BIOLOGY OF REPRODUCTION **53,** 525–531 (1995)

Evidence for Follicle-Stimulating Hormone Mediation in the Hemiorchidectomy-Induced Compensatory Increase in the Function of the Remaining Testis of the Adult Male Bonnet Monkey (*Macaca radiata*)[1]

R. Medhamurthy, R. Suresh, S.S. Paul, and N.R. Moudgal[2]

Primate Research Laboratory, Centre for Reproductive Biology & Molecular Endocrinology
Indian Institute of Science, Bangalore 560 012, India

ABSTRACT

Hemiorchidectomy (HO) in the adult male bonnet monkey results in a selective increase in circulating concentrations of FSH and testosterone, and this is accompanied by compensatory increase in sperm production by the remaining testis. We investigated the possible role of increased FSH concentration that occurs after HO in the compensatory increase in the activity of the remaining testis. Of eight adult male bonnet monkeys that underwent HO, four received i.v. injections every other day for 30 days of a well-characterized ovine FSH antiserum (a/s) that cross-reacts with monkey FSH. The remaining four males received normal monkey serum (NMS) as control treatment in a protocol similar to that employed for a/s-treated males. Blood samples were collected between 2100 and 2200 h before and 1/2, 1, 3, 5, 7, 14, 22, and 29 days after HO. Testicular weight, number of 3β-hydroxy steroid dehydrogenase-positive (3β-HSD+) cells, and DNA flow cytometric analysis of germ cell populations were obtained for testes collected before and at the termination of NMS or a/s treatment.

In NMS-treated males, circulating serum FSH concentrations progressively increased to reach a maximal level by Day 7 after HO (1.95 ± 0.3 vs. 5.6 ± 0.7 ng/ml on Days −1 and 7, respectively). Within 30 min of a/s injection, FSH antibodies were detected in circulation, and the antibody level was maintained at a constant level between Day 7 and end of treatment (exhibiting 50–60% binding to ^{125}I-hFSH).

Although circulating mean nocturnal serum testosterone concentration showed an initial decrease, it rose gradually to pre-HO concentrations by Day 7 in NMS-treated males. In contrast, nocturnal serum testosterone concentrations in a/s-treated males remained lower than in NMS-treated controls ($p < 0.05$) up to Day 22 and thereafter only marginally increased. Testicular weights increased ($p < 0.05$) over the pre-HO weight in NMS- but not in a/s-treated males. After HO, the number of 3β-HSD+ cells (Leydig cells) was markedly increased but was significantly ($p < 0.05$) higher in NMS-treated males compared to a/s-treated males. A significant ($p < 0.05$) reduction in the primary spermatocyte population of germ cells was observed in a/s-treated compared to NMS-treated males. These results suggest that the increased FSH occurring after HO could be intimately involved in increasing the compensatory functional activity of the remaining testis in the male bonnet monkey.

INTRODUCTION

In farm animals and the laboratory rat, hemiorchidectomy (HO) before puberty leads to an increase in circulating serum FSH concentrations and to testicular weight gain; this is generally referred to as compensatory hypertrophy [1–10]. In primates, the hypothalamo-pituitary-testicular axis is quiescent during the greater part of prepubertal development [11], and HO has no effect before the onset of puberty on the function of the testis left in situ [12]. In a recent study, it was observed that HO in the adult male bonnet monkey results in a selective increase in serum levels of FSH and a rapid compensation in the concentration of secreted nocturnal testosterone (T) [13]. In addition, an increase in testicular volume and dynamic changes in germ cell turnover in the testis remaining in situ were also observed [13]. The mechanism by which rapid compensation in T secretion occurs in monkeys immediately after HO without any concomitant increase in circulating LH concentrations is not known.

