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A role for nocturnal serum testosterone surge in regulating spermatogenesis in the adult non-human primate

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Adult male bonnet monkeys exhibit nychthemeral rhythms in testosterone (T) secretion but the precise role of this heightened level of T secretion in regulating spermatogenesis is not known. Intranasal administration of microdoses (500 μg or 250 μg/day) of Norethisterone (IN-NET) to adult monkeys ($n = 6$) at 1600 h each day selectively and completely suppressed the nocturnal surge levels of serum T. Concomitant with this was a significant reduction ($P < 0.01$) in serum LH but not FSH levels. DNA flow cytometric analysis of testicular biopsy tissue showed by week 10 of IN-NET treatment an arrest in meiotic transformation of primary spermatocytes (4C) to round/elongate (1C/HC) spermatids and by week 20 there was a complete absence of 4C, 1C and HC cells (with a relative accumulation in 2C cells). The accumulated meiotic (4C) cells at week 10 showed an increase (> 80%, $P < 0.01$) in coefficient of variation and a decrease in intensity of DNA-bound ethidium bromide fluorescence, parameters characteristic of degenerating 'apoptotic' subpopulation of germ cells. While two monkeys exhibited acute oligozoospermia 4 became azoospermic by 20 weeks of IN-NET treatment. A complete, qualitative reversal in the regressive changes in spermatogenesis and near-normal sperm output were apparent at the end of a 20-week recovery phase. These data demonstrate that prolonged, selective suppression of nocturnal surge levels of serum T secretion exerts a primary effect on meiosis in spermatogenesis leading to oligo/azoospermic status in adult bonnet monkeys.

Keywords: androgen; rhythms; spermatogenesis; meiosis; spermatid; monkey

Introduction

A distinct diurnal rhythmicity in serum testosterone (T) secretion has been reported for the adult rhesus (Goodman *et al.*, 1974; Michael *et al.*, 1974; Plant, 1981) and bonnet (Mukku *et al.*, 1981) monkeys. It has also been shown that the elevation in nocturnal level of T is due to LH stimulating an increase in T secretion from the testes (Mukku *et al.*, 1981; Plant, 1981) rather than change in metabolic clearance of androgen or increase in adrenal androgen secretion *per se* (Goodman *et al.*, 1974). Experimentally-induced alterations in gonadotropic hormone levels in the adult bonnet monkey (Ravindranath *et al.*, 1992; Moudgal *et al.*, 1992; Aravindan *et al.*, 1993; Medhamurthy *et al.*, 1993) has been shown by us earlier to lead to marked changes in nocturnal serum T levels indicating that this is a parameter highly sensitive to the level of gonadotropin support the testis receives. Unlike the rhesus monkey which exhibits virtual cessation of testicular activity during the non-breeding summer months, the bonnet monkey has been observed not to exhibit overt changes in testicular activity during summer, both sperm production and testosterone rhythms being present throughout the year (Murty *et al.*, 1979). However, more recently, we have observed that a clear correlation exists between heightened sperm output during breeding season (Sept-Jan) and annual rhythms in serum prolactin and heightened nocturnal serum T concentrations of the bonnet monkey (Medhamurthy *et al.*, 1994). Although this study suggested a positive correlation between increase in nocturnal serum T concentrations and heightened spermatogenic activity (sperm output) during season it is hitherto not clear if the nocturnal surge level of T (indicating an increased intratesticular T production during dark hours) exerts any specific influence on germ cell transformations during spermatogenesis in the non-human primate.

In the present study in adult bonnet monkeys we have been successful in selectively suppressing the nocturnal surge levels of T alone by intranasal administration of microdoses of Norethisterone (IN-NET) at 1600 h daily over a 20-week treatment period. Using DNA flow cytometry to quantitate the testicular germ cell transformations it has been possible, in this model system, to demonstrate that selective suppression of nocturnal surge levels of T does primarily affect meiotic transformation of primary spermatocytes to spermatids.

