Article

Evaluation of relative role of LH and FSH in restoration of spermatogenesis using ethanedimethylsulphonate-treated adult rats

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Abstract

Spermatogenesis is a complex process, and previous studies have clearly established the role for testosterone in its maintenance. However, the role of FSH remains controversial, although several lines of evidence suggest its importance in initiation of spermatogenesis. In the present study, the relative roles of FSH and LH have been evaluated using specific antisera capable of neutralizing endogenous hormones in adult male rats following ethanedimethylsulphonate (EDS) treatment. Restoration of spermatogenesis in EDS-treated rats was monitored following FSH or LH deprivation by histological analysis and flow cytometry. Deprivation of FSH resulted in a reduction of seminiferous tubule diameter and spermatogonial number, which was much more drastic than that observed following LH deprivation. More importantly, FSH deprivation was associated with a significant reduction in the number of pachytene spermatocytes. These results provide evidence for a definite role for FSH in regulation of spermatogenesis, in addition to confirming the role of LH in spermatogenesis via testosterone.

Keywords: *ethanedimethylsulphonate, FSH, LH, spermatogenesis*

Introduction

FSH and LH are principal regulators of testicular function. LH acts on Leydig cells to stimulate testosterone production, and testosterone is known to act on various cell types in testis including Sertoli cells. Sertoli cells are the target for FSH action, and they produce number of factors which can modulate Leydig cell and germ cell function. It is well documented that testosterone is indispensable for maintenance of normal spermatogenesis, and available evidence suggests that testosterone exerts its effects on spermatogenesis at a specific stage (stage VII) of the spermatogenic cycle by regulation of protein synthesis (Sharpe *et al*., 1992). FSH signalling is considered to be important for pubertal initiation of spermatogenesis, and its maintenance in adults rat (Sharpe, 1994). This function is thought to be exerted through the close association between Sertoli cells and germ cells. While the role of testosterone in maintenance of spermatogenesis is well accepted, the precise role of FSH in male reproduction stills remains a matter of debate (Sharpe, 1989; Moudgal and Aravindan, 1993; Zirkin *et al*., 1994; Kumar *et al*., 1997; Moudgal and Sairam, 1998). Although the role of FSH in regulating spermatogenesis in adult rats still remains unresolved (Russell, 1999; Wreford *et al*., 2001), studies employing immunoneutralization have revealed that FSH is absolutely essential for spermatogenesis in primates. Thus, there appears to be a species difference in the requirement for FSH and the obligate requirement of FSH in primates is suggested to be due to the longer pubertal period (Sharpe *et al*., 1992; Moudgal and Sairam, 1998).

Earlier studies have documented FSH can fully maintain spermatogonia and to partially maintain spermatocytes and round spermatids in short-term maintenance studies using a

hypophysectomized rat model (Russell *et al*., 1993) and gonadotrophin-releasing hormone (GnRH) antagonist-treated rats (Sinha Hikim and Swerdloff, 1995). Immunization of rodents with GnRH reduced the germinal cell types and subsequent therapy with recombinant FSH partially restored the spermatogenesis leading to increase in the number of spermatogonia and germ cells at subsequent maturation stages (McLachlan *et al*., 1995). In addition, available evidences also support a role for FSH in spermatogenesis in primates (Matsumoto *et al*., 1986; Moudgal *et al*., 1992), and it is to be noted that the oligozoospermia observed following suppression of endogenous FSH in humans by chronic administration of human chorionic gonadotrophin (HCG) could be reversed by simultaneous administration FSH with testosterone and not by testosterone alone (Matsumoto *et al*., 1986).

In contrast to the above conclusions, results of studies by Dym *et al*. (1979) and Awoniyi *et al*. (1989) suggested that FSH is not essential for adult rat spermatogenesis. Dym *et al*. (1979) reported that passive immunization of FSH in normal rats had little effect on spermatogenesis, even though a small reduction in testis weight and preleptotene spermatocyte numbers was observed. Awoniyi *et al*. (1989) reported that testosterone alone in the absence of FSH was capable of restoring sperm output in GnRH immunized animals. However, with a similar model, McLachlan *et al*. (1994) reported that FSH concentrations rapidly normalize following testosterone treatment that could support spermatogenesis along with testosterone. Studies carried out with FSHβ knockout mice demonstrate that FSH-deficient males are fertile despite having small testes with significant reduction in sperm count and sperm motility, and concluded that FSH is not required for male fertility (Kumar *et al*., 1997).