Several studies carried out in the male monkey suggest an important role for FSH in the initiation and maintenance of spermatogenesis [14–17]. In the present study, we examined the role of FSH in the compensatory increase in testicular function, with emphasis on the rapid T compensation that occurs in adult monkeys after HO as well as on the compensatory spermatogenic activity of the remaining testis. To this end, a well-characterized FSH antiserum (a/s) was employed to immunoneutralize circulating FSH concentrations in hemiorchidectomized monkeys.

MATERIALS AND METHODS

General Methodology

Thirteen intact and two long-term castrated adult male monkeys aged 8–10 yr weighing 7.0–9.0 kg were used. The general care and maintenance of monkeys under controlled photoperiod (12L:12D) has been described previously [18]. The present study was cleared by the institutional ethical committee for use of laboratory animals for biomedical research. Before initiation of the study, all the intact monkeys were screened for normal T secretory rhythms as reported earlier [19].

Production and Characterization of a/s

Ovine (o)FSH antiserum was raised in adult male monkeys as previously described [14, 20]. Monkeys that had

Accepted April 13, 1995.
Received October 21, 1994.
[1]Supported by INSA and DBT (INDO-USAID Program).
[2]Correspondence: FAX: 91-80-3341683.

been actively immunized previously were administered s.c. booster injections of purified oFSH (~100 μg) in aluminum hydroxide gel adjuvant (Alugel Superfos, Vedback, Denmark) once every 3 mo. Blood was collected for a/s 14 days after each booster injection.

The a/s was checked for FSH antibody titers with ^{125}I-hFSH as tracer as described earlier [21]. The a/s from several monkeys with sufficiently high antibody titer were pooled, characterized, passed through 0.44-μm sterile membrane filters (Millipore Corp., Bedford, MA) [22], and stored frozen as 5-ml aliquots until use. The specificity of a/s was checked by determining its ability to bind ^{125}I-hFSH [21, 22] as well as ^{125}I recombinant monkey FSH (results not reported). The extent of cross-reactivity of the a/s with human (h) LH was less than 4%.

Determination of Minimum Effective Dosage of a/s Necessary to Immunoneutralize FSH

A pilot experiment was carried out to determine the minimum dosage of a/s capable of effectively neutralizing circulating concentrations of endogenous FSH. For this purpose, doses of 0.5, 1, or 2 ml of pooled a/s were injected i.v. into two long-term castrated adult males on three separate occasions. Blood samples were collected before and 1/2, 2, 4, 8, 12, 24, 48, 72, and 96 h after each a/s injection to estimate circulating free FSH in the serum. The concentration of FSH not bound to FSH antibodies was determined by a solid-phase RIA routinely employed in this laboratory [23], with the modification that neat serum (700 μl) was preincubated in the FSH antibody-coated plastic tubes. The serum was decanted, and the tubes were washed twice with RIA buffer before ^{125}I-hFSH was added. The rationale for including the above step in the RIA method was to allow free FSH (i.e., the residual FSH remaining after FSH-antibody complex formation), if present in the serum, to bind to antibodies that had previously been adsorbed onto the wall of the plastic tubes and to remove the hormone-antibody complex present in the serum, thus preventing any interference in the RIA. Circulating serum concentrations of FSH in the two castrated male monkeys ranged from 80 to 110 ng/ml before injection of different dosages of a/s, but serum concentrations of FSH were below assay detectability (< 0.4 ng/tube) within the first 24 h for all dosages of a/s injected. Serum FSH concentrations remained below assay detectability for 48 h in the 1-ml dose and for as long as 72 h in the 2-ml dose of a/s. On the basis of this result, a dose of 1 ml a/s was administered every 48 h for immunoneutralizing endogenous FSH. This dosage of a/s has previously proved effective in our laboratory [14].

Earlier studies from this laboratory have established that injection of a/s into the adult intact male monkey does not result in alteration of serum T concentrations [14, 16, 20]. In order to ensure that this particular batch of a/s also did not alter serum T concentrations, five intact adult males were administered 1 ml a/s i.v. for 7 consecutive days. Analysis of serum T concentration in blood samples collected on Days −1, 1, 3, 5, and 7 of a/s injection revealed no significant change in serum T concentrations (T: 26.2 ± 3.6, 23.4 ± 4.8, 27.3 ± 4.4, 24.6 ± 2.8, and 23.5 ± 3.8 ng/ml on Days −1, 1, 3, 5, and 7, respectively).