Results

Effect of IN-NET on serum hormone profiles

Intranasal administration of the vehicle/solvent alone to three adult males did not result in any alterations in the nocturnal (overnight) serum T concentrations (Figure 1A). In these control monkeys ($n = 3$), the serum T levels rose from a basal concentration of 5.2 ± 1.2 ng/ml (at 1600 h) to a peak concentration of 27.8 ± 3.2 ng/ml (at 2400 h) and, thereafter, gradually decreased to reach the basal concentration (6.7 ± 1.9 ng/ml) by 1000 h. In contrast, IN-NET treated males ($n = 6$) showed a significant ($P < 0.01$) and complete suppression of the typical nocturnal serum T surge profile; the serum T concentrations throughout the night, however, remained at basal levels (range: 2-6 ng/ml; Figure 1A). Computation of the area under the curve (AUC - between 1600 h-1000 h; Figure 1A) revealed a significant decrease (> 85%; $P < 0.01$) in the total nocturnal T secretion as a consequence of IN-NET treatment. Within one week of initiation of IN-NET administration the normal increase in nocturnal serum T secretion (at 2100-2200 h, 20.6 ± 3.8 ng/ml) was prevented, but the basal concentration of serum T (range: 1.8 to 4.9 ng/ml) was maintained till the end of the 20-week treatment phase (Figure 1B). It should be noted here that suppression in nocturnal surge levels of T could be effectively maintained even when the dose of IN-NET was reduced from 500 μg/day (between weeks 0-10) to 250 μg/day (between weeks 11 to 20) during the treatment phase. Once IN-NET treatment was stopped, the serum T concentrations gradually rose to 50% of pretreatment levels by week 10 and the nocturnal T level (2100-2200 h) had reverted to complete normalcy by week 20 of recovery phase (Figure 1B).

There was no significant change in the serum FSH levels of

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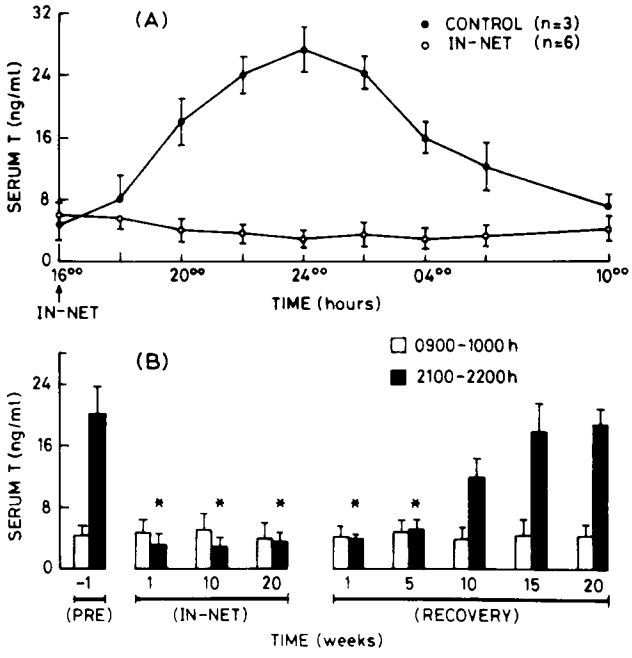

Figure 1 Serum T concentrations in control and IN-NET treated adult male bonnet monkeys. (**A**) represents the overnight profile while (**B**) represents the AM/PM levels of serum T. Values are mean ± SEM. $*P<0.01$

IN-NET treated monkeys throughout the period of study (pretreatment: 2.6 ± 0.4 vs IN-NET: 3.1 ± 0.6, 2.9 ± 0.3 and 3.3 ± 0.8 ng/ml on weeks 1, 10 and 20, respectively). Serum LH level, in contrast, was significantly reduced ($P<0.01$) from a pretreatment concentration of 24.7 ± 2.4 ng/ml to 5.6 ± 1.2 ng/ml by week 1 of IN-NET treatment and remained within this low range (4.8 ± 1.1, and 5.8 ± 1.3 ng/ml on weeks 10 and 20, respectively) throughout the treatment phase. Upon withdrawal of IN-NET administration, a significant increment in serum LH levels was observable by week 10 (16.3 ± 3.6 ng/ml), which reached the pretreatment concentrations by week 20 (21.6 ± 3.8 ng/ml) of recovery phase.

Effect of IN-NET on spermatogenesis

Whereas the histological profile of seminiferous tubules revealed qualitatively normal spermatogenesis during pretreatment phase (Figure 2a), by week 10 of IN-NET treatment a reduction in tubular diameter, complete absence of round and elongate spermatids, occurrence of loose, degenerating spermatocytes obliterating the lumen, and vacuolation of the seminiferous epithelium were apparent (Figure 2b). An increase in the interstitial space, presumably due to reduction in seminiferous tubule size was also apparent (Figure 2b). Owing to the limited availability of biopsy tissue, histological analyses of testis on week 20 of IN-NET treatment and on week 20 of recovery phase could not be undertaken. Further, a detailed quantitation of germ cell populations or analysis of the interstitial (Leydig) cells was not possible with the limited number of tissue sections available.

