lack of FSH affects spermiogenesis and not epididymal maturation per se. In mammals, progressive oxidation of sulfhydryls between and within protamine molecules of sperm chromatin takes place not only during spermiogenesis, but also

Table 1. *Effective concentration of DTT required to elicit 50% of maximal decondensation of control and oFSH-immunized monkey sperm studied using various parameters (ED_{50})*

Parameter	Sperm population	ED_{50} of DTT effect on sperm	
		Control	Immunized
% Cells in channels 0–192	Ejaculated	3.4 ± 0.31	0.06 ± 0.001*
	CAU	0.33 ± 0.002	0.05 ± 0.001*
	COR	0.1 ± 0.002	0.033 ± 0.001*
	CAP	0.06 ± 0.001	0.024 ± 0.002*
% Cells in channels 193–256	Ejaculated	>50 ± 0	8.55 ± 0.61*
	CAU	>50 ± 0	1.0 ± 0.23*
	COR	>50 ± 0	0.42 ± 0.009*
	CAP	9.8 ± 0.73	0.31 ± 0.002*
Channel of maximum cell concentration (CMC)	Ejaculated	4.6 ± 0.41	0.13 ± 0.001*
	CAU	0.76 ± 0.003	0.13 ± 0.001*
	COR	0.37 ± 0.003	0.12 ± 0.001*
	CAP	0.19 ± 0.001	0.1 ± 0.001*
[^{14}C]-IA binding	Ejaculated	>2.5 ± 0	0.033 ± 0.001*

* Significantly different from corresponding controls ($P < 0.001$).

during migration from caput to cauda epididymidis (Calvin et al, 1973; Marushige and Marushige, 1975b; Balhorn, 1982; Zirkin et al, 1985; Rousseaux and Rousseaux-Prevost, 1995). In the light of observations that even spermatozoa forced to remain in the caput following caput ligation acquire a significant degree of protamine thiol oxidation and manage to gain partial fertilizing ability (Seligman et al, 1992), the failure of oFSH-immunized monkey sperm to achieve nuclear compaction even equivalent to caput sperm of normal monkeys despite unhindered epididymal transit indicated not so much an impaired epididymal maturation as a distinctly reduced potential for disulfide formation, suggestive of defective spermiogenesis. Since thiol oxidation is a prerequisite not only for the characteristic morphological shaping of the head (Ford and Huggins, 1963), but also for the acquisition of progressive motility (Cornwall et al, 1988; Pasteur et al, 1992), our earlier observation that FSH deprivation results in reduced sperm motility (Moudgal et al, 1992) supports our present conclusions. Abnormal persistence of histones (Haidl and Schill, 1994) as well as acquisition of variable ratio of protamines (Lung, 1972; Sobhon et al, 1981; Perreault et al, 1987) specifically due to deficient spermiogenesis have been implicated in the reduced capacity of spermatozoa in forming -S-S- bridges. It is possible that abnormal alterations in the kinetics of germ cell transformations during spermiogenesis induced by lack of FSH (Aravindan et al, 1993; Moudgal and Aravindan, 1993) may have resulted in incomplete nuclear protamine transitions during spermiogenesis, leading to impaired chromatin thiol oxidation. Although normal uncompacted spermatocytes (Kimura and Yanagimachi, 1995b) as well as round and elongate spermatids (Kimura and Yanagimachi, 1995a), when microinjected directly into oocytes, thereby bypassing the intrauterine sperm selection processes, are indeed capable of fertilization, abnormal nuclear chromatin condensation caused by defective spermiogenesis has been correlated to infertility in humans (Bedford et al, 1973; Bedford, 1983; Meistrich, 1989; Haidl and Schill, 1994). The current studies suggest a role for FSH in the qualitative maintenance of spermatogenesis as well as spermiogenesis in the adult male bonnet monkey apart from its well-known critical need in quantitative maintenance of spermatogenesis (Moudgal, 1981; Srinath et al, 1983; Matsumoto et al, 1986; Van Alphen et al, 1988; Aravindan et al, 1993; Marshall et al, 1995).

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