

Increased numbers of Sertoli and germ cells in adult rat testes induced by synergistic action of transient neonatal hypothyroidism and neonatal hemicastration

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The combined effects of transient neonatal hypothyroidism and neonatal hemicastration were investigated to see whether they were additive. Hypothyroidism was induced in litters of ten male rats for 25 days from the day of birth by administration of 0.1% (w/v) 6-propyl-2-thiouracil in the mother's drinking water; hemicastration was performed on the day of birth. Controls included both normal and sham-operated animals. Numbers of Sertoli cells and round spermatids were quantified at age 135 days using stereological methods. Sham-operation had no effect on testis mass, or numbers of Sertoli or germ cells. Transient neonatal hypothyroidism resulted in an increase in testicular mass of 27% ($P < 0.05$), whereas neonatal hemicastration resulted in a 33% ($P < 0.05$) increase over control; the combination of the two procedures resulted in a 62% ($P < 0.05$) increase. There were corresponding significant increases in the number of Sertoli cells: 82% with hypothyroidism, 18% with hemicastration and 123% with the combination of the two procedures. Numbers of round spermatids showed similar increases: 59% with hypothyroidism, 45% with hemicastration and 95% with the combination of the two procedures. It is concluded that the effects of the combination of transient neonatal hypothyroidism and hemicastration are additive with respect to testicular mass, and numbers of Sertoli and germ cells.

Introduction

There is increasing evidence that Sertoli cells, by their involvement in the formation of the blood–testis barrier, control the intratubular environment in which spermatogenesis proceeds (de Kretser, 1987). Normally, the Sertoli cells cease proliferating by day 20 postnatally and constitute a stable cell population in the seminiferous epithelium (Orth, 1982; Wang *et al.*, 1989; van Haaster *et al.*, 1992). Several studies have suggested that the number of Sertoli cells limits the spermatogenic output of the testis, raising the concept that each individual Sertoli cell can support a finite number of germ cells (Orth *et al.*, 1988; Berndtson and Thompson, 1990). Orth *et al.* (1984) used the model of neonatal hemicastration, which results in a significant increase in serum FSH concentrations and a subsequent increase in the number of Sertoli cells and spermatogenic output from the remaining testis (Ojeda and Ramirez, 1972; Cunningham *et al.*, 1978; Orth *et al.*, 1984; Kosco *et al.*, 1987).

The number of Sertoli cells can be significantly increased (84%) by the induction of transient neonatal hypothyroidism (TNH) between the day of birth and day 25 (van Haaster *et al.*, 1992; Hess *et al.*, 1993). This model results in the extension of the period of Sertoli cell division from day 20 to 30 and leads, in adults, to a 62% increase in testis mass and a marked increase

in daily sperm production (Cooke and Meisami, 1991; Cooke *et al.*, 1991). Although serum concentrations of testosterone are unchanged during the period of hypothyroidism, serum concentrations of FSH, LH, GH and prolactin are decreased (Cooke and Meisami, 1991; Kirby *et al.*, 1992).

To ascertain whether increased concentrations of FSH could further augment the increase in number of Sertoli cells, we increased FSH concentrations by subjecting rats under the TNH regimen to hemicastration on the day of birth. Our studies demonstrate that the increased FSH concentrations that occur after neonatal hemicastration synergise with the transient neonatal hypothyroidism to augment further the number of Sertoli cells and spermatogenic output of adult testes.

Materials and Methods

Animals and treatments

One-day-old male Sprague–Dawley rats were obtained from Central Animal Services of Monash University and raised in litters of ten rats with one mother in individual boxes. The rats were maintained under controlled conditions of temperature (21°C) and lighting (12 h light:12 h dark). The rats were assigned to six groups ($n = 7$) as (I) control, (II) sham-operated alone, (III) hemicastrated alone, (IV) TNH, (V) sham-operated and TNH and (VI) hemicastrated and TNH.