Hormone RIAs

Serum T was assayed in duplicate 10–20-μl aliquots without chromatography as previously described [24]. The sensitivity of the assay was 10 pg/tube (0.5–1 ng/ml), and the inter- and intraassay coefficients of variation were 10.9% and 9.9%, respectively.

Serum FSH was assayed in 400-μl aliquots by means of a solid-phase RIA as reported previously [23]. This system employs hFSH (hFSH-AFP-4822 B) both for iodination and as standard; the antibody used was an a/s to oFSH raised in an adult male monkey. The sensitivity of the assay was 0.4 ng equivalent of hFSH-AFP-4822B/tube. The inter- and intraassay coefficients of variation were 13.8% and 11.1%, respectively.

Quantification of Testicular Germ Cells by DNA Flow Cytometry

Testicular tissue was processed from one testis removed from each control and a/s-treated monkey on the day of hemicastration and from the other testis removed on Day 30 after HO. Procedures for dispersal of testicular tissue to obtain single cells, and for ethanol-fixation, pepsin treatment (for 10 min at 37°C), staining of cellular DNA with ethidium bromide, and analysis by flow cytometry have been described earlier [25]. The different populations of cells analyzed include 2C (spermatogonia and nongerminal cells), 4C (primary spermatocytes and cells in G2 phase), 1C (round spermatids), and 1 CC (elongated spermatids). Numbers of different cell populations are expressed as a percentage of the total number of cells analyzed (on average 10 000 cells per sample).

Statistical Analyses

All data are expressed as mean ± SEM. The significance of differences for mean hormone concentrations between sera of control and a/s-treated male monkeys was determined by use of two-way analysis of variance followed by the Student-Newman-Keuls multiple range test [26]. The percentages of germ cell populations are expressed as mean ± SEM. The significance of differences between control and treated groups was determined by Student's *t*-test.

Experimental Protocol

The purpose of this experiment was to examine the effect of immunoneutralization of endogenous FSH on the

HO-induced compensatory activity of the remaining testis left in situ. Eight adult males were subjected to HO as reported previously [13]. Surgery was performed between 0900 and 1200 h. Four monkeys were administered 1 ml a/s i.v. immediately and again ~6 h after surgery and thereafter once every 48 h for 30 days. The remaining four monkeys were treated with normal monkey serum (NMS; control serum) according to a protocol identical to that employed for a/s-treated monkeys. Blood samples were collected one day before and on 0.5, 1, 3, 5, 7, 14, 21, and 29 days after HO. Additional blood samples were collected 30 min before and 30 min after surgery, and on Days 30, 32, 36, and 40 of HO. Blood was collected by means of a vacutainer tube (Becton-Dickinson, Rutherford, NJ) via femoral vein/arterial punctures. To collect blood between 2100 and 2200 h, the 24-h L:D cycle was briefly interrupted with the aid of a 2-cell (1.5-volt) flashlight. Nocturnal samples were taken because T secretion is maximal at this point in the 24-h cycle of male bonnet monkeys [19].

