

Response of serum inhibin B and pro- α C levels to gonadotrophic stimulation in normal men before and after steroidal contraceptive treatment

Kati L. Matthiesson^{1,2}, David M. Robertson^{1,2}, Henry G. Burger^{1,2} and Robert I. McLachlan^{1,2,3}

¹Prince Henry's Institute of Medical Research, Monash Medical Centre and ²Department of Obstetrics and Gynaecology, Monash University, Clayton, Victoria 3168, Australia

³To whom correspondence should be addressed at: Prince Henry's Institute of Medical Research, PO Box 5152, Clayton, Victoria 3168, Australia. E-mail: rob.mclachlan@med.monash.edu.au

BACKGROUND: Testicular regulation of inhibin B may be influenced by the germ cell complement. **METHODS:** We examined the effects of gonadotrophin stimulation on serum inhibin B and pro- α C in 25 normal men at (i) control (stimulation test 1), (ii) after spermatogenic suppression induced by testosterone plus progestin treatment (stimulation test 2), and (iii) during spermatogenic recovery induced by FSH and/or hCG treatment (stimulation test 3). For each test, subjects received a single injection of 1200 IU FSH or 5000 IU hCG or both. **RESULTS:** Inhibin B and pro- α C fell with spermatogenic suppression (75 and 51% of pre-treatment baseline respectively, $P < 0.05$). Inhibin B response to FSH (130–144%) was similar in controls and after germ cell suppression. Pro- α C response after germ cell suppression compared with control was significantly increased ($P < 0.05$) with both FSH (210–229% versus 140–185%) and hCG (254–261% versus 145%). All treatments partially restored spermatogenesis with no clear relationship apparent between inhibin B and sperm count. **CONCLUSIONS:** We conclude that: (i) serum inhibin B and pro- α C are only partially gonadotrophin dependent, (ii) spermatogenic suppression does not modify inhibin B response to FSH but enhances pro- α C response to both FSH and hCG, and (iii) inhibin B is a poor marker of spermatogenesis in this model of gonadotrophic manipulation in normal men.

Key words: FSH/inhibin/LH/Sertoli cell/testosterone

Introduction

Inhibin exists as two biologically active forms, inhibin A and B, which share a common α -subunit linked to either a β A- or β B-subunit by a disulphide bond (Vale *et al.*, 1988). Inhibin B is the primary form found in the adult male (Anawalt *et al.*, 1996). It is now recognized that there is a reciprocal relationship between serum FSH and inhibin B (Anawalt *et al.*, 1996; Illingworth *et al.*, 1996; Anderson, 2001; Hayes *et al.*, 2001; Meachem *et al.*, 2001). In addition to the dimeric inhibin forms, the partially processed form of the free α -subunit (pro- α C) is found in the circulation. The testis is the primary circulatory source of both inhibin B and pro- α C as orchidectomy results in very low or non-detectable levels (Anawalt *et al.*, 1996).

In-situ hybridization and immunocytochemical studies have revealed different patterns of α - and β B-subunit expression in the tissue depending on the developmental age and species studied (Bergh and Cajander, 1990; Forti *et al.*, 1992; Vannelli *et al.*, 1992; Majdic *et al.*, 1997; Andersson *et al.*, 1998). Using immunocytochemical methods, the α -subunit is localized to human spermatocytes,

Sertoli and Leydig cells, while the β B-subunit is localized to Sertoli and Leydig cells and more controversially to spermatocytes and early spermatids (Bergh and Cajander, 1990; Forti *et al.*, 1992; Vannelli *et al.*, 1992; Majdic *et al.*, 1997; Andersson *et al.*, 1998).

The secretion of inhibin is controlled by many factors including gonadotrophins and intrinsic factors involving Sertoli, Leydig and germ cells. In normal men, a single large dose (3000 IU) of recombinant human FSH (rhFSH) led to a doubling of serum inhibin B levels, reaching a peak within 72 h (Anawalt *et al.*, 1996) with more recent studies showing that a dose of 225 IU was ineffective (Kinniburgh and Anderson, 2001). A dose-response study showed that administration of rhFSH (1000, 2000 and 3000 IU) led to significant increases in inhibin B, with the later two doses showing a significantly greater area under the curve than placebo or 1000 IU rhFSH (Kamischke *et al.*, 2001). Both FSH and hCG have been shown to significantly increase pro- α C levels (Kamischke *et al.*, 2001; Kinniburgh and Anderson, 2001).

It is also recognized that the relationship between serum FSH and inhibin B is modulated by other factors. Several

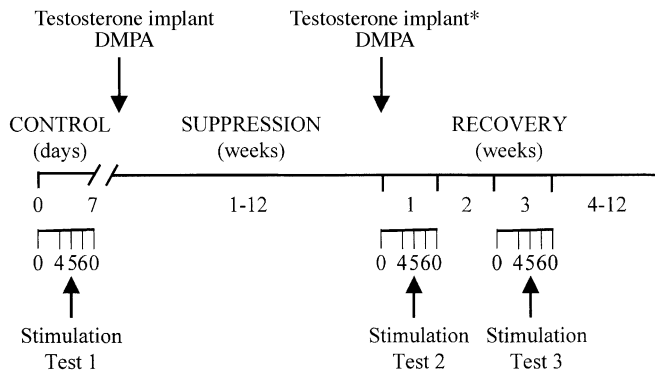


Figure 1. Study protocol: 25 men enrolled in control phase, 15 men re-randomized for remainder of study protocol. Six week minimum interval between control and suppression phases. *Only FSH-alone-treated men received a second testosterone (T) implant at the onset of the recovery phase. Recovery was achieved over a 12 week period with either FSH 300 IU twice weekly or hCG 5000 IU weekly or a combination of FSH plus hCG. DMPA = depot medroxyprogesterone acetate.

studies have shown that inhibin B levels fall markedly following severe testicular insults (chemotherapy, irradiation) correlating with the disappearance of germ cells (Wallace *et al.*, 1997; Foppiani *et al.*, 1999; Petersen *et al.*, 1999). Similar findings have been observed in rats following methoxyacetic acid treatment (Allenby *et al.*, 1991). On the other hand, gonadotrophic suppression following exogenous sex steroid administration led to little or only a partial suppression of serum inhibin levels (Anderson *et al.*, 1997; Zhengwei *et al.*, 1998; Buchter *et al.*, 1999; Martin *et al.*, 2000; McLachlan *et al.*, 2002) with no relationship with either the duration of treatment or the extent of suppression of sperm count (Anderson *et al.*, 1997). The basis for this differential effect may be that the extent of inhibin B suppression is related to the degree of damage to the spermatogenic process with toxic agents leading to a more profound loss of germ cells, compared with that achieved with gonadotrophin suppression.

To examine the potential influence of germ cells on inhibin release from the testis (of both Sertoli and Leydig cell origin) we examined the acute effects of FSH and/or LH (using hCG as an LH substitute) administration on serum inhibin B and pro- α C levels in normal men, and compared this response with that seen following spermatogenic suppression induced by a regimen developed for the purpose of hormonal contraception (Handelsman *et al.*, 1996). In addition we wished to examine the relationships between spermatogenic recovery, induced by FSH and/or hCG treatment, and serum inhibin B.