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Hemicastration was performed on the day of birth under hypothermia-induced anaesthesia (Orth *et al.*, 1984). The right testis in each rat was removed via a small incision immediately above the superficial inguinal ring. In the sham-operated group, rats were similarly anaesthetized and a comparable incision was made without interference to the testis. Wounds were sutured using 6–0 silk suture (Ethicon, North Ryde, NSW).

All rats were provided with dry pellets (GR-2; Barstoc Stockfeeds Pty Ltd, Pakenham, Victoria) and water *ad libitum*. TNH groups received 0.1% (w/v) 6-propyl-2-thiouracil (PTU; Sigma Chemical Co., St Louis, MO) in their mother's drinking water from day 1 to day 25 as described by Cooke and Meisami (1991). The drinking water was sweetened with the artificial sweetener, aspartame (18 mg l⁻¹; Equal, Searle, Crows Nest, NSW) to offset the bitter taste due to PTU. All rats were weighed weekly and weaned at 26 days of age for controls and 30 days of age for TNH groups. Rats were killed at 10, 20, 30 and 135 days of age.

Tissue preparation

Rats were injected subcutaneously with 15 units g⁻¹ body mass of heparin (Heparin injection BP, Commonwealth Serum Laboratories, Melbourne) 30 min before they were anaesthetized by ether inhalation. Testes were fixed by vascular perfusion with Bouin's fixative. The descending thoracic aorta was cannulated and flushed with normal saline and, when all the testicular vasculature was clear, fixation was achieved with Bouin's fixative. The testes were then removed and weighed. Tissues were postfixed in the same solution for about 24 h, cut into 2–3 mm thick transverse slices and kept in 70% (v/v) ethanol until processed. Slices were divided in half and a hemislice from each alternate slice was sampled and embedded in hydroxyethylmethacrylate (Technovit 7100; Heraeus Kulzer GmbH, Wehrheim), sectioned at 25 µm and stained with periodic acid–Schiff/haematoxylin.

Histology and stereology

Three sections per animal were analysed using the 'optical disector' method (Braendgaard *et al.*, 1990). Sections were viewed using a Panasonic WV-F15E video camera (Matsushita Electric Industrial Co. Ltd, Osaka) mounted on an Olympus (BH2) microscope. Fields were sampled using a systematic uniform random scheme generated using a motorized stage (Lang GMBH & Co., Huttenberg). Starting at the upper left corner of the section, the stage was moved in 675 µm steps along the *x*-axis until the section was no longer in the field. The stage was then moved 675 µm along the *y*-axis and the process repeated until the section was completely sampled. Sections were viewed using a 100× S. Plan Apo (1.4 NA) objective at a final screen magnification of × 2708. GRID 1.2 (software by Graffiti data, Silkeborg) was used, and a set of four unbiased counting frames (Gundersen, 1977) of total area 1468 µm² was superimposed on each field image using an Amiga 2000 computer equipped with an Impact Vision 24 Professional video adapter (Great Valley Products Inc, King of Prussia, PA). The microscope was equipped with a microcator (Heidenhain-

Metro MT12, Traunreut) and a bidirectional counter (Heidenhain VRZ 401, Traunreut). Germ and Sertoli cells were counted by focusing through the section, starting approximately 2 µm from the top (guard volume). Nuclei were counted as their equatorial region came into clear focus. A depth of 12 µm was counted. The relationship between testis mass and processed volume was determined as described by Yang *et al.* (1990). In practice, 1 g of Bouin's fixed testis was equivalent to 1000 mm³ of processed tissue.

Hormonal measurements

Blood was collected by cardiac puncture. After clotting, serum was separated by centrifugation (500 g, 10 min) and stored at -20°C. Total serum thyroxine concentrations were measured with a coated tube radioimmunoassay ([¹²⁵I]T₄-CT RIA, ICN Biomedical Inc., Costa Mesa, CA). The lower limit of detection in this assay ranged between 3.63 and 5.22 ng ml⁻¹ and the intra- and interassay coefficients of variation were 6.9–10.6 and 10.1%, respectively.