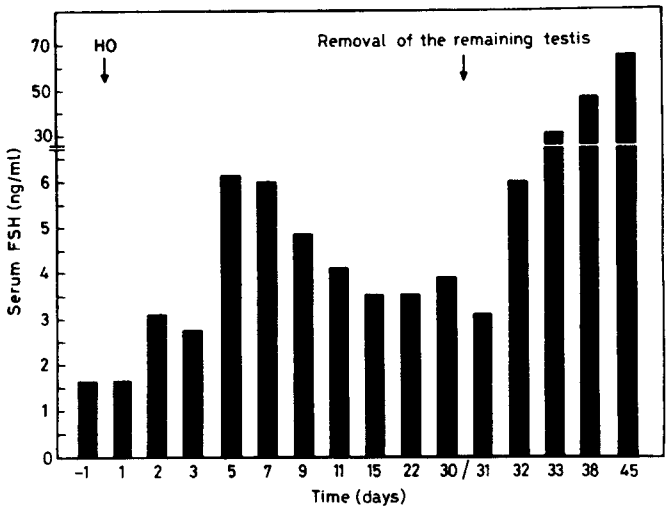

FIG. 1. Concentrations of circulating FSH in representative adult male bonnet monkey before HO, at various times after HO, and after removal of the remaining testis. Note that FSH concentrations became further elevated upon total castration.

Determination of Serum FSH Antibody Activity After Injection of a/s

The FSH binding activity of circulating antibody in serum of monkeys that received a/s treatment was determined as previously described [20]. Briefly, 100 µl of sera (diluted 1:50 with 0.01 M PBS, pH 7.4, containing 0.1% BSA) was incubated overnight with ^{125}I-hFSH (~100 000 cpm/tube) in a total volume of 500 µl. Bound tracer was precipitated with sheep anti-monkey IgG and 12.5% of polyethylene glycol (PEG 6000), and FSH binding activity was expressed as a percentage of total cpm added.

Processing of Testicular Tissue In Vitro

Testes were obtained at the time of HO and at the end of 30 days of HO according to a surgical procedure described earlier [13]. Upon removal from a monkey, each testis was placed in a beaker containing ice-cold saline, cleared free of the adhering fat tissue and the epididymis, and wiped with a filter paper to remove excess saline before being weighed. Testicular weight was expressed as grams wet weight. The testis was transferred to a Petri dish containing M-199 medium (Gibco Laboratories, Grand Island, NY), decapsulated, minced using a surgical blade, and subjected to collagenase cell dispersion essentially similar to the procedure previously reported by Klinefelter et al. [27]. The testicular cell suspension was washed several times with the medium to remove collagenase before being incubated with the necessary substrate and cofactor essential to identifying 3β-hydroxy steroid dehydrogenase (3β-HSD) activity in the Leydig cells, which was carried out according to the method described by Payne et al. [28]. The cells positive for 3β-HSD (3β-HSD+; blue-purple formazan granules) were counted in a Neubauer cell-counting chamber (Fein-Optik, Blankenburg, Germany) with use of a light microscope. The total number of 3β-HSD+ cells was expressed per gram wet weight of testicular tissue.

RESULTS

Serum FSH Concentration in HO Monkeys Treated with a/s or NMS

Before HO, mean serum FSH concentrations in a/s- and NMS-treated monkeys were 2.8 ± 0.3 and 1.95 ± 0.3 ng/ml, respectively. From the profile of serum FSH concentration in a representative adult monkey before HO, after HO, and after NMS treatment, it is evident that the serum FSH level increased within two days of HO, and by Day 5 was ~6-fold greater than pre-HO concentration (Fig. 1). Removal of the remaining testis led to a further (20-fold) increase in serum FSH concentration (Fig. 1). Mean circulating FSH concentrations before and on different days after HO in four monkeys that received NMS treatment showed a sustained increase in FSH concentration within two days of HO, and a concentration on Day 7 after HO 3–4-fold higher ($p < 0.05$) than the pre-HO concentration (1.95 ± 0.3 vs. 5.6 ± 0.7 ng/ml on Days −1 and 7, respectively, of HO). Although the mean serum FSH concentration declined by Day 30 of HO, it was still significantly higher ($p < 0.05$) than the pre-HO concentration (Fig. 2).