In view of the controversies that still persist, the relative contribution of LH and FSH in spermatogenesis was studied in adult rats by assessing the effect of deprivation of these individual hormones on restoration of spermatogenesis following androgen deprivation. Towards this end, an ethanedimethylsulphonate (EDS)-treated rat model was employed and this model has been widely used to study the development of Leydig cells. EDS is an alkylating agent, which destroys Leydig cells specifically (Kerr *et al*., 1985; Molenaar *et al*., 1986; Morris *et al*., 1986). Destruction of Leydig cells following EDS treatment leads to a temporary arrest in spermatogenesis due to androgen deprivation. Within 2–3 weeks after EDS treatment, Leydig cell regeneration occurs due to high concentrations of gonadotrophins, with increase in serum testosterone and testosterone-producing capacity of the new population of Leydig cells (Molenaar *et al*., 1986; Teerds, 1996). Endogenous neutralization of LH within this period results in loss of steroidogenic capacity of Leydig cells with undetectable concentrations of serum testosterone, in contrast to the moderate reduction in steroidogenic capacity and serum testosterone following FSH deprivation (Sriraman *et al*., 2003). With this background, the relative role of testosterone and FSH in initiation of spermatogenesis was assessed using a passive neutralization approach during this period of recovery.

Materials and methods

Chemicals

EDS was synthesized in the laboratory of Professor SriKrishna, Department of Organic Chemistry, Indian Institute of Science, Bangalore from ethylene glycol and methanesulphonyl chloride as described by Jackson and Jackson (1984). Collagenase was purchased from Worthington, NJ, USA. Ethidium bromide, Hank's balanced salt solution (HBSS), dimethylsulphoxide and haematoxylin were procured from Sigma, St Louis, MO, USA. RNase was obtained from Boehringer Manneheim Ltd, Germany. Ovine LH and FSH were prepared at the Institute of Clinical Research and Reproduction, Montreal, Quebec, Canada. Rat LH was a kind gift from Dr AF Parlow, National Hormone and Pituitary Program, NIDDK, USA.

Production and characterization of antiserum to ovine LH (oLH) and FSH (oFSH)

LH antiserum

Antiserum to highly purified oLH was raised in monkeys as described earlier (Sriraman *et al*., 2000). The ability of this antiserum to neutralize endogenous LH in rats was established by demonstrating that single intraperitoneal injection of 200 µl of LH antiserum reduced the serum testosterone by 85–90% in 24 h when compared with the preimmune serum-treated animals (0.33 ± 0.04 ng/ml versus 2.87 ± 0.4 ng/ml, $n = 6$). Administration of this high titre LH antiserum to adult male rats for 7 days leads to 30% reduction in testes weight and decreases the serum and testicular testosterone to undetectable concentrations, establishing the complete neutralization of LH.

FSH antiserum

Highly purified oFSH was used for immunization in adult bonnet monkeys (Sriraman *et al*., 2003). The FSH preparations were affinity purified to remove the trace quantities of LH by passing through a LH antibody affinity column and the absence of LH contamination was established by Leydig cell testosterone production assay. Actively immunized monkeys with the purified FSH preparations did not show alterations on nocturnal surge of serum testosterone, which is an LH dependent parameter. Similar results were obtained with adult bonnet monkeys that were passively neutralized with the FSH antiserum in contrast to the LH antiserum treatment, which completely abolishes the nocturnal surge in serum testosterone. The ability of the antiserum to neutralize endogenous FSH in rats was established by the observation that when neonatal rats (10 days old) were administered 100 µl of FSH antiserum by intraperitoneal route for 7 days, there was a decrease in the testicular weight by 50% with significant reduction in seminiferous tubule diameter and the steady state mRNA levels of androgen binding protein (Sriraman *et al*., 2003).

Animals and antiserum treatment

Adult Wistar rats of 90 days old were obtained from National Institute of Nutrition, Hyderabad, India. The animals were housed in an environmentally controlled facility with 12 h of light and 12 h of dark and allowed free access to food and water. Animal procedures employed in the study were conducted according to a protocol approved by the Institutional Animal Ethical Committee. Animals were administered EDS (75 mg/kg body weight, intraperitoneal route) in 50% DMSO and from day 4 after EDS treatment, the rats were injected 500 µl of LH antiserum (E + L) or FSH antiserum (E + F) for 18 days by the intraperitoneal route. Control groups received equal volume of normal monkey serum (E + N) and vehicle-treated rats also received normal monkey serum (V + N). On day 23, rats were killed, blood was collected for serum testosterone estimation (V + N = 3.1 ± 0.4 ng/ml, E + N = 1.6 ± 0.2 ng/ml, E + L = undetectable) and testes were weighed (V + N = 2.8 ± 0.3 g, E + N = 1.3 ± 0.2 g, E + L = 0.68 ± 0.2 g) (E + F – serum testosterone = 1.15 ± –0.2 ng/ml; testes weight = 0.63 ± –0.15 g) (Sriraman *et al*., 2003). In order to ensure that the observed effects of LH or FSH neutralization are under a condition at which the endogenous hormone is completely neutralized, the presence of excess antibody in circulation, was monitored to determine the efficiency of neutralization (Sriraman *et al*., 2003). The testes were fixed for histological analysis in Bouin's fluid, and 5 μm sections were obtained from paraffin embedded tissues and stained with haematoxylin (Drury and Wallington 1980). Stained sections were visualized by light microscopy and spermatogonia were counted manually that were identified by its dark staining with haematoxylin. A minimum of 15–20 tubules were counted in each slide to determine the spermatogonial number.