The typical flow cytogram of testicular germ cell populations before, during IN-NET treatment and during recovery phase presented in Figure 3 represent the results of a longitudinal study carried out in a group of six adult male bonnet monkeys. Use of such a model system was taken recourse to as an earlier investigation from this laboratory has clearly demonstrated that no significant variation in percent germ cell population is seen following flow cytometric analysis of repeat testicular biopsies taken over a length of

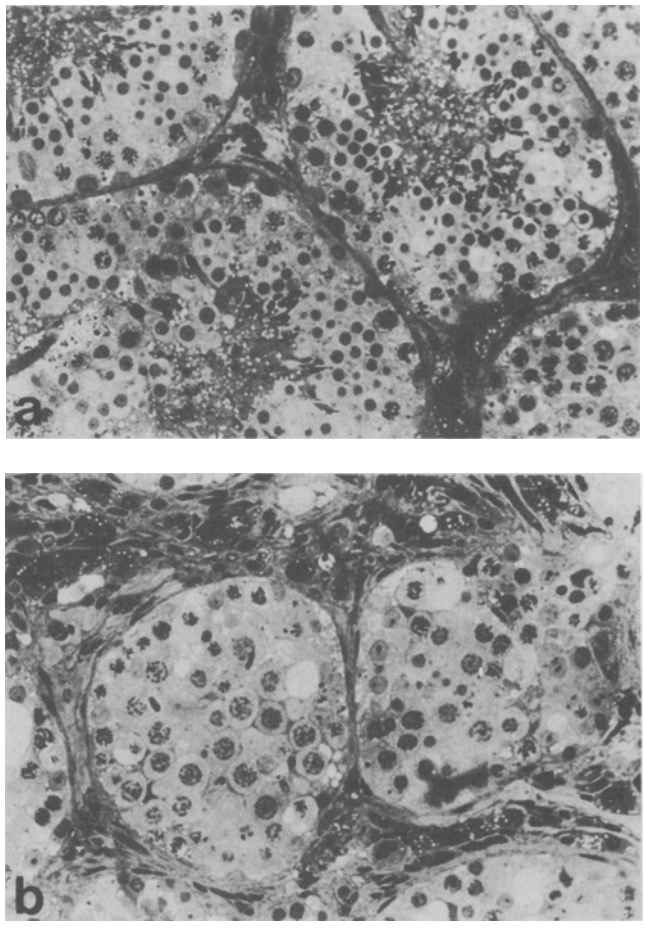

Figure 2 Representative histological profiles of seminiferous tubules from control (**a**) and after 10-week IN-NET treated (**b**) testis of adult male bonnet monkeys (× 600)

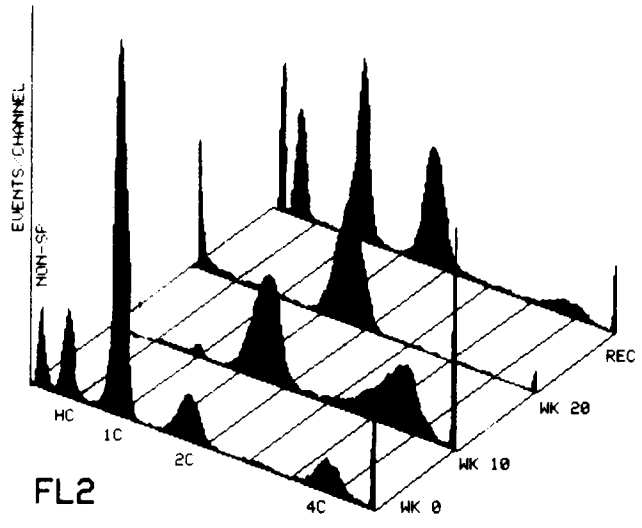

Figure 3 Representative DNA flow cytograms of adult male bonnet monkey testicular germ cells during pretreatment (wk 0), IN-NET treatment (wk 10, 20) and recovery (REC) phase. FL2 represents the channel of fluorescence intensity. 2C, spermatogonia and other diploid cells; 4C, primary spermatocytes and cells in synthetic phase; 1C, round spermatids; HC, elongate spermatids

time (Aravindan *et al.*, 1993). By week 10 of IN-NET treatment the primary spermatocytes (4C) population showed a significant ($P<0.01$) accumulation and this was accompanied by a near-complete absence of round (1C) and a total absence of elongate (HC) spermatids (Figure 4), essentially similar to the histological profile seen at this time point. By

week 20 of treatment, however, a marked reduction in the 4C population and a relative accumulation in the spermatogonial (2C) population could be seen (Figure 4). The decrease in spermatid (1C/HC) populations could be correlated to the arrested, accumulated meiotic (4C) cells ($r = -0.84$; $P < 0.037$ and $r = -0.94$; $P < 0.006$ for 1C and HC, respectively). Similarly, the relative accumulation in 2C cells was correctable to reduction in 1C ($r = -0.98$; $P < 0.0006$) and HC ($r = -0.96$; $P < 0.002$) populations. Thus, in both qualitative and quantitative terms, these data indicate that the primary and immediate effect of blocking nocturnal surge levels of T is on meiotic/post-meiotic germ cell transformations of spermatogenesis.