In monkeys that received a/s treatment, serum FSH concentrations could not be determined because circulating antibodies in the FSH RIA system interfered. The modified FSH assay for detecting free FSH in the presence of antibodies as outlined in *Materials and Methods* could not be employed since intact adult male bonnet monkeys normally have very low circulating FSH concentrations in contrast to

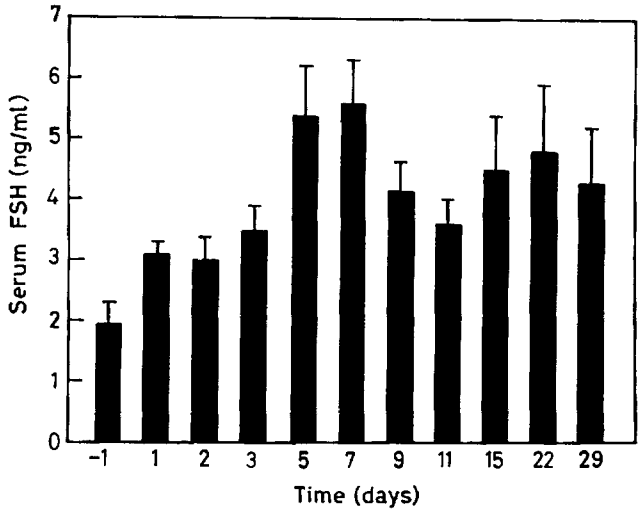

FIG. 2. Circulating FSH concentrations in adult male bonnet monkeys before and at various times after HO. NMS was injected i.v. immediately after HO every other day for 30 days. Two-way ANOVA followed by Student-Newman-Keuls test indicated that mean FSH concentrations were significantly ($p < 0.05$) higher than pre-HO levels on all days beginning Day 1 of HO. Values are mean ± SEM.

the very high concentrations in castrated males. After a/s treatment, FSH binding activity of serum, used here as a measure of antibody concentration, increased within 30 min of injection. The antibody level reached a plateau by Day 7 and was maintained at this concentration for the duration of treatment (Fig. 3). After discontinuation of a/s treatment on Day 30, FSH binding activity declined and became undetectable by Day 40 (Fig. 3).

Effect of a/s Treatment on HO-Compensating T Production

Circulating mean nocturnal serum T concentration before HO was 25.4 ± 1.4 and 22.8 ± 1.6 ng/ml for NMS- and a/s-treated groups of monkeys, respectively (Fig. 4). In monkeys that received NMS treatment after HO, although serum T concentration was decreased by 50% ($p < 0.05$) on Day 1, it rose to pre-HO levels by Day 7 of HO (25.4 ± 1.4 vs. 22.8 ± 2.6 ng/ml on Day −1 and Day 7 of HO, respectively; $p > 0.05$; Fig. 4). In striking contrast, in monkeys that received a/s treatment, circulating serum T concentration was lower ($p < 0.05$) than pre-HO values on Days 1, 2, 5, 7, 9, 11, and 15 of HO (Fig. 4). By Day 22, however, the mean T concentration, although still lower, was not significantly different from the pre-HO level ($p > 0.05$; 22.7 ± 1.6 vs. 19 ± 1.6 ng/ml on Days −1 and 22, respectively; Fig. 4). Serum T concentrations on Days 2, 5, 7, 9, 11, and 15 of HO in NMS-treated monkeys were higher ($p < 0.05$) than those observed on corresponding days in the a/s-treated monkeys (Fig. 4).