Serum concentrations of FSH were measured using a specific radioimmunoassay using reagents supplied by NIDDK (Bethesda, MD). This assay uses NIDDK-anti-rFSH-S-11 antiserum, NIDDK-rFSH-RP-2 as standard and iodinated NIDDK-rFSH-I-8 as tracer. The sensitivity of the assay ranged between 2.01 and 2.19 ng ml⁻¹ and the intra- and interassay coefficients of variation were 4.1–4.5% and 7.4%, respectively.

Serum concentrations of immunoreactive inhibin were measured by a radioimmunoassay as described by Robertson *et al.* (1988). The antiserum (No. 1989), raised to bovine 31 kDa inhibin, crossreacts 288% with pro α_c (Robertson *et al.*, 1988) and thus measures both dimeric inhibin and α subunit products. This assay uses iodinated 31 kDa bovine inhibin as tracer. Results are expressed in terms of a rat ovarian extract standardized against bovine inhibin, such that 1 unit = 0.37 ng human recombinant inhibin. The sensitivity of this assay ranged between 0.91 and 0.97 U ml⁻¹ and the intra- and interassay coefficients of variation were 4.0–4.1% and 9.5%, respectively.

Statistical analyses

Statistical comparisons were made using SIGMASTAT V.1 (Jandel Scientific Software, San Rafael, CA). Data were compared using a one-way ANOVA in conjunction with the Bonferroni *t* test for all pairwise multiple comparisons. Differences were considered significant when *P* < 0.05. Data are presented as the mean ± SEM.

Results

Body and testis masses

Body masses of the PTU-treated groups were significantly lower (*P* < 0.05) than controls during PTU administration and failed to reach control values even at 135 days of age, at which time their body masses were still approximately 20% lower

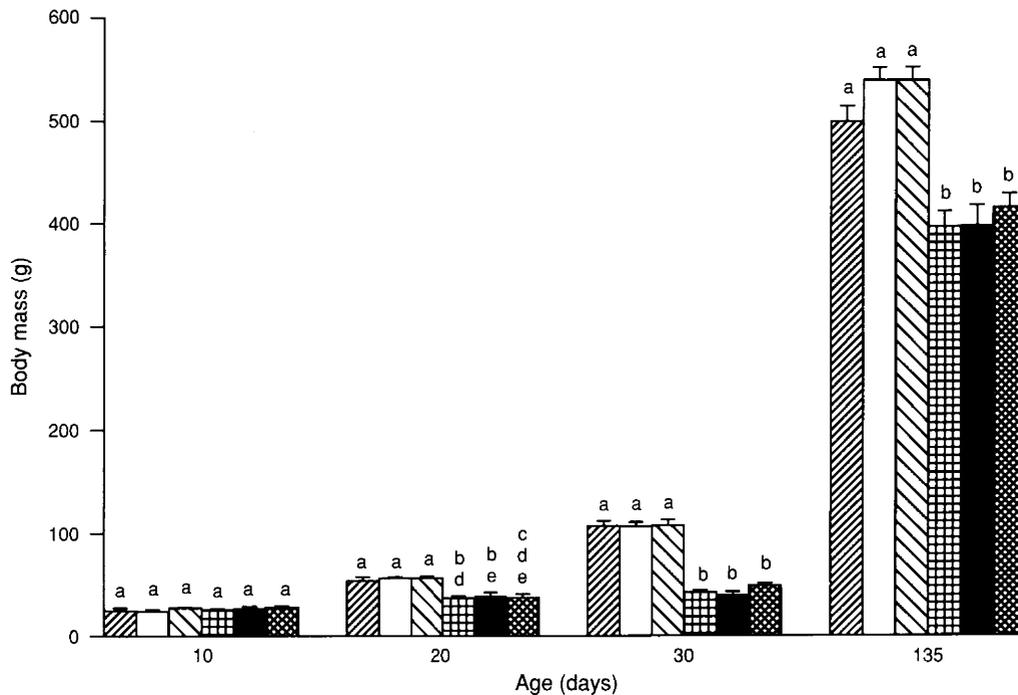


Fig. 1. Body mass as a function of age in (▨) control, (□) sham-operated, (▩) hemicastreated, (▧) TNH (transient neonatal hypothyroidism), (■) sham-operated–TNH and (▦) hemicastreated–TNH rats. All TNH groups had reduced body mass at 20, 30 and 135 days. Within each age group, groups with different letters are significantly different ($P < 0.05$).