Preparation of germ cells and flow cytometry

Germ cells from the testes were prepared by a protocol described by Vaishnav and Moudgal (1994) with minor modifications. Testis was excised and minced into fine pieces with scissors in HBSS and minces were subjected to collagenase digestion in 50 ml HBSS at 34°C in shaking water bath for 1 h. The dispersed testicular tissue was centrifuged at 250 *g* for 10 min at 4°C. The pellet was resuspended in HBSS and filtered through 100 µm nylon mesh twice to remove the undigested tissue and larger aggregates. Subsequently, it was passed through glass wool twice to remove the spermatozoa, and the filtrate was centrifuged at 250 *g* for 10 min to obtain a pellet rich in germ cells. The cells were then suspended in a minimal volume of HBSS and fixed with 70% ice-cold ethanol at 4°C overnight. For flow cytometry, cells were washed thrice

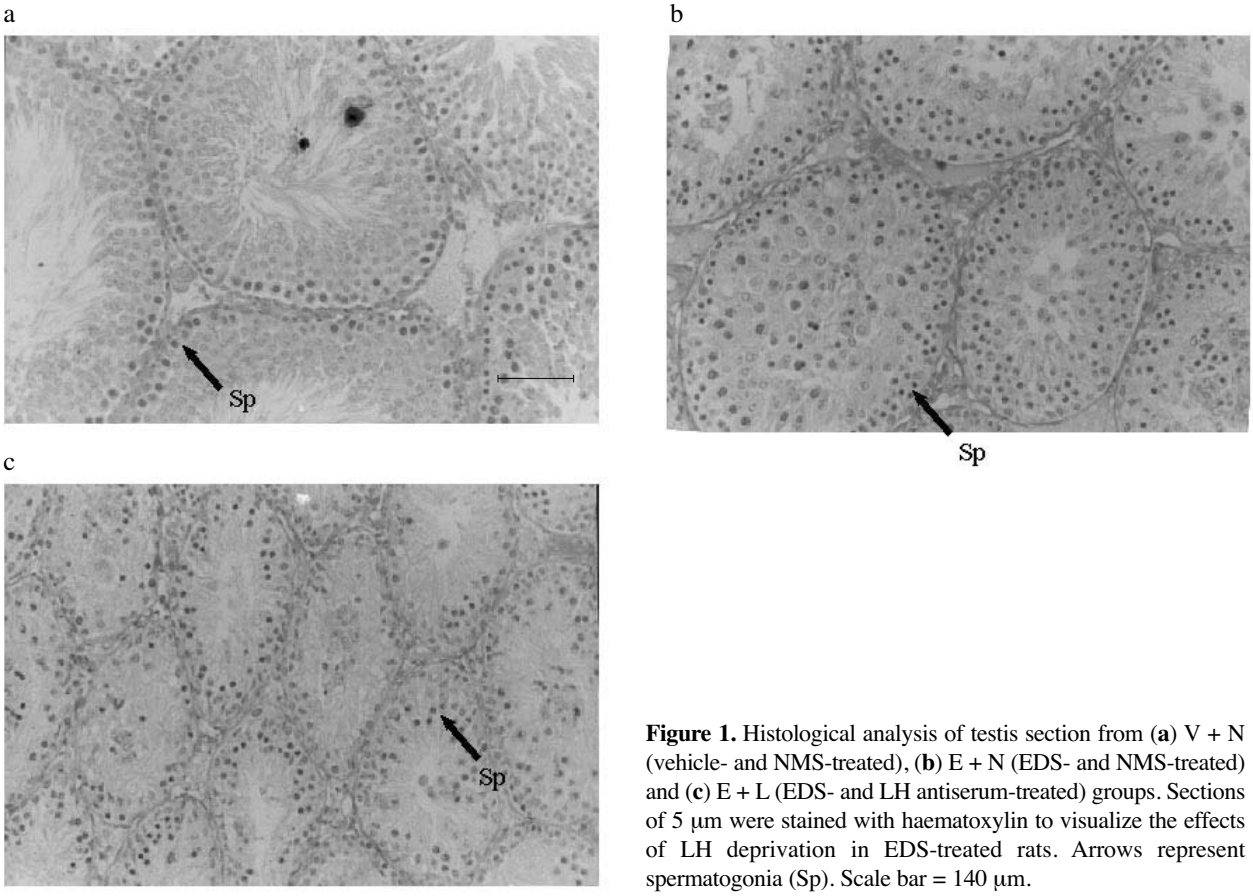

Figure 1. Histological analysis of testis section from (**a**) V + N (vehicle- and NMS-treated), (**b**) E + N (EDS- and NMS-treated) and (**c**) E + L (EDS- and LH antiserum-treated) groups. Sections of 5 µm were stained with haematoxylin to visualize the effects of LH deprivation in EDS-treated rats. Arrows represent spermatogonia (Sp). Scale bar = 140 µm.

with PBS and then subjected to pepsin digestion in saline (pH = 2) at 37°C for 5–10 min. Subsequently, cells were washed, cellular DNA was stained with ethidium bromide and analysed in FACS (Becton Dickinson, USA) following calibration with peripheral blood lymphocytes (2n).

Statistical analysis of data

The data were analysed by Kruskal–Wallis ANOVA and a *P*-value <0.05 was considered to be statistically significant. The data presented in figures and graphs were representative of at least three independent experiments.

Results

Effect of administration of LH and FSH antiserum in EDS-treated rats on testicular histology and germ cells

Histological analysis of the testis from EDS-treated rats on day 23 post-EDS treatment revealed early stages of re-initiation of spermatogenesis indicating the gradual recovery from androgen loss in contrast to the vehicle-treated groups, which exhibited normal stages of spermatogenesis (**Figure 1**). Deprivation of endogenous LH in EDS-treated rats resulted in significant reduction in tubular diameter (**Figure 1**) and spermatogonia when compared with the EDS- and NMS-treated controls (**Figure 2**). Administration of FSH antiserum also resulted in significant