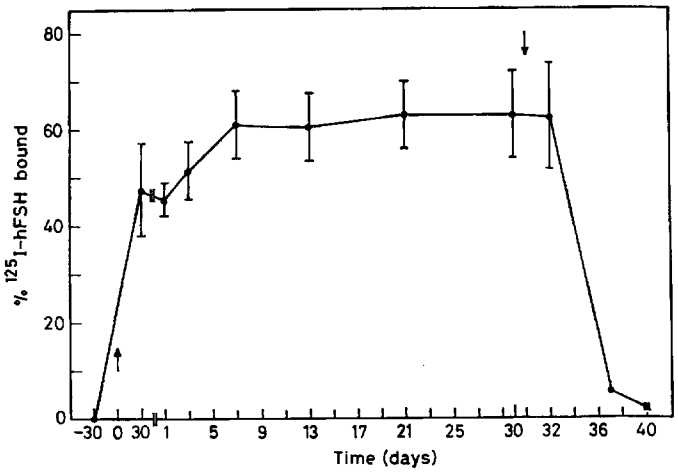

FIG. 3. FSH binding activity in serum of adult male bonnet monkeys before, during, and after 30 days of i.v. administration of FSH antiserum, initiated on Day 0 of HO and terminated on Day 30. Values are mean ± SEM.

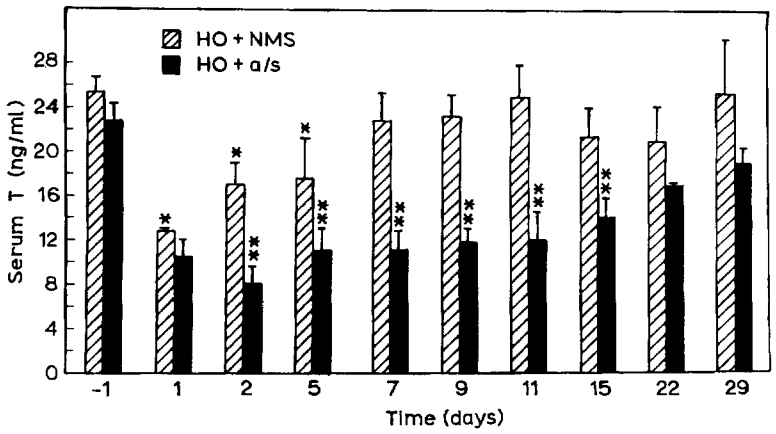

FIG. 4. Circulating serum T concentrations in adult male bonnet monkeys that received NMS or oFSH antiserum before and at various times after HO. For days shown, blood samples were collected between 2100 and 2200 h. * Values in NMS-treated monkeys differ significantly ($p < 0.05$) from pre- and post-HO values. ** Values in a/s-treated monkeys differ significantly ($p < 0.05$) from those in NMS-treated monkeys.

Effect of a/s Treatment on Testicular Weight and 3β-HSD+ Cell (Leydig Cell) Number in HO Monkeys

The testicular weights of individual monkeys within a group showed considerable variation. For comparison, the testicular weight of each monkey before HO (computed from the weight of the testis removed on Day 0) was set as 100, and the percentage of change that occurred by Day 30 of HO was calculated. The weight of the testis left in situ increased 36% ($p < 0.05$) within 30 days of HO in the NMS-treated group, both within the group as well as when compared to that of the corresponding testis of a/s-treated monkeys (Table 1).

In the NMS-treated monkeys, by Day 30 of HO the number of 3β-HSD + cells (Leydig cells) expressed per gram wet weight had increased threefold ($p < 0.05$) compared to that of the Day 0 (intact control) values. An increase in Leydig

cell number was also seen in HO + a/s-treated males, but this increment was markedly less ($p < 0.05$) than in the testis of HO + NMS-treated monkeys (Table 1).

Effect on Testicular Germ Cell Transformation Following a/s Treatment

Because no variation was observed in the percentage values of any of the germ cell populations between the NMS- and a/s-treated groups of monkeys on Day 0 of HO, for convenience the values for Day 0 from these groups were combined (Fig. 5). While no significant changes were observed between cell percentages of 2C, 1C, and 1 CC populations in the NMS- and a/s-treated groups, a significant reduction in the 4C population was observed at the end of 30 days of a/s treatment (Day 0, 13.1 ± 0.74; Day 30, 14.3 ± 0.9 and 6.3 ± 0.4 for NMS- and a/s-treated groups, respectively; $p < 0.05$; Fig. 5).