Table 1. Testis masses (g) in each experimental group are shown (mean \pm SEM; $n = 7$ rats per group)

Group	Age (days)			
	10	20	30	135
Control	0.023 \pm 0.002 ^a	0.098 \pm 0.008 ^a	0.423 \pm 0.016 ^a	1.95 \pm 0.045 ^a
Sham-operated	0.024 \pm 0.002 ^{ac}	0.110 \pm 0.005 ^a	0.394 \pm 0.034 ^a	2.05 \pm 0.089 ^{ad}
Hemicastreated	0.036 \pm 0.001 ^b	0.176 \pm 0.009 ^b	0.603 \pm 0.039 ^b	2.59 \pm 0.045 ^{bf}
TNH	0.022 \pm 0.001 ^a	0.054 \pm 0.002 ^{cd}	0.098 \pm 0.007 ^c	2.48 \pm 0.059 ^{bde}
Sham-operated–TNH	0.023 \pm 0.002 ^a	0.065 \pm 0.003 ^{cc}	0.087 \pm 0.004 ^c	2.41 \pm 0.144 ^{acf}
Hemicastreated–TNH	0.034 \pm 0.003 ^{bc}	0.081 \pm 0.009 ^{ade}	0.185 \pm 0.013 ^c	3.16 \pm 0.176 ^c

Within a column, groups without a common superscript are significantly different ($P < 0.05$).
TNH: transient neonatal hypothyroidism.

than control values. Neither hemicastration nor sham-operation had any effect on body mass (Fig. 1).

The testicular masses of the TNH and the sham-operated–TNH groups showed a similar pattern in that they were significantly lower than control values at 20 and 30 days of age. Hemicastration of TNH rats resulted in slightly (but not significantly) higher testis masses at 20 and 30 days of age compared with the TNH and the sham-operated–TNH groups, but they were still below the control value (not significantly at 20 days of age, but significantly at 30 days of age). At 135 days of age, the testis masses of the TNH and the sham-operated–TNH groups were significantly greater ($P < 0.05$), being 127 and 124% of control and sham-operated control masses, respectively. The hemicastration of TNH-treated rats resulted in greater mean testis masses, 162% of control

(3.16 \pm 0.18 g) ($P < 0.05$) at 135 days of age. Hemicastration alone resulted in significant increases of 57% at 10 days after surgery, and 81%, 42% and 33% at 20, 30 and 135 days of age, respectively, compared with controls (Table 1).

Effects on numbers of Sertoli cells and round spermatids

Number of Sertoli cells. The total number of Sertoli cells per testis in control rats at 135 days of age was $38.0 \pm 1.45 \times 10^6$ per testis. Animals that had experienced TNH had an 82% increase ($P < 0.05$) in Sertoli cells ($69.3 \pm 0.92 \times 10^6$ per testis), while the hemicastreated animals had an 18% increase ($P < 0.05$) ($44.7 \pm 0.41 \times 10^6$ per testis) in comparison with controls. The combination of neonatal hemicastration and TNH resulted in a greater increase of 123% ($P < 0.05$) to $84.90 \pm 2.50 \times 10^6$ per