reduction of tubular diameter (**Figure 3**) and drastic reduction in spermatogonial number (**Figure 2**) in the testis. It should be noted that the observed reduction in number of spermatogonia is much more in the E + F group compared with the decrease seen in the E + L group ($P < 0.001$), despite the fact that in the E + F group serum testosterone was 1.15 ± 0.2 ng/ml, which was a moderate reduction when compared with E + N controls (1.6 ± 0.2 ng/ml); in contrast, serum testosterone was not detectable in the E + L-treated group.

Flow cytometric analysis of germ cell preparations revealed

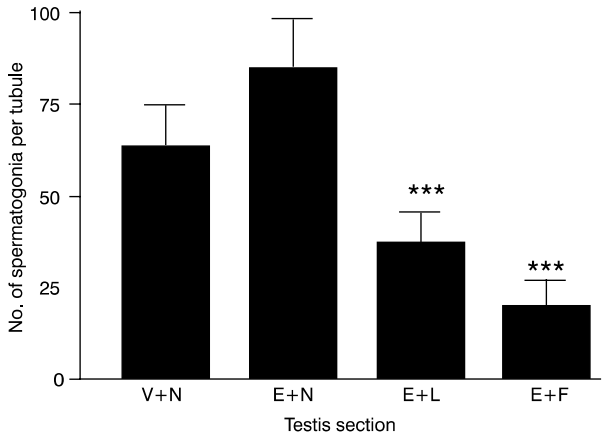

Figure 2. Effect of neutralization of LH and FSH in EDS-treated rats on the number of spermatogonia in seminiferous tubules as judged by staining of the testis sections with haematoxylin. Values presented are mean ± SE from three separate experiments and 8–10 fields were examined in each section (***$P < 0.001$).

that the vehicle-treated rats had a higher number of haploid cells (n) compared with the diploid (2n) and tetraploid (4n) population of cells which is a characteristic pattern of germ cell population in normal adult rats due to normal progression of spermatogenesis. However, analysis of germ cells in EDS-treated rats after 23 days of treatment revealed a decrease in haploid population (n) of germ cells and increase in both diploid and tetraploid cells, as seen in the case of immature rats (**Figure 4**). Deprivation of LH in EDS-treated rats also revealed a similar distribution of germ cell population as seen with the E + N group, although the total numbers of germ cells were reduced (**Figure 4**). Administration of FSH antiserum to EDS-treated rats (E + F) also resulted in a decrease in the total number of germ cells, and flow cytometric analysis revealed reduction in both diploid and tetraploid population of cells when compared with the E + N group (**Figure 5**). It is also important to note that the observed reduction in the diploid and tetraploid population of cells in the E + F group is quite drastic when compared with E + L group though serum testosterone was detectable. Light microscopic analysis of the germ cell preparation from E + F group also revealed the reduction in germ cells along with a tetraploid population (4n, pachytene spermatocytes) of cells (**Figure 6**). These results clearly establish the requirement of FSH and LH (via testosterone) in restoration of spermatogenesis in EDS-treated rats.