Methods and materials

Subjects

Twenty-five healthy men were recruited through media advertisement to participate in this study conducted at Prince Henry's Institute of Medical Research in accordance with the

guidelines of the Southern Healthcare Network Human Research Ethics Committee. Of these 25 men, 15 were enrolled to participate in both the suppression and recovery phases. All men underwent medical interview, physical examination and biochemical investigations. Subjects were required to fulfil each of the following criteria: (i) age 21–45 years, (ii) normal physical findings and normal testicular volumes, (iii) two normal semen analyses (World Health Organization, 1999); (iv) normal serum testosterone, sex hormone-binding globulin, FSH, LH, prolactin and estradiol levels, (v) normal liver and renal function, serum lipids and full blood examination. Men with a past history of hypertension, significant cardiovascular, renal, hepatic, prostatic and testicular disease or infertility were excluded. Men with a past history of drug abuse or who were taking significant prescribed medications were also excluded, as were men undertaking competitive sport who were subject to testing for androgen usage. Informed consent was obtained from all participants.

Study design

Phase 1: Responses of serum inhibin B and pro- α C to single dose gonadotrophin administration. Development of stimulation test protocol. Stimulation test 1

The overall study design is displayed in Figure 1. Twenty-five men were randomized into five groups ($n = 5/\text{group}$) to receive a single injection of one of the following: rhFSH (either Gonol-F, Serono, Sydney, Australia; or Puregon, Organon, Sydney, Australia) 600, 1200, 2400 IU s.c.; hCG (Profasi, Serono; or Pregnyl, Organon) 5000 IU s.c.; or FSH 1200 IU plus hCG 5000 IU. Blood was drawn (0800–1100 h) daily for 8 days beginning on the day prior to the injections in order to cover the likely period of inhibin rise (Anawalt *et al.*, 1996).

Phases 2 and 3: Effects of spermatogenic suppression and recovery on serum inhibin B and pro- α C responses to exogenous gonadotrophins. Stimulation tests 2 and 3

After an interval of ≥ 6 weeks, 15 men went on to be randomized into three groups ($n = 5/\text{group}$). All men initially received an 800 mg testosterone implant (4 \times 200 mg subdermal, Organon) and depot medroxyprogesterone acetate (DMPA) 300 mg i.m. (2 ml at 150 mg/ml, Upjohn Pharmaceuticals, Sydney, Australia). Following a 12 week suppression phase, all men received a second dose of DMPA (150 mg) to ensure continued gonadotrophin suppression during a further 12 weeks of recovery treatment with either FSH (300 IU twice weekly s.c.) alone, hCG alone (5000 IU weekly s.c.) or a combination of both. Those men receiving only FSH also received a second 800 mg testosterone implant at the beginning of the restoration phase in order to maintain androgen levels in the normal range for the duration of the 12 week-induced recovery period.

For assessing the stimulatory response of serum inhibin, single doses of 1200 IU FSH alone or 5000 IU hCG or a combination of the two were administered by the investigators on day 0 in weeks 1 and 3 of the recovery phase. These doses were chosen based on the data from phase 1 with blood being obtained for each stimulation test on days 0, 4, 5 and 6. The

Table I. Baseline characteristics of the subjects at enrolment

Group	No. of men	Age (years)	Combined testis volume (ml)	Semen volume (ml)	Sperm concentration ($\times 10^6/ml$)	Serum FSH (IU/l)	Serum LH (IU/l)	Serum testosterone (nmol/l)	Serum B inhibin B (ng/l)	Serum pro- α C (ng/l)
Phase 1										
FSH 600 IU	5	27.0 \pm 3.2	51.0 \pm 3.9	4.6 \pm 1.0	81.2 \pm 7.5	3.6 \pm 0.5	5.0 \pm 0.5	20.1 \pm 2.2	125 \pm 12.4	35.6 \pm 5.6
FSH 1200 IU	5	38.4 \pm 3.5	61.0 \pm 4.0	3.5 \pm 0.6	103 \pm 39.5	4.2 \pm 1.2	2.8 \pm 0.5	16.5 \pm 3.3	191 \pm 43.5*	42.8 \pm 8.5
FSH 2400 IU	5	28.8 \pm 3.4	48.6 \pm 2.0	3.4 \pm 0.6	114 \pm 18.0	2.7 \pm 0.6	4.2 \pm 1.0	17.8 \pm 3.2	162 \pm 18.4	34.1 \pm 6.0
hCG 5000 IU	5	36.6 \pm 3.1	52.6 \pm 3.8	3.1 \pm 0.7	60.6 \pm 13.1	3.4 \pm 0.3	2.5 \pm 0.3	14.5 \pm 1.7	116 \pm 16.4	46.5 \pm 15.0
FSH 1200 IU plus hCG 5000 IU	5	35.4 \pm 3.6	52.4 \pm 5.9	2.9 \pm 0.7	97.0 \pm 24.5	3.6 \pm 0.4	4.3 \pm 0.6	17.3 \pm 2.1	141 \pm 20.9	26.1 \pm 3.1
Phase 2										
FSH 1200 IU	5	32.4 \pm 4.4	50.8 \pm 2.3	2.7 \pm 0.45	106 \pm 19.4	3.5 \pm 0.8	2.8 \pm 0.2	13.8 \pm 2.6	127 \pm 21.7	31.1 \pm 4.8
hCG 5000 IU	5	39.8 \pm 1.7	50.4 \pm 4.8	4.4 \pm 1.0	70.6 \pm 11.3	3.5 \pm 0.5	3.6 \pm 0.9	17.6 \pm 6.4	73.3 \pm 21.4*	36.8 \pm 7.7
FSH 1200 IU plus hCG 5000 IU	5	31 \pm 4.2	51.6 \pm 3.5	3.8 \pm 0.40	74.0 \pm 20.5	3.6 \pm 1.18	5.3 \pm 0.8	16.3 \pm 2.4	65.0 \pm 16.3*	34.2 \pm 5.4

Values are mean \pm SEM.* $P < 0.05$, baseline inhibin B was significantly higher in phase 1.

FSH 1200 IU treatment group compared with phase 2

FSH 1200 IU plus hCG 5000 IU and hCG 5000 IU treatment groups.

control response (stimulation test 1) was used for comparison with those obtained after spermatogenic suppression (stimulation test 2) and week 3 in recovery (stimulation test 3). In the remaining weeks of the recovery phase (i.e. 2 and 4–12), men receiving FSH had their FSH doses administered on day 0 by the investigators and self-administered their second FSH dose on day 4 of each week. Men receiving hCG had their dose administered on day 0 of each week by the investigators. Semen analyses and blood samples were obtained weekly throughout the recovery phase.

Baseline characteristics for participants are shown in Table I. Following 12 weeks of gonadotrophin stimulation, men were followed at regular intervals to monitor recovery of FSH, LH and sperm counts.

Assays

Serum FSH and LH were measured by microparticle enzyme immunoassay (Abbott Diagnostics, North Chicago, Illinois, USA). The sensitivity of the FSH assay was 0.5 IU/l with an inter-assay variation of 4.4–4.9%. The sensitivity of the LH assay was 0.5 IU/l with an inter-assay variation of 5.2–6.4%. Serum FSH and LH were also determined using sensitive immunofluorometric assays (IFMA) (Robertson *et al.*, 2001) with assay sensitivities of 0.01 and 0.005 IU/l for FSH and LH respectively. All samples were assayed in single assays.