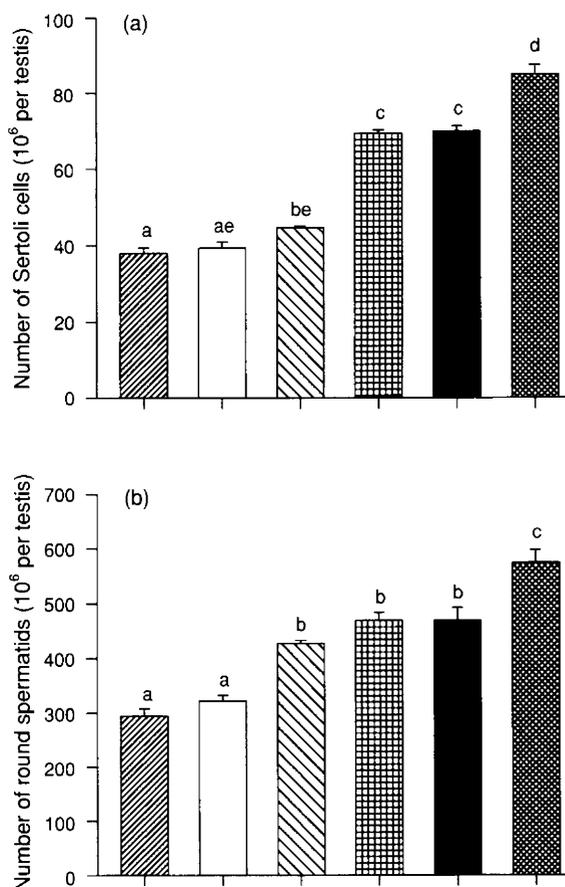


Fig. 2. (a) Number of Sertoli cells at 135 days in (▨) control, (□) sham-operated, (▧) hemicastreated, (▩) TNH (transient neonatal hypothyroidism), (■) sham-operated-TNH and (▤) hemicastreated-TNH rats. Sertoli cell number was elevated in all TNH and hemicastreated groups. (b) Number of round spermatids at 135 days in control, sham operated, hemicastreated, TNH, sham-operated-TNH and hemicastreated-TNH rats. The number of round spermatids was increased in all TNH and hemicastreated groups. Groups with different letters are significantly different ($P < 0.05$).

testis compared with controls. Sham-operation and the combination of sham-operation and TNH had no significant effects on numbers of Sertoli cells (39.4 ± 1.55 and $70 \pm 1.27 \times 10^6$ per testis) compared with control and TNH rats, respectively (Fig. 2a).

Number of round spermatids. The same pattern of increases was observed for the number of round spermatids per testis as for Sertoli cells. The number of round spermatids per testis in control rats at 135 days of age was $294.5 \pm 13.33 \times 10^6$ per testis. Rats that had experienced TNH had a 59% increase ($P < 0.05$) in the number of round spermatids ($468.5 \pm 15.08 \times 10^6$ per testis), while the hemicastreated animals had a 45% increase ($P < 0.05$) ($426.7 \pm 14.2 \times 10^6$ per testis) compared with controls. The combination of neonatal hemicastration and TNH resulted in a higher increase of 95% ($P < 0.05$) to $574.7 \pm 22.36 \times 10^6$ per testis. Sham operation and the combination of sham operation and TNH had no significant effects on numbers of round spermatids (322.2 ± 10.23 and $469.6 \pm 21.80 \times 10^6$ per

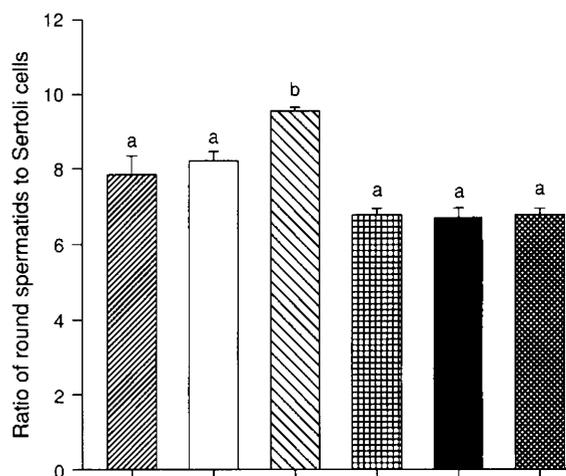


Fig. 3. Ratio of round spermatids to Sertoli cells at 135 days in (▨) control, (□) sham-operated, (▧) hemicastreated, (▩) TNH (transient neonatal hypothyroidism), (■) sham-operated-TNH and (▤) hemicastreated-TNH rats. The ratio was significantly elevated in hemicastreated rats. Groups with different letters are significantly different ($P < 0.05$).

testis) compared with control and TNH rats, respectively (Fig. 2b).