Discussion

In the present study, a passive neutralization approach has been employed to delineate the role of FSH and LH in restoration of spermatogenesis using treated EDS rats. Passive neutralization has the unique advantage of specific neutralization of a hormone for a desired period of time (Sriraman *et al*., 2003), over the other methods that have been employed to suppress the concentrations of LH and FSH by hypophysectomy or administration of gonadal steroids or GnRH analogues.

Neutralization of endogenous of FSH resulted in a significant reduction in tubular diameter and spermatogonial number, despite the fact that testosterone was detectable in the serum of these animals. Flow cytometric and microscopic analysis clearly revealed a significant decrease in number of pachytene spermatocytes. There was also a significant reduction in total number of germ cells as seen in the histological analysis and microscopic analysis of germ cells, which was more severe than observed following LH neutralization. Deprivation of endogenous LH in EDS-treated rats resulted in reduction of serum testosterone to undetectable concentrations. As a consequence of androgen deprivation, there was a significant reduction in tubular diameter and spermatogonial number, though there was no significant change in relative population of cells when compared with EDS- and NMS-treated controls. This could be explained due to the presence of FSH and the chosen time-point was very early to observe the effect of androgen deprivation on spermatogenesis. It is to be noted that the effects observed following LH deprivation are distinctly different from FSH deprivation providing proof for the involvement of FSH in regulation of spermatogenesis in the rodents. The studies also confirm and extend the previous observation that pachytene spermatocytes are more sensitive to FSH deprivation by passive neutralization (Shetty *et al*., 1996; Meachem *et al*., 1999). In this connection, it is pertinent to note the observation that administration of testosterone fails to