The contour plots of the flow cytograms of 4C population of cells at 10 weeks of IN-NET treatment reveals, compared to pre-treatment controls (Figure 5a and b), a significant increase (>80%; $P < 0.01$) in the coefficient of variation (CV) of 4C cells as well as a reduction in the ethidium bromide fluorescence intensity, perhaps a reflection of the reduced DNA content of these cells. This is typical of degenerating cells (also seen with dexamethasone-treated rat thymocytes used here as internal control as per the procedure of Nicoletti *et al.*, 1991) and could represent 'apoptotic' sub populations of germ cells. This degeneration in 4C cells seen as early as 10 weeks is perhaps responsible for the virtual disappearance of this cell population by 20 weeks of IN-NET treatment. Upon withdrawal of IN-NET treatment, however, a complete reversal in qualitatively normal spermatogenesis could be seen by week 20 of recovery phase (Figures 3 and 4).

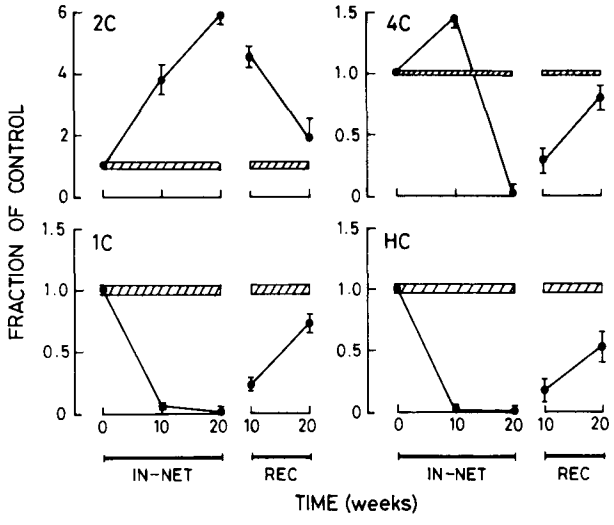

Figure 4 Changes in the quantity of testicular germ cell populations in adult male bonnet monkeys during IN-NET treatment and recovery phases. Values are Mean ± SEM of six monkeys. The cross-hatched bar parallel to the abscissa denotes the control levels, wherein the mean values have been normalized to the week 0 value. For details of germ cell populations, see Figure 3

Effect of NET treatment on seminal parameters

A significant decrease in testicular volume was recorded by week 10 of IN-NET treatment (>50%; $P < 0.05$) which was further pronounced at the end of 20-week treatment phase (>70%; $P < 0.01$); a gradual reversal to near-pretreatment volume was observed by week 20 of recovery phase. Consistent with and concomitant to the arrest in meiosis of spermatogenesis seen in the testis, acute oligozoospermia ($n = 2$) or azoospermia ($n = 4$) was evident by week 20 of IN-NET treatment. The reversal in this effect, however, was only upto 70% of pretreatment sperm counts even at the end of 20-week recovery phase. Per cent motility and viability of the few sperm voided by IN-NET treated, acutely oligozoospermic monkeys were significantly reduced (>95%; $P < 0.01$); but an almost complete reversal in these parameters was observed by week 20 of recovery phase (Table 1).

Discussion

The primary objective of the current study was to determine whether the marked increase in testicular T production during the night hours (nocturnal T surge) has any specific regulatory significance during spermatogenesis in the adult bonnet monkey. To this end, we have been able to demonstrate that a selective and complete suppression in the nocturnal surge levels of serum T could be achieved by IN-NET treatment (on a daily basis) in bonnet monkeys. While intratesticular T levels could not be measured owing to the limited biopsy tissue available, it could be safely presumed at this juncture that the suppression in nocturnal serum T surge levels is a primary consequence of the reduced availability of LH to the Leydig cells in the testes. Several earlier studies have indeed clearly shown the direct relationship extant