Ratio of round spermatids to Sertoli cells. Although there were increases in numbers of both Sertoli cells and round spermatids, the ratio of round spermatids to Sertoli cells at 135 days of age was not significantly different between the control, the TNH, the sham-operated-TNH and the hemicastreated-TNH groups (7.84 ± 0.50 , 6.77 ± 0.18 , 6.70 ± 0.25 and 6.77 ± 0.17 , respectively). However, in the hemicastreated group, the ratio of round spermatids to Sertoli cells was significantly ($P < 0.05$) higher (9.55 ± 0.10) in comparison with other groups (Fig. 3).

Serum concentrations of hormones

Hypothyroidism was confirmed by the demonstration of significantly ($P < 0.05$) decreased serum thyroxine in TNH, hemicastreated-TNH, and sham-operated-TNH groups at 10, 20 and 30 days of age in comparison with control rats. At 135 days of age, thyroxine concentrations of all groups were similar (Fig. 4a).

Serum concentrations of immunoreactive inhibin were significantly lower in the three hypothyroid groups (TNH, hemicastreated-TNH, and sham-operated-TNH) at 10 and 20 days in comparison with control rats. Subsequently, at 30 and 135 days there were no significant differences between groups. The concentrations in all groups at 135 days were lower than at 10, 20 and 30 days (Fig. 4b).

Serum concentrations of FSH at 10 days were significantly ($P < 0.05$) greater in the hemicastreated and the hemicastreated-TNH groups than in all other groups at that age, but not significantly different between those two groups. The FSH concentrations in the hemicastreated alone group remained high at 20 and 30 days, but the FSH concentrations in the hemicastreated-TNH group declined to the concentrations