Serum hCG was measured in an automated immunoassay carried out on a Dade Behring Dimension RxL Clinical Chemistry system with reagents and calibrators supplied by Dade Behring Diagnostics (Sydney, Australia). The assay sensitivity was 1.0 IU/l with an inter-assay variation of 3.9–6.1%. Testosterone was measured by an automated chemiluminescent immunoassay (Chiron Diagnostics, East Walpole, MA, USA) with a sensitivity of 0.3 nmol/l and inter-assay variations of 5.5–9.9%.

The pro- α C enzyme-linked immunosorbent assay (ELISA) (Groome *et al.*, 1995) was employed using reagents [INPRO monoclonal antibody (Mab) as capture and R1 Mab as label] and a standard reference preparation provided by Oxford Bio-Innovation Ltd (Upper Heyford, Oxon, UK). The sensitivity of the assay was 2 ng/l. The within-assay variation and the between-assay variation were 8–9% (eight assays) and 16.5% (eight assays) respectively. The inhibin B ELISA method (Groome *et al.*, 1996) was employed using kit reagents and inhibin B standard reference provided by Oxford Bio-Innovation. The sensitivity of the assay was 4 ng/l. The within-assay and between-assay variations were 5–8% (eight assays) and 15% (eight assays) respectively.

Statistical analyses

Data are shown as mean \pm SEM or mean percentage baseline. Statistical comparisons were made using Sigma Stat (SPSS Inc., Chicago, IL, USA). All data were log-transformed prior to analysis. Non-parametric statistics were used

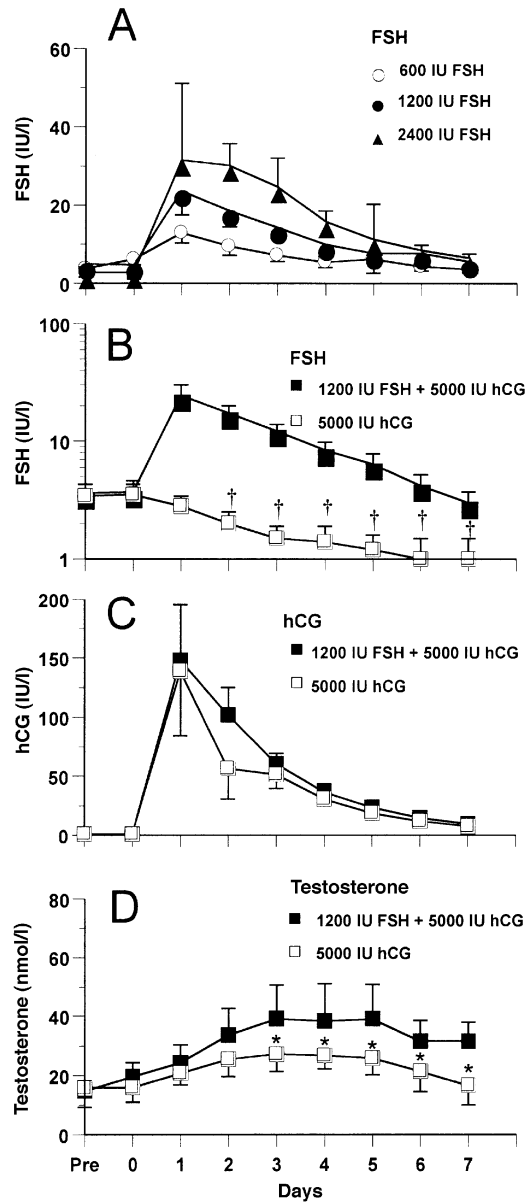


Figure 2. Changes in serum hormone levels following a single injection of FSH (600, 1200, 2400 IU), hCG (5000 IU) or combination FSH (1200 IU) plus hCG (5000 IU). Serum FSH (A, B), hCG (C) and testosterone (D) are shown daily over the week following injection (day 0). Pre-treatment levels were taken during initial evaluation. Serum FSH significantly decreased over days 2–7 with hCG treatment ($\dagger P < 0.001$). Serum testosterone increased ($*P < 0.05$) in FSH plus hCG group compared with hCG-alone group. Note log scale for the y-axis in B. Data are shown as mean \pm SEM, $n = 5$ /group.

when equal variance testing failed. Serum hormone and semen data were analysed by either repeated measures analysis of variance (ANOVA) or Friedman repeated measures ANOVA followed by Tukey test to examine differences across time between treatment groups. Differences in percentage baseline inhibin B (day 0 compared with days 4–6) and pro- α C (day 0 compared with days 4–5) in stimulation tests 1, 2 and 3 in each treatment group was evaluated using one-way ANOVA.

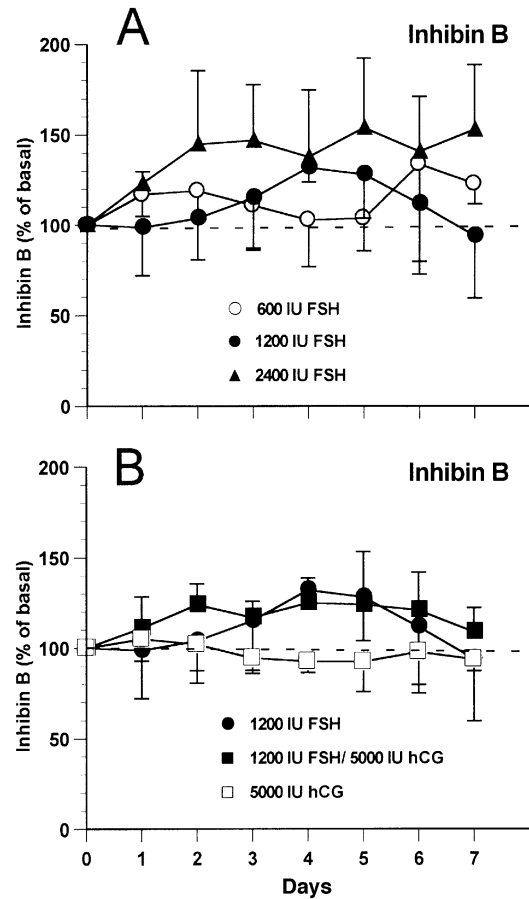


Figure 3. (A) Changes in serum inhibin B levels in subjects administered a single dose of FSH (600, 1200, 2400 IU). A significant increase was observed between day 0 and days 2–7 ($P < 0.05$) for FSH (2400 IU) treatment group and day 0 and day 4–5 ($P < 0.05$) for FSH (1200 IU) treatment group. Data shown as percentage baseline mean \pm SEM, $n = 5$ /group. (B) Changes in serum inhibin B levels in subjects administered a single dose of FSH (1200 IU), FSH (1200 IU) plus hCG (5000 IU) or hCG (5000 IU). No significant differences were seen in the FSH (1200 IU) plus hCG (5000 IU) or hCG (5000 IU)-alone groups. Data shown as percentage baseline mean \pm SEM, $n = 5$ /group.