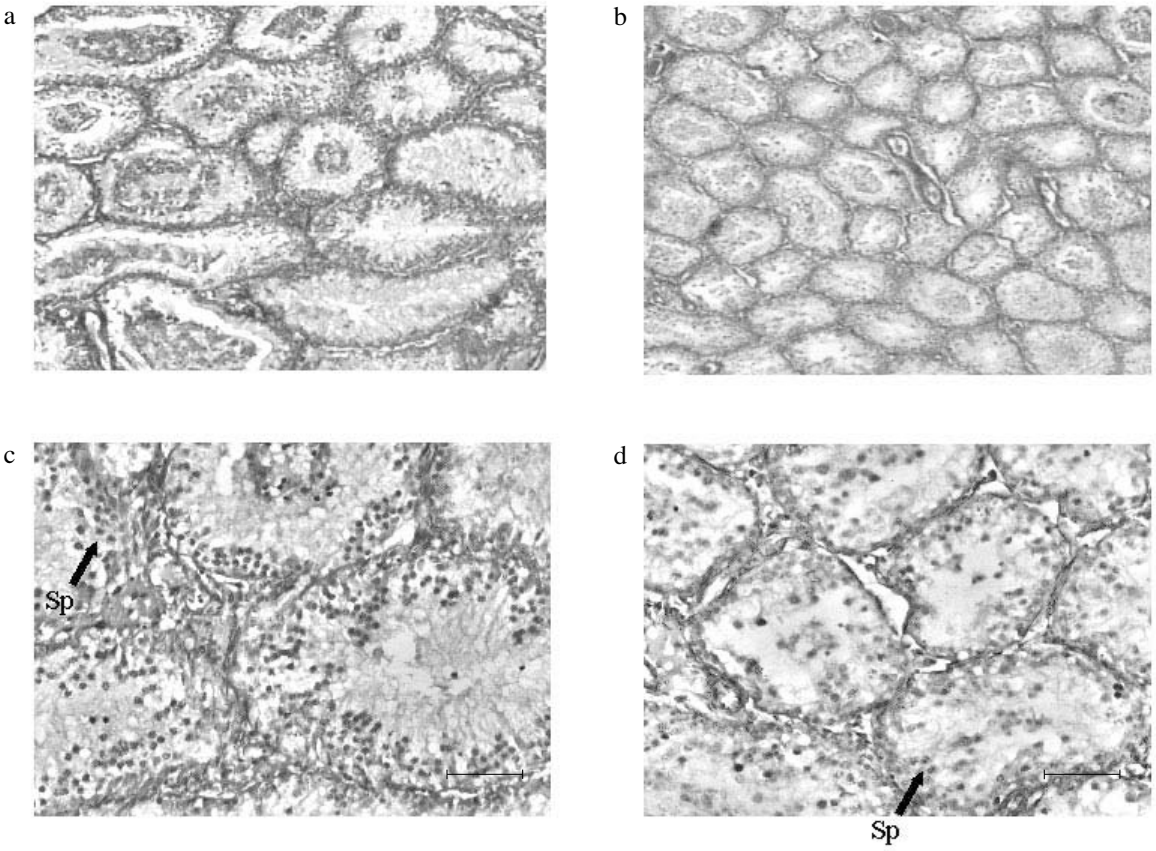

Figure 3. Histological analysis of testis section from (**a**, **b**) E + N (EDS- and NMS-treated) and (**c**, **d**) E + F (EDS- and FSH antiserum-treated). Sections of 5 μm were stained with haematoxylin to visualize the effects of FSH deprivation in EDS treated rats. Arrows represent the spermatogonia (Sp). Scale bars: a, c = 300 μm; b, d = 140 μm.

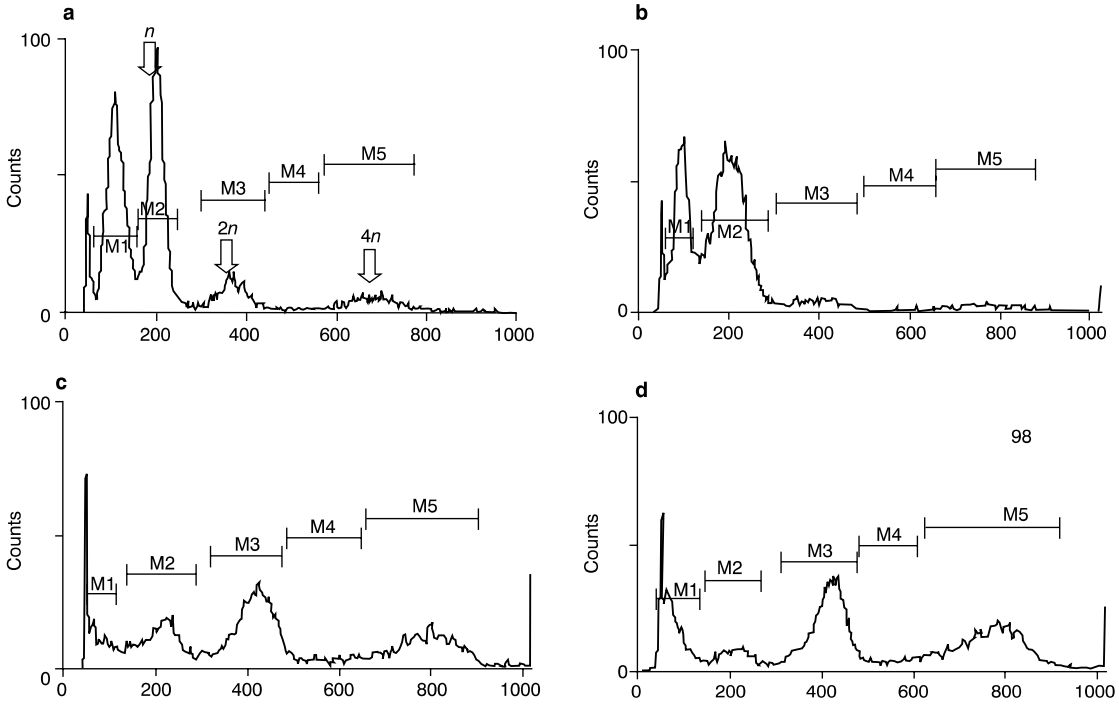

Figure 4. Flow cytometric analysis of germ cells. Germ cells prepared from the testis were stained with ethidium bromide and analysed by flow cytometry to assess the effect of LH neutralization in EDS-treated rats. (**a**) Control germ cells, (**b**) V + N (vehicle- and NMS-treated), (**c**) E + N (EDS- and NMS-treated) and (**d**) E + L (EDS and LH antiserum-treated). M1–M5 indicates the distinct peak areas that could be detected. The M1 peak represents the damaged cells that arise during the process of preparation of germ cells.