DISCUSSION

In species that respond to HO by alterations in the functional activity of the remaining testis left in situ, one of the key events that occurs after HO is the consistent increase in circulating FSH concentrations; serum LH concentrations are either unaffected [13] or elevated only transiently [29]. The results of the present study provide evidence that hypersecretion of FSH is involved in the rapid compensation of testicular function that occurs after HO. The role of FSH in enhancing testicular activity was assessed by administering to hemiorchidectomized monkeys a well-characterized FSH a/s in sufficient amounts to continuously neutralize endogenous FSH. We have successfully employed such an approach earlier to determine the role of endogenous FSH in regulating spermatogenesis [14, 16, 20] and follicular maturation [22] in adult bonnet monkeys. The dosage of a/s used was sufficiently high that during the entire period of a/s treatment, the presence of excess of antibody was titratable. Furthermore, the FSH-neutralizing ability of this particular batch of a/s was assessed by injecting it into castrated adult male monkeys. That the a/s used was biologically effective and the dosage was sufficient to immunoneutralize FSH in HO monkeys is further illustrated by a significant decrease (> 50%, $p < 0.05$) in the primary spermatocyte (4C) cell population observed upon a/s treatment. Our earlier study in intact adult male monkeys has shown this event to be highly sensitive to FSH deprivation [30]. In contrast, specific LH immunoneutralization leading to a drastic reduction in serum T levels was seen to immediately block the meiotic transformation of primary spermatocytes (4C) to round spermatids (1C) in intact adult male monkeys [31].

The current study has clearly shown that the compensatory increase in T production that occurs after HO is blocked to a marked extent after deprivation of endogenous FSH. The mechanism(s) by which FSH could stimulate the increase in T production from the remaining testis is not yet clear. It is open to question whether the increased FSH enhances Leydig cell responsiveness via paracrine factors secreted by the Sertoli cell. Peritubular cells and macrophages in the testis are also believed to have FSH receptors. We are currently not aware how the function of these cells are affected by FSH deprivation, and it is not clear whether the factors secreted by these cells have any regulatory role in Leydig cell function.

The observation in the current study that the 3β-HSD+ cell (Leydig cell) population increased markedly after HO, though based on subjective analysis, is perhaps important. The increased T concentration correlates with the increase in the number of these cells after HO. The fact that upon neutralization of endogenous FSH the number of 3β-HSD-stainable cells and T production were significantly reduced suggests that FSH has a role in enhancing Leydig cell num-

TABLE 1. Day 30 testicular weight and 3β-HSD+ cell numbers from intact control, HO + NMS-, and HO + a/s-treated adult male bonnet monkeys.

Treatment	Testicular weight (% change over control)	3β-HSD+ cells (expressed per g wet wt of the tissue; × 10^3)
Pre-HO control		4515 ± 1448
HO + NMS	+ 35.8 ± 9.3[a]	13 426 ± 1813[c]
HO + a/s	− 07.2 ± 3.5[b]	8959 ± 542[d]

Values are mean±SEM. Values with different superscripts differ ($p < 0.05$) from each other.

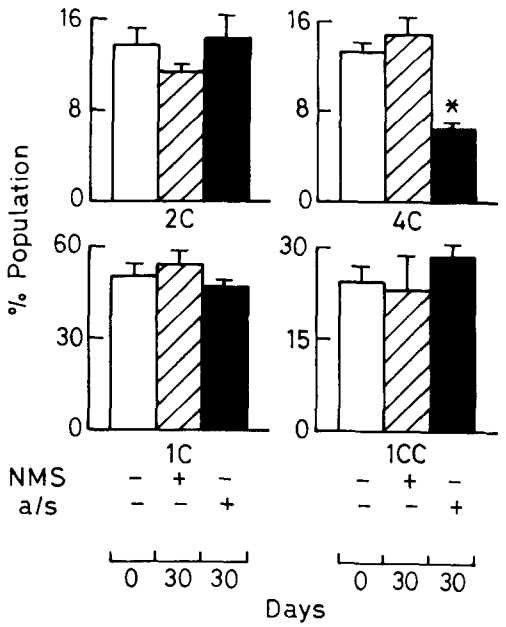

FIG. 5. Changes in percentages of testicular germ cell populations in adult bonnet monkeys before (open bars) and on Day 30 following HO + NMS (cross-hatched bars) or HO + a/s (solid bars) treatment. Values are mean ± SEM. * Indicates a significant reduction ($p < 0.05$) in 4C population. For details of germ cell populations, see *Materials and Methods.*