Results

Phase 1

Serum gonadotrophins and testosterone levels

Dose-related increases were seen in serum FSH in response to FSH stimulation (Figure 2A). Serum FSH levels fell significantly over days 2–7 in response to hCG stimulation ($P < 0.001$), presumably reflecting increased inhibition from the rise in serum testosterone (Figure 2B). There was a significant augmentation of the serum testosterone response to hCG by the co-administration of 1200 IU FSH with both a higher peak level (27.2 ± 2.6 versus 39.1 ± 5.1 nmol/l on day 3) and a more prolonged period of elevation (hCG plus FSH group elevated above baseline at day 7) (Figure 2D).

Serum inhibin B and pro- α C

Serum inhibin B and pro- α C responses to treatments are shown in Figures 3 and 4 with data expressed as percentage of

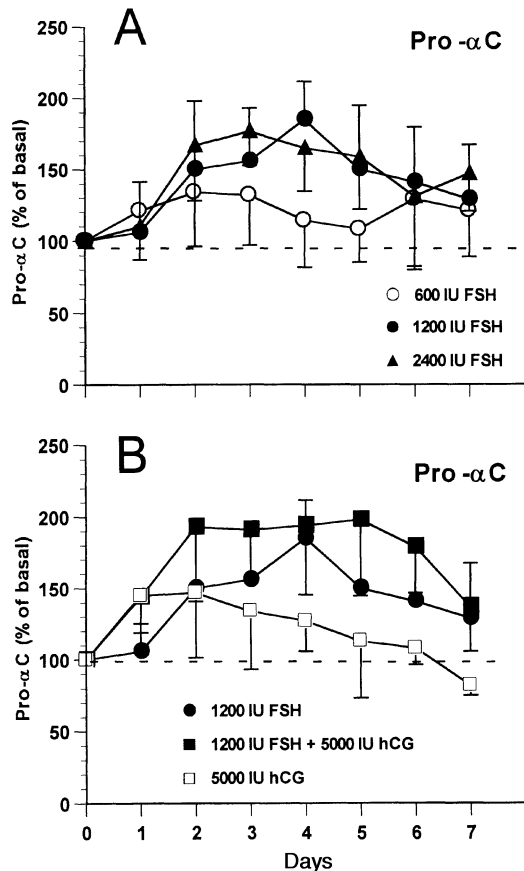


Figure 4. (A) Changes in serum pro- α C levels in subjects administered a single dose of FSH (600, 1200, 2400 IU). Significant increases were observed between day 0 and days 2–6 ($P < 0.05$) for FSH 1200 and 2400 IU treatment groups. Data shown as percentage baseline, mean \pm SEM, $n = 5$ /group. (B) Changes in serum pro- α C levels in subjects administered a single dose of FSH (1200 IU), FSH (1200 IU) plus hCG (5000 IU) or hCG (5000 IU). Pro- α C levels were significantly increased relative to day 0 in the FSH (1200 IU) plus hCG (5000 IU) group between days 1–5 ($P < 0.01$) and days 6–7 ($P < 0.05$), and in the hCG (5000 IU) treatment group between days 0 and days 1–2 ($P < 0.01$) and 3–4 ($P < 0.05$). Data shown as percentage baseline mean \pm SEM, $n = 5$ /group.

baseline. Serum inhibin B was significantly increased with 1200 and 2400 IU FSH on days 4–5 (130% of baseline) and 2–7 (138–154% baseline) respectively. Pro- α C levels significantly increased with 1200 and 2400 IU FSH on days 2–6 (140–185% baseline, Figure 4A, B). hCG alone did not affect inhibin B levels but led to a rapid rise in pro- α C by day 1 (145% baseline, Figure 4B). Co-administration of FSH and hCG led to an apparent additive increase in pro- α C levels on days 4–5 (195–198% baseline, Figure 4B). No change in the inhibin B profile was seen by co-administration of hCG to 1200 IU FSH (Figure 3B).

Phases 2 and 3

Serum gonadotrophins and testosterone levels

Testosterone plus DMPA treatment markedly suppressed serum FSH and LH levels ($P < 0.05$) to the limit of assay detection (only FSH data shown, Figure 5A). Similar peak FSH and hCG levels were seen with stimulation tests 2 (5.4–5.8 and

27.0–28.6 IU/l) and 3 (5.1–5.6 and 31.0–33.5 IU/l) on day 4 (Figure 5A, B). FSH levels measured by the sensitive FSH IFMA in the hCG treatment group were markedly suppressed but remained detectable across the recovery phase with mean levels ranging from 0.025 IU/l (0.79% control baseline) in week 3 to 0.13 IU/l (4% control baseline) by week 7. LH levels determined by the sensitive LH IFMA in the FSH treatment group fell to the limit of the assay (0.005 IU/l, 0.16% control baseline) and remained suppressed for the duration of the study (sensitive assay FSH and LH data not shown). Testosterone levels remained within the physiological range except at some time-points in the FSH-alone-treated group which received a second testosterone implant at the onset of the recovery phase in order to prevent hypogonadism (Figure 1). Co-administration of FSH with hCG as compared with hCG alone appeared to augment the testosterone response with a statistically significant higher testosterone level being seen with FSH plus hCG treatment ($P < 0.05$) in week 7. Comparison of testosterone levels in the FSH-alone group was not analysed due to the use of a second testosterone implant in this group (Figure 5C).

Serum inhibin B and pro- α C

In all 15 men receiving testosterone plus DMPA treatment for 12 weeks, significant falls ($P < 0.001$) were seen in both mean serum inhibin B (88.7 ± 13.5 versus 61.6 ± 6.9 ng/l, 75% pre-treatment baseline) and pro- α C levels (34.0 ± 3.4 versus 14.8 ± 1.9 ng/l, 51% pre-treatment baseline). Changes in serum inhibin B and pro- α C for each treatment group are shown as a percentage of pre-treatment levels (Figure 6A, B). Inhibin B levels declined significantly with hCG stimulation to a nadir by week 3, day 5 (41% pre-treatment baseline, $P < 0.001$). On the other hand, FSH alone and FSH plus hCG led to a prompt increase in serum inhibin B levels (Figure 6A). All treatments led to a rise in serum pro- α C (Figure 6B).

Inhibin B and pro- α C responses to gonadotrophic stimulation following spermatogenic suppression (stimulation test 2) and week 3 in recovery (stimulation test 3) are shown in Figure 7A, B. Inhibin B and pro- α C, day 0 test values in each stimulation test were used for assessment of the gonadotrophic response. In stimulation test 2, only FSH-alone treatment led to a significant rise in inhibin B by day 5 (144%, $P = 0.038$). Pro- α C was significantly raised over days 4–6 with both hCG (254–261%, $P < 0.001$) FSH-alone (210–229%, $P < 0.001$) and treatments thereafter remaining stable. Combined FSH plus hCG treatment also resulted in an increase in pro- α C although not reaching significance. In stimulation test 3, no significant changes in either inhibin B or pro- α C levels were seen for any treatment group.