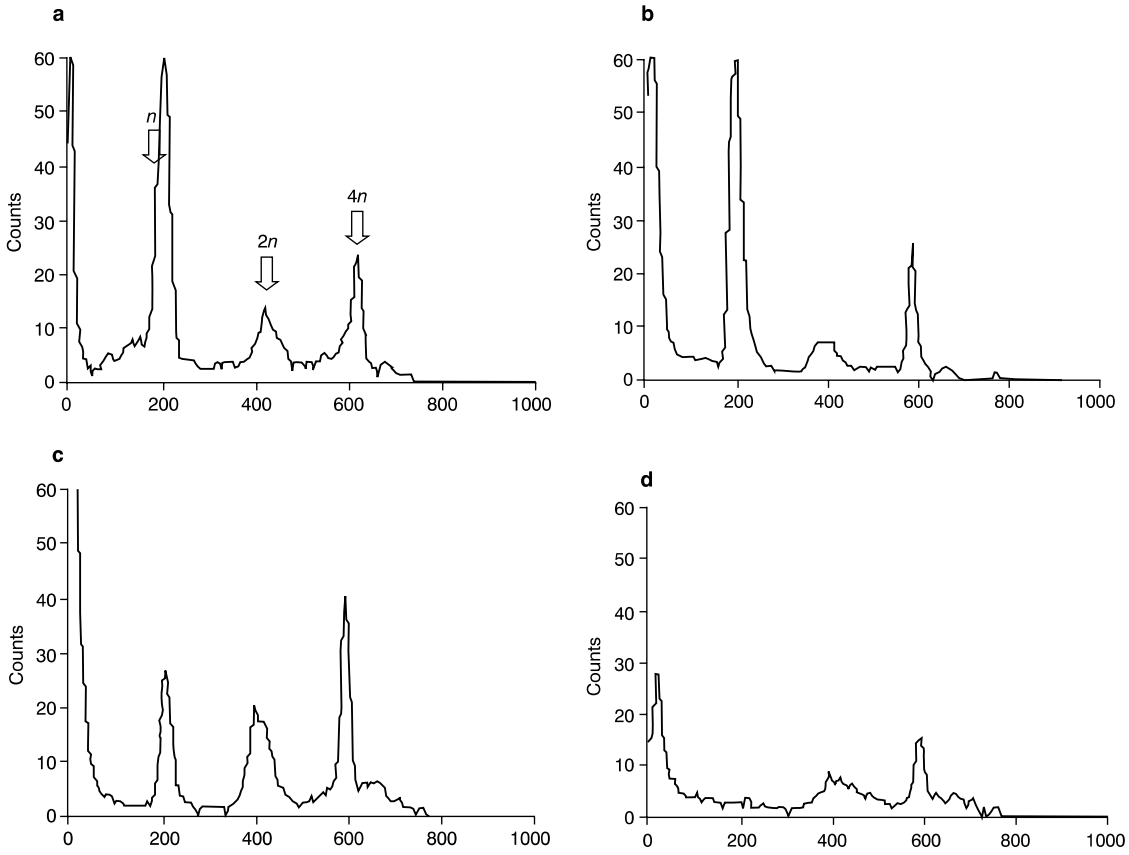

Figure 5. Flow cytometric analysis of germ cells. Germ cells prepared from the testis were stained with ethidium bromide and analysed by flow cytometry to assess the effect of FSH neutralization in EDS-treated rats. (**a**) Control germ cells, (**b**) V + N (vehicle- and NMS-treated), (**c**) E + N (EDS- and NMS-treated) and (**d**) E + F (EDS and FSH antiserum-treated).