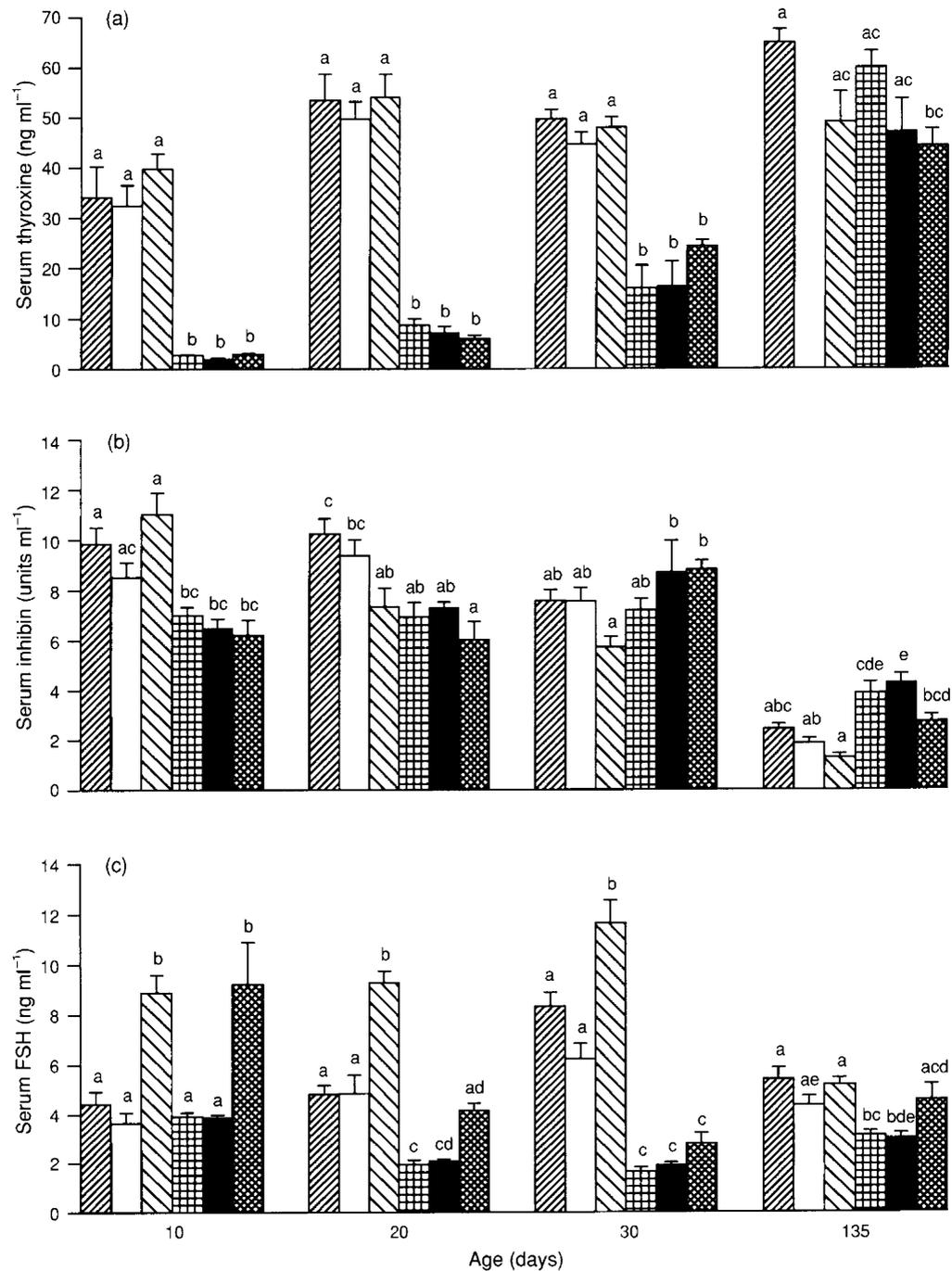


Fig. 4. Serum concentrations of (a) thyroxine, (b) inhibin and (c) FSH as a function of age in (▨) control, (□) sham-operated, (▤) hemicastrated, (▥) TNH (transient neonatal hypothyroidism), (■) sham-operated-TNH and (▩) hemicastrated-TNH rats. Within each age group, groups with different letters are significantly different ($P < 0.05$). The serum thyroxine concentrations in the TNH groups at 10 days are below the sensitivity of the assay. Serum thyroxine concentrations were significantly lower in all TNH-treated animals at 10, 20 and 30 days. Measurements of thyroxine in the sham-operated group at 135 days were not available. Serum inhibin concentrations were lower in all TNH groups at 10 and 20 days. Serum FSH concentrations were lower in all TNH intact rats at 10, 20 and 30 days. Hemicastration resulted in a significant increase of FSH concentration compared with the corresponding control at all ages.

in the control and the sham-operated groups at 20 days and were significantly ($P < 0.05$) lower than these groups at 30 days. At 20 and 30 days, the serum concentrations of FSH in

the TNH and the sham-operated-TNH groups were significantly lower ($P < 0.05$) than in the controls and the sham-operated controls. At 135 days, the FSH concentrations in the

TNH and the sham-operated–TNH groups were significantly lower than all other groups (Fig. 4c).

Discussion

The study demonstrates that increased concentrations of FSH resulting from neonatal hemicastration can synergize with the effect of neonatal hypothyroidism to increase the number of Sertoli cells and the spermatogenic output of adult testes.

Transient neonatal hypothyroidism alone can raise the number of Sertoli cells by extending the duration of Sertoli cell proliferation from about 20 days to about 30 days after birth. This extended duration of Sertoli cell proliferation is responsible for a larger testicular mass and greater testicular sperm production in adult life (van Haaster *et al.*, 1992). However, these changes are achieved in the presence of significantly lower FSH concentrations, for reasons that are not clear (Kirby *et al.*, 1992; van Haaster *et al.*, 1992). The data presented here confirm the effects of transient neonatal hypothyroidism on numbers of Sertoli cells and, although the degree of testicular hypertrophy was not as marked, the measurements of thyroxine concentrations confirmed the achievement of neonatal hypothyroidism. Furthermore, these data support the observation that the increase in numbers of Sertoli cells and round spermatids occurs in the presence of lower than normal serum FSH concentrations (Kirby *et al.*, 1992; van Haaster *et al.*, 1992). In these experiments numbers of Sertoli cells and round spermatids were quantified at 135 days. Cooke and Meisami (1991) observed maximum testis size at day 160. However, in the experience of the authors of this report, there was no further increase in testis mass in TNH rats between 135 (2.48 ± 0.16 g) and 160 days (2.56 ± 0.24 g).