Comparison of the gonadotrophin stimulatory responses of serum inhibin B (days 4–6) and pro- α C (days 4–5) were calculated using the baseline day 0 value for each of the three stimulation tests. Administration of hCG alone had no stimulatory effect on inhibin B at any time-point. Pro- α C responses to hCG (119 ± 10 versus 279 ± 53 versus $127 \pm 31\%$) and FSH (158 ± 21 versus 225 ± 20 versus $113 \pm 10\%$) stimulation were significantly ($P < 0.05$) greater following spermatogenic suppression (stimulation test 2) compared

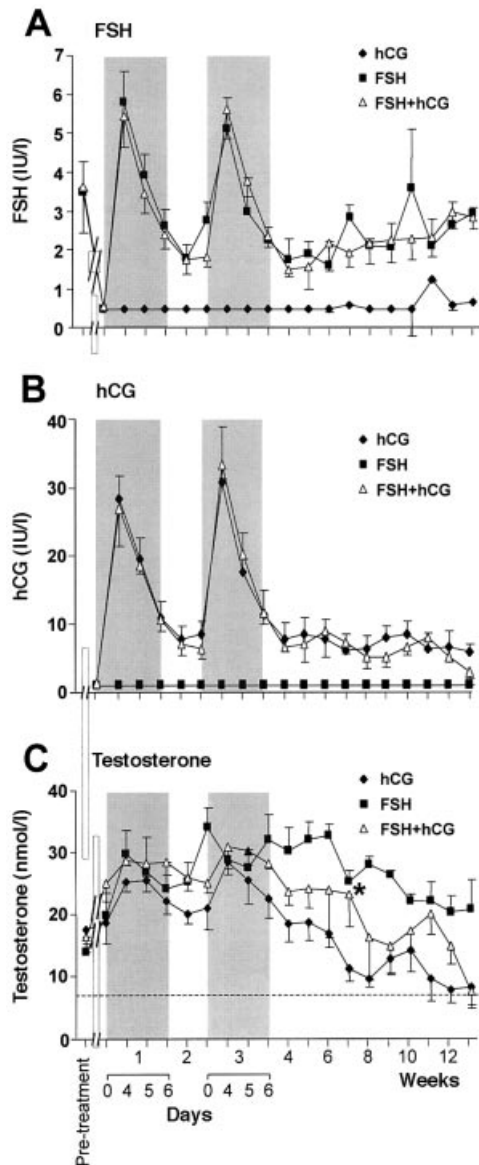


Figure 5. Changes in serum FSH (A), hCG (B) and testosterone (C) during induced spermatogenic recovery with FSH, hCG or combined FSH plus hCG treatment. FSH-alone treatment group received a second testosterone implant at the onset of the recovery phase. * $P < 0.05$ between the testosterone levels in the FSH plus hCG and hCG treatment groups. Shaded portion indicates stimulation tests 2 and 3. The horizontal dashed line refers to the lower limit of the normal reference range for testosterone. Data shown as mean \pm SEM, $n = 5$ /group.

with control (stimulation test 1) and week 3 in induced recovery (stimulation test 3).

Spermatogenic suppression and recovery

Testosterone plus DMPA treatment led to marked suppression of spermatogenesis (0.08 – 0.21×10^6 /ml) in all 15 men (Figure 6C). All recovery treatments led to a partial restoration in spermatogenesis with sperm counts in the hCG group ranging from 0.21 to 8.4×10^6 /ml, in the FSH group between 0.07 and 10×10^6 /ml and in the combined

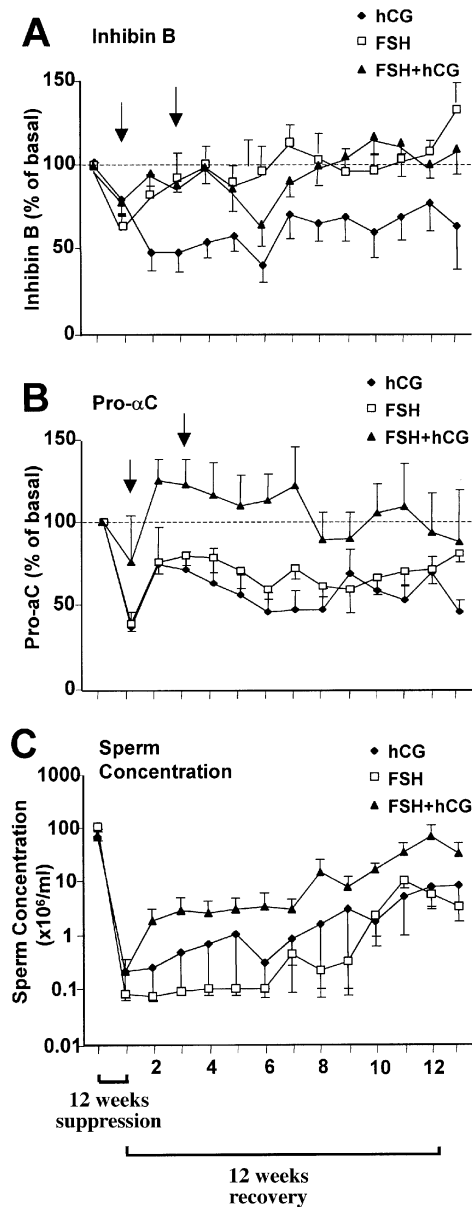


Figure 6. (A and B) Changes in serum inhibin B (A) and pro- α C (B) during 12 weeks induced recovery of spermatogenesis with FSH, hCG or combined FSH plus hCG treatment. Vertical arrows refer to stimulation tests 2 and 3. The week 1 time-point represents day 0, prior to gonadotrophin stimulation. Only day 0 data shown. Data shown as percentage of baseline, mean \pm SEM, $n = 5$ /group. (C) Changes in sperm concentration following testosterone plus depot medroxyprogesterone acetate (12 week suppression phase) and either FSH, hCG or combined FSH plus hCG treatment for 12 weeks to induce spermatogenic recovery. Data shown as percentage baseline, mean \pm SEM, $n = 5$ /group.

group between 0.21 and 69×10^6 /ml. However, despite all groups showing partial restoration of spermatogenesis, serum inhibin B levels either fell with hCG, or returned toward control levels with FSH alone or combined with hCG. No clear relationship was apparent between serum inhibin B levels and sperm counts over the 12 week-induced recovery phase (Figure 6A, C).