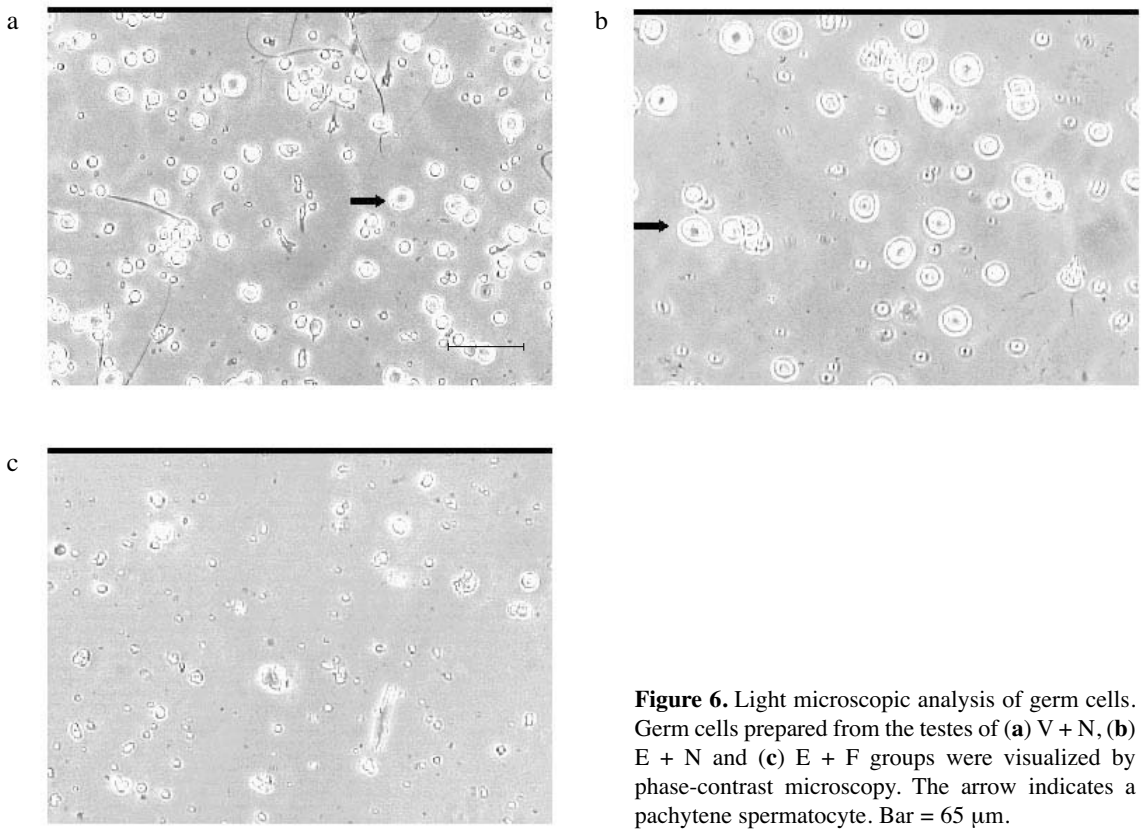

Figure 6. Light microscopic analysis of germ cells. Germ cells prepared from the testes of (**a**) V + N, (**b**) E + N and (**c**) E + F groups were visualized by phase-contrast microscopy. The arrow indicates a pachytene spermatocyte. Bar = 65 µm.

maintain or restore spermatogenesis quantitatively in hypophysectomized rats which suggest that pituitary factors participate in regulation of spermatogenesis (Roberts and Zirkin, 1991; Zirkin *et al*., 1994). The results also demonstrate the requirement of FSH for mature Sertoli cells which was speculated to secrete factors independent of FSH (Molenaar *et al*., 1986). The results of the present study and earlier studies unequivocally establish a role for FSH in regulation of Sertoli cell function in adult rats.

Careful analysis of the results of recent studies with the FSHβ and FSH receptor knockout (FORKO) mice establishes the importance of FSH in spermatogenesis and provides support for these conclusions. Male mice were subfertile with reduction in serum testosterone concentrations (Dierich *et al*., 1997). Furthermore, studies in FORKO mice also reveal that critical phases of spermatogenesis are affected with significant increase in 2C cells (spermatogonia and testicular somatic cells) and decrease in percentage of elongated spermatids (Krishnamurthy *et al*., 2000a). Also, there was a decrease in the ratio of 4C (primary spermatocyte and G2 spermatogonia) to 2C cells, suggesting that a low number of spermatogonia are entering the process of spermatogenesis (Krishnamurthy *et al*., 2001). The spermatozoa also exhibited aberrant morphology with abnormal chromatin structure. Recent studies with FSHβ knockout mice have revealed that the conversion of germ cell ratios are consistently below normal at many of the steps of spermatogenesis emphasizing that spermatogenesis is impaired in the absence of FSH (Wreford *et al*., 2001). Importantly, there was increased attrition in the conversion of spermatogonia through to pachytene spermatocytes in the FSH-β null mice, with decrease in ratio of spermatogonia and early stages of first meiotic prophase (pre-leptotene, leptotene and zygotene) to the later stages of meiotic prophase (pachytene and diplotene) (Wreford *et al*., 2001). Interestingly, the bonnet monkeys (*Macaca radiata*) used for active immunization with purified FSH showed a reduction in semen volume and sperm count. Neutralization of endogenous FSH by active immunization of FSH (Moudgal *et al*., 1992) or blocking FSH action by active immunization of FSH receptor peptides in monkeys (Moudgal *et al*., 1997; Rao *et al*., 2003) leads to formation of poor quality spermatozoa, oligozoospermia and infertility. Similar effects were observed in humans who were actively immunized with FSH (Krishnamurthy *et al*., 2000b). Collectively, the present studies indicate a definite role for FSH in regulation of spermatogenesis in the rat, although the exact mechanism by which it supports the spermatogenic process still remains unclear.

Acknowledgements

The authors wish to thank Ayesha Joshi and F Martin for their valuable help in germ cell preparation. The Central Facility for FACS analysis and Dr Omana Joy are also gratefully acknowledged. Our thanks are also due to TS Raviprakash and G Ramesh in histological sectioning. Financial assistance from Department of Science and Technology, Indian Council of Medical Research, Department of Biotechnology, New Delhi, CONRAD and Mellon Foundation, USA and MRC of Canada are greatly acknowledged. The authors also wish to wish to thank Dr Ramachandra, Mr Ramesh, Mr Krishnamoorthi, Primate Research Laboratory for their help in handling the monkeys and Professor Sri Krishna for providing EDS.

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Received 29 August 2003; refereed 23 September 2003; accepted 29 October 2003.