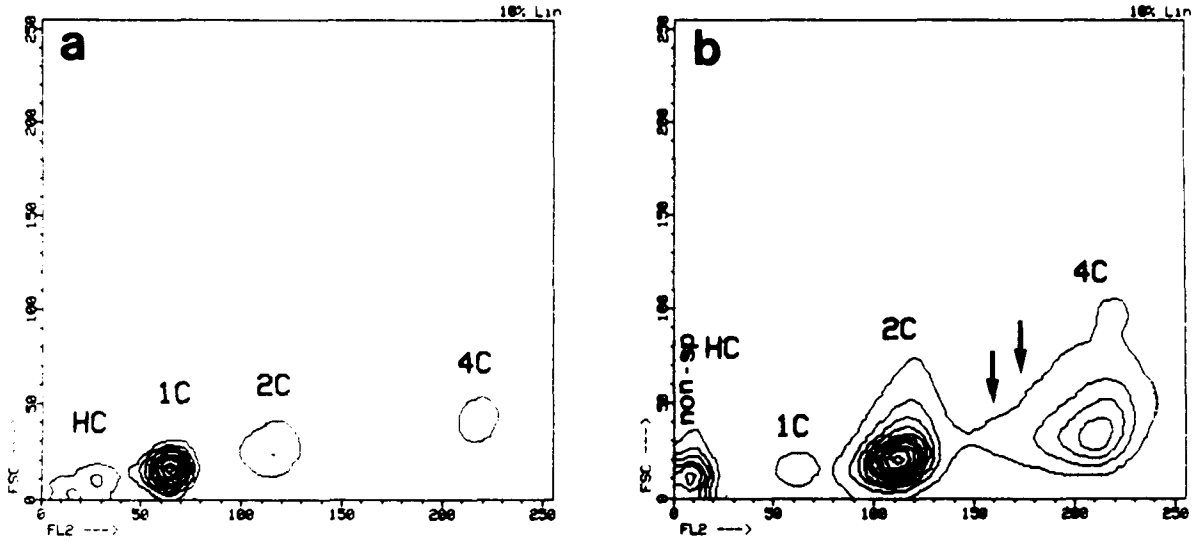

Figure 5 Flow cytometric contour plots of testicular germ cells before (**a**) and after (**b**) IN-NET treatment. Note that a G1/S phase arrest (4C) is followed by the formation of degenerating 'apoptotic' sub-populations (arrows) and a significant increase in the CV value. The contour plots were generated using LYSIS II DNA histogram analysis software (Becton-Dickinson). For details of germ cell populations, see Figure 3

Table 1 Total counts, motility and viability of ejaculated sperm from adult bonnet monkeys during IN-NET treatment and recovery phase

	In-net treatment phase			*Recovery phase*	
Parameters[b]	*Week 0*	*Week 10*	*Week 20*[a]	*Week 10*[a]	*Week 20*
Counts (millions/ejaculate)	536 ± 36	113 ± 41*	26;18**	65;138**	310 ± 104
% Motility[c] (rapid & linear, progressive)	85	25*	5**	40*	75
% Viability[c] (supra-vital staining)	70	20*	5**	35*	60

[a]Data of two monkeys only are provided as four monkeys at this time were azoospermic.
[b]Unless otherwise stated, the values are Mean ± SEM of 6 monkeys (*$P<0.05$; **$P<0.01$).
[c]Median values only

between intratesticular T and serum levels of T in non-human primates (Mukku *et al.*, 1981; Plant, 1981).

Although earlier studies have shown that intranasal administration of hormonal steroids like estrogen, progesterone or progestins could suppress spermatogenesis in the non-human primate (Anand Kumar *et al.*, 1980; Moudgal *et al.*, 1985), no conclusions were drawn with regard to the role, if any, of the nocturnal surge level of T in regulating specific germ cell transformation steps during spermatogenesis.