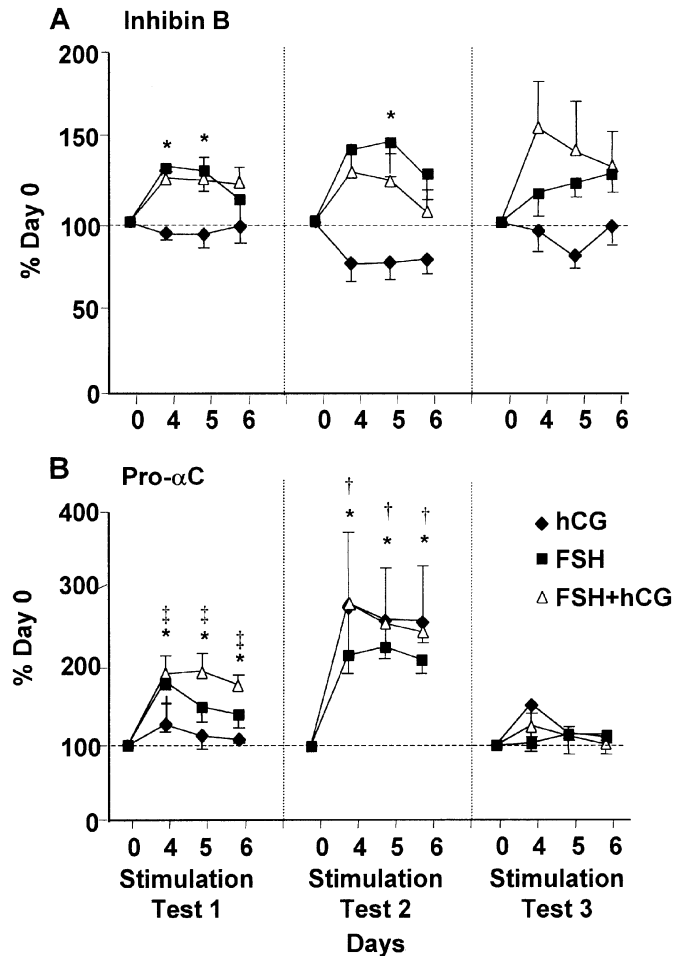


Figure 7. (A and B) Gonadotrophin stimulation of serum inhibin B (A) and pro- α C (B) during the control phase (stimulation test 1), after 12 weeks of spermatogenic suppression (stimulation test 2) and during the third week of gonadotrophic stimulation (stimulation test 3). In stimulation tests 1 and 2, a significant increase in inhibin B with FSH was observed between day 0 and days 4–5 ($P < 0.05$) and between day 0 and day 5 ($P = 0.038$) respectively. FSH treatment in stimulation tests 1 and 2 produced significant increases in serum pro- α C between day 0 and days 4–6 ($P < 0.05$ and 0.001 respectively). hCG treatment in stimulation test 1 showed a significant increase in pro- α C levels between day 0 and 4 ($P < 0.05$) and in stimulation test 2 between day 0 and days 4–6 ($P < 0.001$). Pro- α C levels were significantly increased relative to day 0 in the FSH plus hCG group between days 4–5 ($P < 0.01$) and day 6 ($P < 0.05$) for stimulation test 1 only. Data shown as percentage day 0, mean \pm SEM, $n = 5$ /group. * $P < 0.05$ versus day 0 of respective test for FSH group, † $P < 0.05$ versus day 0 of respective test for hCG group and ‡ $P < 0.01$ – 0.05 versus day 0 of respective test for FSH plus hCG group.

Recovery and adverse events

Recovery to pre-study baseline was monitored in 13 of the 15 participants, one of the subjects in the FSH plus hCG group discontinued at 6 weeks for personal reasons. Thirteen men recovered normal sperm counts by 12 months. One man in the hCG group discontinued follow-up with a last recorded sperm count of 13×10^6 /ml at 14 months post treatment. FSH, LH and testosterone levels recovered in all 14 men between 1 and 12 months. Three men required additional testosterone replace-

ment (1–2 injections of testosterone esters 100–250 mg) in the recovery phase. Two participants in the suppression phase developed mild gynaecomastia, one transiently, the other having persistent 1 cm non-tender unilateral enlargement. No other serious adverse events occurred.

Discussion

This study has investigated the serum inhibin B and pro- α C responses of the normal testis to gonadotrophin stimulation, and is the first to explore the impact of the suppression of germ cell number on their release. We have shown that the suppression of spermatogenic cells does not modify the acute inhibin B response to FSH. Indeed serum inhibin B appears to be a poor marker of spermatogenesis in this model of experimental gonadotrophic manipulation. Only a 25% decline in serum inhibin B was observed even when germ cell numbers were markedly reduced, resulting in severe oligo- or azoospermia (McLachlan *et al.*, 2002), and serum FSH levels were suppressed to $<4\%$ of baseline levels. In terms of the maximal inhibin B secretory response of the normal testis to gonadotrophin treatment, only an $\sim 40\%$ increase over baseline was seen and this increment was not modified by spermatogenic suppression. Others have also shown that inhibin B levels are only partially FSH dependent (Anawalt *et al.*, 1996; Nachtigall *et al.*, 1996; Seminara *et al.*, 1996; Anderson *et al.*, 1997; Buchter *et al.*, 1999; Martin *et al.*, 2000).

There is considerable evidence that germ cells modify inhibin responses in both animal (Pineau *et al.*, 1989, 1990; Maddocks *et al.*, 1992) and human models (Carreau, 1995; Andersson *et al.*, 1998). The mechanism of this effect is unclear but germ cells may regulate the synthesis and incorporation of the β B subunit into dimeric inhibin by the Sertoli cell, the rate-limiting step in inhibin B synthesis (Clifton *et al.*, 2002).

In normal men, a positive relationship is recognized between serum inhibin and sperm output (Jensen *et al.*, 1997; Klingmuller and Haidl, 1997). Therefore in our model of spermatogenic suppression in normal men, one could postulate several possible impacts of germ cells on the Sertoli cell inhibin B secretory response. The most likely relationship would be that germ cells potentiate the ability of the Sertoli cell to secrete inhibin B, resulting in decreased secretion in the setting of germ cell depletion. Alternatively, if germ cells inhibit Sertoli cell inhibin B secretion, then in the setting of germ cell depletion, there may be an increase in its secretion, either basally or in response to FSH. Finally if there are non-germ cell factors modulating the ability of the Sertoli cell to secrete inhibin B, then there may be no significant change in Sertoli cell inhibin B response.

We have shown that the combined reduction in germ cell number and suppression of gonadotrophins resulted in significant falls in inhibin B and pro- α C. However, germ cell depletion in normal young men did not modify the ability of the Sertoli cell inhibin B response to gonadotrophins. Comparison of the inhibin B response with FSH showed no significant differences between control (stimulation test 1) and following germ cell suppression (stimulation test 2).

The hypothesis that specific germ cell types might regulate inhibin in normal men (Pineau *et al.*, 1990; Allenby *et al.*, 1991; Guitton *et al.*, 2000; Clifton *et al.*, 2002) is difficult, if not impossible, to assess as specific and complete loss of germ cell subtypes cannot be achieved. Germ cell types are suppressed to a variable degree by chronic gonadotrophin suppression in man (McLachlan *et al.*, 2002). Type A and B spermatogonia were suppressed to ~70 and ~20% of control levels respectively, with spermatocytes, round and elongated spermatids remaining at 20%, indicating that the type A pale to B spermatogonial maturation is the key site of effect. The reduction of sperm counts to levels <1% of control is in large part attributed to the failure of sperm release (McLachlan *et al.*, 2002). In contrast, iatrogenic spermatogenic failure, e.g. by cancer treatments, generally induces total spermatogenic failure, although these treatments may also alter Sertoli/Leydig cell function and interaction.

Alteration in Sertoli cell inhibin secretion in our model could also have resulted from either a chronic FSH deficiency modifying the ability of the Sertoli cells to respond to acute FSH stimulation and/or to the effect of changes in the germ cell complement. We attempted to address this issue by repeating the stimulation test in week 3 of FSH-induced recovery, by which time we speculated that any FSH-related defect in Sertoli cell function would have been corrected and that there would still be a low germ cell complement. However, there was no suggestion of a 'priming effect' of FSH treatment leading to an increased inhibin B response in the third week.