These results also confirm the FSH rise that occurs transiently after neonatal hemicastration of TNH rats, and the resultant increase in the number of Sertoli cells that results in testicular hypertrophy and an increase in the spermatogenic output of the adult testis. The present study, in which hemicastrated rats were treated with PTU, demonstrates that a further increment in the number of Sertoli cells and spermatogenic output is achieved in adults. It is likely that this effect is the result of the transient increase of FSH concentration that occurs after hemicastration and emphasizes two important points. First, the Sertoli cells in hypothyroid rats can still respond to FSH by a further increment in number of cells; second, the hypothalamo–hypophyseal axis in the hypothyroid rat can respond, with an increase in FSH concentrations, to a reduction of the feedback signal from the testis as achieved by hemicastration. This increased FSH concentration in the hemicastrated–TNH group is apparent at 10 days, reduced but still significantly higher than the hypothyroid rats at 20 days, but no different at 30 days. The last observation suggests that the prolonged hypothyroid state (30 days) interferes to a greater extent with FSH secretion, since the FSH concentrations of hemicastrated rats are still significantly high at day 30. The reason for the impairment of FSH secretion in TNH rats remains unclear.

These results confirm the lowered serum concentrations of immunoreactive inhibin present at days 10 and 20 of TNH, but

the increased FSH concentrations in the hemicastrated state are not reflected by higher inhibin concentrations in the hemicastrated–TNH state. This result suggests that the hypothyroid state impairs the ability of the Sertoli cells to secrete inhibin even in the presence of greater FSH concentrations. The nature of this impaired Sertoli cell function is not known, but it is suggested that triiodothyronine influences directly the state of Sertoli cell differentiation acting through the thyroid hormone receptors (Bunick *et al.*, 1994). Cooke *et al.* (1994) demonstrated that the addition of triiodothyronine to Sertoli cell cultures derived from 5-day-old animals inhibits Sertoli cell division and facilitates their maturation, as indicated by the pattern of the mRNA production. The ability of FSH to stimulate Sertoli cell division in normal animals depends on the time at which its concentration increases. Hemicastration experiments indicate that there is a relatively short period corresponding to the period during which Sertoli cell division is occurring. In the hypothyroid animal, the delay in Sertoli cell maturation apparently extends the period during which this stimulus can operate.

Although the total spermatogenic output of the TNH groups was increased, the ratio of the number of round spermatids to Sertoli cells was significantly lower in the groups that underwent TNH treatment in comparison with hemicastration alone (9.55 versus 6.77, 6.70 and 6.77). This raises the possibility that, although TNH can increase the number of Sertoli cells, the capacity of the Sertoli cell to ‘nurture’ germ cells may be impaired by the neonatal disturbance. Alternatively the delayed maturation of the Sertoli cell associated with TNH has resulted in a submaximal ratio at this time.

This was the first stereological study of the testis in which the ‘optical disector’ approach has been used to quantify the number of Sertoli and germ cells. Hardy *et al.* (1989) and Mendis-Handagama and Ewing (1990) used the ‘physical disector’ approach to quantify interstitial cells, and van Haaster *et al.* (1992, 1993) used the same approach to quantify the number of Sertoli cells in the hypo- and hyperthyroid model, in which they obtained comparable numbers of Sertoli cells per testis in the control animals. The ‘physical disector’ approach involves matching fields on sequential physical sections and is extremely time consuming. The ‘optical disector’ approach is much more efficient, as thick plastic sections are simply optically sectioned to count cells.

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