The marked difference in the effective dose of IN-NET administered in the earlier studies (30 μg/day; Anand Kumar *et al.*, 1980; Moudgal *et al.*, 1985) compared to that required to be administered in the current study (500 and 250 μg/day) perhaps could be ascribed to the large variation in the efficiency of the nebulizers adopted in the former vs present studies (<2% *vs* >90%, respectively). Pharmacokinetic studies of IN-NET in monkeys have shown that a dose of 300 μg of IN-NET is required to provide a maximal serum concentration of 10.7 nM of NET, wherein a spray device similar to the one used in the current study was employed (Puri *et al.*, 1987). It is possible that the low efficiency of the nasal spray device used earlier might have resulted in an underestimation of the actual effective dose of steroids administered. Though there is a 10 to 15-fold difference in the dose of IN-NET administered between the two studies, interestingly, both have shown an uniform effect on spermatogenesis. However, while earlier studies have monitored the serum T levels at a specific time of the day (Anand Kumar *et al.*, 1980) or night (Moudgal *et al.*, 1985) to determine the effects of IN-NET treatment, in the present study, we have monitored the serum T concentrations at multiple time points throughout the night to conclude that a complete suppression of the nocturnal surge level of T is indeed achieved by this procedure. This fact assumes importance since in a recent related study we observed that intranasal administration of bromocriptine at 1600 h to adult male bonnet monkeys although suppressed the increase in serum T levels as measured at 2100–2200 h, caused only ~30–40% suppression in the overnight serum T profile (Suresh & Moudgal, 1992). That the suppression in nocturnal surge of T secretion occurs as a consequence of IN-NET blocking pituitary LH secretion is evident from the significant reduction in serum LH levels seen in the current study. Unlike in the female bonnet monkey where IN-NET treatment significantly suppressed the mid-cycle surge of both FSH and LH (Moudgal *et al.*, 1985), in the male monkey, serum FSH levels were unaffected by such a treatment. This differential effect observed in the male monkey is perhaps due to the microdoses of IN-NET treatment as administration of high doses of steroids or progestins (for eg. in male contraception clinical trials) is known to suppress the secretion of both LH and FSH (Handelsman *et al.*, 1992; Wallace *et al.*, 1993; WHO, 1993).

The suppression in nocturnal surge levels of serum T alone appears adequate to adversely affect overall testicular function as evident from the significant decrease in testicular volume and the occurrence of acute oligozoospermia or even azoospermia in the bonnet monkey. The histological and flow cytometric data are essentially supportive of each other and provide clear evidence for a blockade in spermatogenesis. In particular, the flow cytometric data indicates that suppression of nocturnal surge levels of T alone brings about an arrest in meiosis, the regulatory transformation step during production of spermatids. It should be noted here that the DNA flow cytograms of germ cells (Figure 3) represent the relative per cent of 2C, 4C, 1C and HC populations at each time point. Consequently, when compared with the pre-treatment (wk 0) profile, the changes occurring in percentages of each of these germ cell populations (upon IN-NET treatment) become significant. Furthermore, although a significant 'increase' in the 2C population is apparent (by wk 10 and 20 of IN-NET), this increase is only relative and does not necessarily indicate an actual increase and accumulation of spermatogonial (2C) cells in the testis.

Active immunization against ovine LH leads to a total suppression of both basal and nocturnal surge levels of T secretion and this has also been shown to result in an immediate arrest of meiosis during spermatogenesis in adult rabbits and bonnet monkeys (Suresh *et al.*, 1993b). Further, in pituitary desensitized (GnRH agonist-treated) monkeys where near-complete suppression in testicular T production was achieved, exogenous supplementation with basal levels of T alone did not result in any significant reversal of the regressive changes seen in spermatogenesis (Aravindan *et al.*, 1993). It thus appears that any disturbance in the normal diurnal rhythmicity of T production would result in disruption of the spermatogenic process in the non-human primate.

It is well known that withdrawal of gonadotropins/testicular androgens enhances the degeneration of both somatic and germ cells in the mammalian testis (review: Zirkin *et al.*, 1994). Several studies have focused attention on the nature of the degeneration process in gonadal cells under normal/experimental situations employing sensitive biochemical or molecular parameters and have shown this degeneration or atresia to be closely associated with the genomic DNA fragmentation termed apoptosis (Hughes & Gorospe, 1991; Tilly *et al.*, 1991; Kerr, 1992; Coucouvanis *et al.*, 1993). Further, *in vivo* deprivation of gonadotropins and testicular androgens by hypophysectomy or by selective withdrawal of T production with ethane dimethane sulphonate treatment has been shown to result in apoptotic cell death of germ cells in adult rats (Tapanainen *et al.*, 1993; Troiano *et al.*, 1994); these effects being reversible to a significant extent by exogenous gonadotropin/androgen treatment (Tapanainen *et al.*, 1993). More recently, DNA flow cytometry has been shown to provide a simple, rapid, quantitative and reliable method of analysis of apoptotic cells (Sgonc *et al.*, 1994). In addition, it has been shown that irradiation of the mouse results in a dose-dependent increase of germ cell CV values on flow cytograms thus reflecting the induction of numerical and structural chromosome aberrations during spermatogenesis (Spano *et al.*, 1987). Although biochemical/

molecular evidences for apoptotic degeneration of germ cells are not forthcoming in the current study, the fact that the primary spermatocytes (4C) arrested from completing meiotic transformation are indeed degenerating has been established from flow cytometric analysis of parameters characterizing the 'apoptosis' phenomenon. An obvious decrease in DNA-bound ethidium bromide fluorescence (a measure of the regressive 'apoptotic' nature of transforming germ cells) and an increase in CV value of the 4C (meiotic) cell population are seen during IN-NET treatment phase; these degenerative changes, however, are prevented once the endogenous hormone levels revert to normalcy during the recovery phase.