An FSH dose-response effect on pro- α C secretion was seen, with ≥ 1200 IU FSH being required for a maximal response. The rise in pro- α C after hCG was more rapid (peak day 1–2) than with FSH, suggesting that the Leydig cell responds more rapidly to its tropic hormone than does the FSH-Sertoli cell axis. Additive effects of FSH and hCG on pro- α C were seen in both the single dose acute setting and during recovery, implying that both the Sertoli and Leydig cells contribute during combined treatment. This would also be in keeping with immunohistochemical data which have shown α -subunit staining in both Sertoli and Leydig cells (Bergh and Cajander, 1990; Forti *et al.*, 1992; Vannelli *et al.*, 1992; Majdic *et al.*, 1997; Anderson *et al.*, 1998; Andersson *et al.*, 1998). As previously shown, hCG (as an LH substitute) does not stimulate inhibin B secretion (Kamischke *et al.*, 2001; Kinniburgh and Anderson, 2001).

In contrast with the inhibin B response, germ cell loss appeared to enhance pro- α C responses to both FSH and hCG. It is possible that germ cells may have an inhibitory effect on the production of the α -subunit by both the Sertoli and Leydig cells which, when removed, results in a greater secretion of this isoform. Equally possible is that the germ cells positively influence the production of the β B-subunit by the Sertoli cell to FSH and that their removal results in less dimerization and thus an apparent increase in the free α -subunit. However, this apparent increase in pro- α C may also relate to the lower baseline value for the test after gonadotrophin suppression (stimulation test 2). It should also be noted that there was a lesser pro- α C response with the presumed return of germ

cells in the third week of recovery. In any event, it is clear that both Sertoli (FSH-dependent) and Leydig cell (hCG/LH-dependent) components respond more vigorously in terms of pro- α C secretion than inhibin B with germ cell depletion.

Germ cell/FSH/inhibin relationships may differ in other settings of testicular dysfunction. Despite severe germ cell depletion, high levels of α -subunit-containing peptides are found in infertile men, including Klinefelter's syndrome (Anawalt *et al.*, 1996), and chemotherapy-induced azoospermia (Wallace *et al.*, 1997), in which settings, inhibin B levels are very low or undetectable. This secretion of pro- α C is gonadotrophin sensitive as suppression of FSH/LH reduces the serum levels (Martin *et al.*, 2000; Kinniburgh *et al.*, 2002). The threshold for FSH and LH/hCG effects on both inhibin B and pro- α C may differ in these disease settings compared with those in the current study. The rise in pro- α C in male infertility may result from the much more severe loss of germ cell number (e.g. Sertoli cell-only syndrome) than that seen in our model, resulting in increased α -subunit secretion, and/or a loss of inhibin B secretory capacity. It has been previously suggested (Wallace *et al.*, 1997) that observed differences could be accounted for by the frequently elevated serum LH levels in these subjects leading to an increased secretion of α subunit by the Leydig cell. However, it is also possible that there is a contribution by preferential secretion of α -subunit by the Sertoli cell in the face of severe germ cell loss and high FSH levels.

One interesting incidental finding was the augmentation of hCG-induced testosterone secretion associated with co-administration of FSH, both in the single dose acute setting and over the weeks of recovery. These data support the previous observation (Levalle *et al.*, 1998) that enhancement of Sertoli cell function by FSH stimulation may result in improved Leydig cell function via paracrine effects. Given that there are no FSH receptors on Leydig cells, it has been suggested that the increase in testosterone observed after the administration by FSH is possibly mediated via a Sertoli cell-released non-steroidal factor with a molecular mass of >50 kDa (Levalle *et al.*, 1998). However, this effect was not seen in subjects with acquired hypogonadotropic hypogonadism in whom no further rise in serum testosterone was seen following 1 month of treatment with FSH with or without hCG (Young *et al.*, 2000). One potential explanation for these differences is that in the latter study, testicular size was markedly reduced (4–10 ml) probably due to spermatogenic failure. In turn this may have altered Sertoli cell function and its ability to augment Leydig cell testosterone secretion which was not restored by 1 month of gonadotrophin treatment.

In conclusion, we found that inhibin B in this setting of experimental germ cell depletion in normal men is a poor marker of spermatogenesis, as germ cell loss led to only minor changes in basal inhibin B levels and no modulation of FSH stimulatory response. Furthermore, during induced gonadotrophin recovery of spermatogenesis, no clear relationship was seen between sperm output and serum inhibin B. However, we caution that this conclusion should not be extrapolated to other settings of genetic or acquired defects of spermatogenesis

wherein different FSH/inhibin B/spermatogenic relationships may exist and account for the observed reciprocal relationship between serum inhibin B and FSH in spermatogenic failure. Thus, while we see no benefit in the use of an FSH stimulation test to assess spermatogenic status in normal men, it may be useful in some settings of male infertility.