bers. A detailed morphometric analysis of the testes from control and a/s-treated monkeys, however, is required to unequivocally establish the actual increment in Leydig cell numbers.

The Leydig cells in the testicular interstitium are generally considered to be terminally differentiated; in this case cell division is rarely, if at all, encountered [32]. Prolonged treatment with gonadotropins has been shown to result in an increase in Leydig cell numbers, but dividing interstitial cells arise from the differentiation of mesenchymal cells [33, 34]. However, more recently it has been shown that while LH maintains Leydig cell volume and differentiated function, it may not be required to maintain Leydig cell numbers in the adult rat testis [35, 36]. Several studies indicate that FSH plays an important role in the transformation of mesenchymal cells to Leydig cells prior to the onset of puberty [37, 38], or in the increase in Leydig cell number in pubertal and adult rats [39, 40]. It is well known that FSH stimulates the function of Sertoli cells, and these in association with germ cells secrete factors/peptides that effect stimulatory action on Leydig cell steroidogenesis [41, 42]. Irrespective of whether FSH increases the Leydig cell number or its steroidogenic capacity, it is clear from this study that FSH plays an important role in the intratesticular control of steroidogenesis, perhaps through a paracrine mechanism.

In the present study, the demonstration that HO-mediated increased testicular weight is also prevented by immunoneutralization of FSH seems to indicate that FSH is involved in the hyperplasia and/or hypertrophy of testicular cells. Studies employing in utero neutralization of FSH as well as ablation of the fetal pituitary have suggested a major role for FSH in proliferation of Sertoli cells in fetal rams [4]. Consistent with the above observation, the increase in testicular weight that occurs in rats and ram lambs subjected to HO prior to the onset of puberty has been reported to be associated with increased division of Sertoli cells [6, 41, 42]. That the increased proliferation of Sertoli cells as measured by [^{3}H]thymidine incorporation indeed occurs after HO is demonstrable as early as 8 h after surgery in prepubertal rats [43]. Furthermore, the earlier the HO is performed after birth in rats, the more pronounced is its effect on testicular weight, a finding consistent with the observation that Sertoli cell proliferation is programmed to continuously decrease after birth [43, 44]. In addition to hyperplasia of Sertoli cells, some studies have also found hypertrophy of Sertoli cells in response to HO in sheep [6, 45], indicating that both hyperplasia and hypertrophy might occur in the remaining testis. Another line of evidence, albeit indirect, suggesting that increased proliferation of Sertoli cells contributes to the increased testicular weight following HO is the observation that passive immunoneutralization of LHRH in either intact or HO prepubertal rats leads to decreased testicular weight, whereas the same treatment is ineffectual when performed after puberty [46]. It is not known whether Sertoli cells in the present study underwent hypertrophy or hyperplasia or both. The proliferative activity of germ cells has been observed to increase in the remaining testis ([^{3}H]thymidine incorporation in the HO + NMS-treated monkeys showed an increment, data not shown) resulting in near-normal sperm output by the single testis [13]. If constancy in relationship between Sertoli and germ cell numbers is to be maintained, hyperplasia of Sertoli cells must be invoked as a means of facilitating development of a larger number of germ cells [47, 48]. In conclusion, the results of the current study indicate that FSH plays a major role in the HO-induced compensatory activity of the remaining testis.

ACKNOWLEDGMENT

Our thanks are due to Dr. S.G. Ramachandra for his assistance in surgery.

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