In conclusion, we have successfully demonstrated that IN-NET can selectively suppress the nocturnal surge levels of T alone and that the abolishment of the surge level of T primarily and adversely affects meiosis in spermatogenesis thus rendering monkeys acutely oligozoospermic or azoospermic. The potential of administering microdoses of IN-NET (an opposed to the high, oral doses; Johansson & Nygren, 1973) to men as an alternative means of achieving male contraception method needs to be explored. Recent, successful clinical trial in men (Christensen *et al.*, 1994) using a combination of a progestin (levonorgestrel) and testosterone enanthate indeed supports this view.

Materials and methods

General methodology

Nine adult male bonnet monkeys, 8–10 years of age and weighing 7–9 kg were screened for normal diurnal T rhythms, spermatogenesis (by DNA flow cytometry) and seminal parameters (sperm count, motility and viability) prior to recruitment for the present study. The general care and maintenance of monkeys under regulated photoperiod (12L:12D) has been described earlier (Ravindranath & Moudgal, 1987). All the monkeys were initially trained upto 30 days to receive the intranasal spray with minimal restraining or stress. This study was cleared by the Ethics Committee of the Institute for use of laboratory animals for biomedical research.

Drug formulation

Micronized NET (particle size – 3 to 5 μm) was dissolved at a concentration of 250 μg/100 μl of a suitable solvent. The solvent ethanol:propylene glycol:distilled water, in a volume ratio of 3:3:4 – has been approved for clinical use and successfully tested both in the monkeys (Anand Kumar *et al.*, 1980; Moudgal *et al.*, 1985; Puri *et al.*, 1986) and in human volunteers (Anand Kumar *et al.*, 1991). The nebulizer used was obtained from Pfeiffer GmBH Co. KG, Radolfzell, Germany, and delivers 100 μl solvent/spray. The efficiency of the nebulizer as tested using a [^{3}H] steroid was $>90\%$ with a variation of $\pm 2\%$ between sprays. Recent studies have shown this spray device to be reliable and highly efficient when tested in hyperprolactinaemic patients with a dopamine agonist (Bromocriptine) to reduce serum prolactin levels (Suresh *et al.*, 1993a).

Blood samples and hormone assays

Blood samples were collected from unanaesthetized monkeys by way of femoral vein/arterial puncture using sterile Vacutainer tubes (Becton-Dickinson, New Jersey, USA) between 0900–1000 h (AM levels) and 2100–2200 h (PM levels). In addition, blood was collected at 2 h intervals between 1600–1000 h through the night on two occasions (see below) for the purpose of computing the nocturnal profile of serum FSH, LH and T levels. To collect blood samples during night, the 24 h L:D cycle was briefly interrupted by using a 2-cell (1.5 volts) flash light. The sera were separated within 12 h and stored at −20°C until analysis.

Testosterone was assayed in duplicate using 10–20 μl aliquots of serum as previously described (Mukku *et al.*, 1981). The sensitivity of the assay was 10 pg/tube (0.5–1 ng/ml). The inter- and intra-assay coefficients of variation were 9.8% and 8.3% respectively.

FSH was measured in 400 μl aliquots of the serum by a solid-phase RIA system developed in our laboratory. This assay employs an immunochemical bridge method (Murthy *et al.*, 1989) to coat polypropylene tubes with an appropriate dilution of a monkey polyclonal antiserum to oFSH. In addition, human FSH (hFSH-AFP- 4822B) was used both for iodination and as standard. The sensitivity of the assay was 0.4 ng equivalent of hFSH (AFP-4822B) per tube. The inter- and intra-assay coefficients of variation were 12.2 and 8.6% respectively.

LH was estimated in 100 μl aliquots of the serum by use of a sensitive radio-receptor assay standardized recently in this laboratory (Selvaraj & Moudgal, 1993). Human LH (NIH-AFP-4745B) was used for iodination and as standard. The source of the receptor (30 μl of an appropriate diluted receptor preparation/tube) was a crude particulate membrane preparation obtained from sheep corpora lutea. The sensitivity of the assay was 0.77 ng equivalent of hLH (NIH-AFP-4745B) per tube. The inter- and intra-assay coefficients of variation were 13.2 and 9.8% respectively. The hLH and hFSH samples used were obtained from NIAMDD, National Hormone and Pituitary Program, NIH, Bethesda, USA.

Quantitation of spermatogenesis by DNA flow cytometry

Testicular biopsy was performed under ketamine anaesthesia using a sterile needle and trochar as described earlier (Medhamurthy *et al.*, 1993). Care was taken to obtain testicular parenchyma from alternate testis during subsequent biopsies to allow sufficient time for the previous wound to heal completely. Biopsies were obtained on weeks 0, 10 and 20 during IN-NET treatment, and on weeks 10 and 20 of the recovery phase. The procedure adopted for preparing single cell suspensions of testicular germ cells has been described previously (Aravindan *et al.*, 1990; Suresh *et al.*, 1992). An aliquot of the ethanol-fixed single cell suspension was washed twice in physiological saline (0.9% NaCl) and treated with 0.5% pepsin (pH 2.0; Serva Feinbiochemica, Heidelberg, Germany) for 10 min at 37°C, centrifuged for 10 min, resuspended in the staining solution (25 μg/ml ethidium bromide, 40 μg/ml ribonuclease A and 0.3% Nonidet P-40; all obtained from Sigma Chemical Co., St.Louis, USA) for 30 min at 4°C in dark before analysis in a flow cytometer (FACScan, Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser for fluorescence excitation at 488 nm. At least 10 000 cells per sample were acquired and analysed using LYSIS II software programme (Becton-Dickinson). Monkey peripheral blood leukocytes were used as the diploid marker to ascertain peak positions and to identify germ cell populations in the flow cytograms. The different cell populations quantitated on the basis of their DNA contents were expressed as 'C' values. Essentially, four distinct populations of germ cells could be determined; spermatogonia and other diploid cells (2C), the cells in S-phase and primary spermatocytes (4C), round spermatids (1C), and elongate spermatids (HC). The numbers of different cell populations were expressed as percent populations of the total number of cells analysed.

Data acquired in list mode and analysed by contour plot using LYSIS II softare programme (Becton-Dickinson) provided a measure of the 'apoptotic' cell sub-population. Reduction in fluorescence intensity of ethidium bromide bound to DNA and an increase in CV values were used as a measure of this cell sub-population. Debris were gated out based on light scatter measurement before the single parameter contour plots were drawn according to procedures

described by Coucouvanis *et al* (1993). Normal and dexamethasone-treated rat thymocytes were used as internal control to monitor the positioning of the cell populations (Nicoletti *et al.*, 1991; Sun *et al.*, 1992) on contour plots. Analysis of DNA fragmentation by electrophoretic methods was not possible as biopsy tissue material was minimal.

Histology

Light microscopic histological analysis (Humason, 1972) of the testicular biopsy samples were performed only during pretreatment (week 0) and during IN-NET treatment phase (at week 10) to randomly compare the results obtained with that of DNA flow cytometry. Since the amount of biopsy tissue available was limiting, detailed morphometric analysis and quantitation of spermatogenesis were not undertaken.

Seminal parameters

Sperm count, per cent motility and viability were assessed on weeks 0, 10 and 20 during treatment and on weeks 10 and 20 during recovery phase of the study according to the procedures described in the WHO manual (1987).

Statistical analyses

All data are expressed as Mean ± SEM. Significance of differences between pretreatment and during treatment profiles of hormone levels, percent germ cell populations and seminal parameters were analysed by use of two-way analysis of varience followed by Student-Knewman-Keuls multiple range test (Winer, 1971). Correlation coefficient values were obtained using Statgraphics software (Version 7.0; Manugistics Corp. (C) 1993).

Experimental protocol

Six of the monkeys received IN-NET at a dose of 500 μg (two sprays of 250 μg/100 μl, one spray/nostril) at 1600 h daily for the first 10 weeks of treatment. Subsequently, from week 11, a maintenance dose of 250 μg NET (one spray/day) was administered until the end of the treatment phase (week 20). All the monkeys were allowed to recover from week 21 (= week 1 of recovery phase) and monitored for a further period of 20 weeks. The remaining three monkeys served as vehicle (solvent) treated controls and were used along with the IN-NET monkeys only for the purpose of comparing the nocturnal serum T profiles (throughout the night) on week 2 of treatment phase and on week 20 of recovery phase.

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