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References

- Allenby, G., Foster, P.M. and Sharpe, R.M. (1991) Evidence that secretion of immunoreactive inhibin by seminiferous tubules from the adult rat testis is regulated by specific germ cell types: correlation between *in vivo* and *in vitro* studies. *Endocrinology*, **128**, 467–476.
- Anawalt, B.D., Bebb, R.A., Matsumoto, A.M., Groome, N.P., Illingworth, P.J., McNeilly, A.S. and Bremner, W.J. (1996) Serum inhibin B levels reflect Sertoli cell function in normal men and men with testicular dysfunction. *J. Clin. Endocrinol. Metab.*, **81**, 3341–3345.
- Anderson, R.A. (2001) Clinical studies: inhibin in the adult male. *Mol. Cell. Endocrinol.*, **180**, 109–116.
- Anderson, R.A., Wallace, E.M., Groome, N.P., Bellis, A.J. and Wu, F.C. (1997) Physiological relationships between inhibin B, follicle stimulating hormone secretion and spermatogenesis in normal men and response to gonadotrophin suppression by exogenous testosterone. *Hum. Reprod.*, **12**, 746–751.
- Anderson, R.A., Irvine, D.S., Balfour, C., Groome, N.P. and Riley, S.C. (1998) Inhibin B in seminal plasma: testicular origin and relationship to spermatogenesis. *Hum. Reprod.*, **13**, 920–926.
- Andersson, A.M., Muller, J. and Skakkebaek, N.E. (1998) Different roles of prepubertal and postpubertal germ cells and Sertoli cells in the regulation of serum inhibin B levels. *J. Clin. Endocrinol. Metab.*, **83**, 4451–4458.
- Bergh, A. and Cajander, S. (1990) Immunohistochemical localization of inhibin- α in the testes of normal men and in men with testicular disorders. *Int. J. Androl.*, **13**, 463–469.
- Buchter, D., von Eckardstein, S., von Eckardstein, A., Kamischke, A., Simoni, M., Behre, H.M. and Nieschlag, E. (1999) Clinical trial of transdermal testosterone and oral levonorgestrel for male contraception. *J. Clin. Endocrinol. Metab.*, **84**, 1244–1249.
- Carreau, S. (1995) Human Sertoli cells produce inhibin *in vitro*: an additional marker to assess the seminiferous epithelium development. *Hum. Reprod.*, **10**, 1947–1949.
- Clifton, R.J., O'Donnell, L. and Robertson, D.M. (2002) Pachytene spermatocytes in co-culture inhibit rat Sertoli cell synthesis of inhibin beta B-subunit and inhibin B but not the inhibin alpha-subunit. *J. Endocrinol.*, **172**, 565–574.
- Foppiani, L., Schlatt, S., Simoni, M., Weinbauer, G.F., Hacker-Klom, U. and Nieschlag, E. (1999) Inhibin B is a more sensitive marker of spermatogenic damage than FSH in the irradiated non-human primate model. *J. Endocrinol.*, **162**, 393–400.
- Forti, G., Vannelli, G.B., Barni, T., Balboni, G.C., Orlando, C. and Serio, M. (1992) Sertoli-germ cells interactions in the human testis. *J. Steroid Biochem. Mol. Biol.*, **43**, 419–422.
- Groome, N.P., Illingworth, P.J., O'Brien, M., Priddle, J., Weaver, K. and McNeilly, A.S. (1995) Quantification of inhibin pro- α C-containing forms in human serum by a new ultrasensitive two-site enzyme-linked immunosorbent assay. *J. Clin. Endocrinol. Metab.*, **80**, 2926–2932.
- Groome, N.P., Illingworth, P.J., O'Brien, M., Pai, R., Rodger, F.E., Mather, J.P. and McNeilly, A.S. (1996) Measurement of dimeric inhibin B throughout the human menstrual cycle. *J. Clin. Endocrinol. Metab.*, **81**, 1401–1405.
- Guitton, N., Touzalin, A.M., Sharpe, R.M., Cheng, C.Y., Pinon-Lataillade, G., Meritte, H., Chenal, C. and Jegou, B. (2000) Regulatory influence of germ cells on Sertoli cell function in the pre-pubertal rat after acute irradiation of the testis. *Int. J. Androl.*, **23**, 332–339.
- Handelsman, D.J., Conway, A.J., Howe, C.J., Turner, L. and Mackey, M.A. (1996) Establishing the minimum effective dose and additive effects of depot progestin in suppression of human spermatogenesis by a testosterone depot. *J. Clin. Endocrinol. Metab.*, **81**, 4113–4121.
- Hayes, F.J., Pitteloud, N., DeCruz, S., Crowley, W.F., Jr and Boepple, P.A. (2001) Importance of inhibin B in the regulation of FSH secretion in the human male. *J. Clin. Endocrinol. Metab.*, **86**, 5541–5546.
- Illingworth, P.J., Groome, N.P., Byrd, W., Rainey, W.E., McNeilly, A.S., Mather, J.P. and Bremner, W.J. (1996) Inhibin-B: a likely candidate for the physiologically important form of inhibin in men. *J. Clin. Endocrinol. Metab.*, **81**, 1321–1325.
- Jensen, T.K., Andersson, A.M., Hjollund, N.H., Scheike, T., Kolstad, H., Giwercman, A., Henriksen, T.B., Ernst, E., Bonde, J.P., Olsen, J. *et al.* (1997) Inhibin B as a serum marker of spermatogenesis: correlation to differences in sperm concentration and follicle-stimulating hormone levels. A study of 349 Danish men. *J. Clin. Endocrinol. Metab.*, **82**, 4059–4063.
- Kamischke, A., Simoni, M., Schrameyer, K., Lerchl, A. and Nieschlag, E. (2001) Is inhibin B a pharmacodynamic parameter for FSH in normal men? *Eur. J. Endocrinol.*, **144**, 629–637.
- Kinniburgh, D. and Anderson, R.A. (2001) Differential patterns of inhibin secretion in response to gonadotrophin stimulation in normal men. *Int. J. Androl.*, **24**, 95–101.
- Kinniburgh, D., Zhu, H., Cheng, L., Kicman, A.T., Baird, D.T. and Anderson, R.A. (2002) Oral desogestrel with testosterone pellets induces consistent suppression of spermatogenesis to azoospermia in both Caucasian and Chinese men. *Hum. Reprod.*, **17**, 1490–1501.
- Klingmuller, D. and Haidl, G. (1997) Inhibin B in men with normal and disturbed spermatogenesis. *Hum. Reprod.*, **12**, 2376–2378.
- Levalle, O., Zylbersztein, C., Aszpis, S., Aquilano, D., Terradas, C., Colombani, M., Aranda, C. and Scaglia, H. (1998) Recombinant human follicle-stimulating hormone administration increases testosterone production in men, possibly by a Sertoli cell-secreted nonsteroid factor. *J. Clin. Endocrinol. Metab.*, **83**, 3973–3976.
- Maddocks, S., Kerr, J.B., Allenby, G. and Sharpe, R.M. (1992) Evaluation of the role of germ cells in regulating the route of secretion of immunoreactive inhibin from the rat testis. *J. Endocrinol.*, **132**, 439–448.
- Majdic, G., McNeilly, A.S., Sharpe, R.M., Evans, L.R., Groome, N.P. and Saunders, P.T. (1997) Testicular expression of inhibin and activin subunits and follistatin in the rat and human fetus and neonate and during postnatal development in the rat. *Endocrinology*, **138**, 2136–2147.
- Martin, C.W., Riley, S.C., Everington, D., Groome, N.P., Riemersma, R.A., Baird, D.T. and Anderson, R.A. (2000) Dose-finding study of oral desogestrel with testosterone pellets for suppression of the pituitary–testicular axis in normal men. *Hum. Reprod.*, **15**, 1515–1524.
- McLachlan, R.I., O'Donnell, L., Stanton, P.G., Balourdos, G., Frydenberg, M., de Kretser, D.M. and Robertson, D.M. (2002) Effects of testosterone plus medroxyprogesterone acetate on semen quality, reproductive hormones, and germ cell populations in normal young men. *J. Clin. Endocrinol. Metab.*, **87**, 546–556.
- Meachem, S.J., Nieschlag, E. and Simoni, M. (2001) Inhibin B in male reproduction: pathophysiology and clinical relevance. *Eur. J. Endocrinol.*, **145**, 561–571.
- Nachtigall, L.B., Boepple, P.A., Seminara, S.B., Khoury, R.H., Sluss, P.M., Lecain, A.E. and Crowley, W.F., Jr (1996) Inhibin B secretion in males with gonadotropin-releasing hormone (GnRH) deficiency before and during long-term GnRH replacement: relationship to spontaneous puberty, testicular volume, and prior treatment—a clinical research center study. *J. Clin. Endocrinol. Metab.*, **81**, 3520–3525.
- Petersen, P.M., Andersson, A.M., Rorath, M., Daugaard, G. and Skakkebaek, N.E. (1999) Undetectable inhibin B serum levels in men after testicular irradiation. *J. Clin. Endocrinol. Metab.*, **84**, 213–215.
- Pineau, C., Velez de la Calle, J.F., Pinon-Lataillade, G. and Jegou, B. (1989) Assessment of testicular function after acute and chronic irradiation: further evidence for an influence of late spermatids on Sertoli cell function in the adult rat. *Endocrinology*, **124**, 2720–2728.
- Pineau, C., Sharpe, R.M., Saunders, P.T., Gerard, N. and Jegou, B. (1990) Regulation of Sertoli cell inhibin production and of inhibin alpha-subunit mRNA levels by specific germ cell types. *Mol. Cell. Endocrinol.*, **72**, 13–22.
- Robertson, D.M., Pruyers, E., Stephenson, T., Pettersson, K., Morton, S. and McLachlan, R.I. (2001) Sensitive LH and FSH assays for monitoring low

- serum levels in men undergoing steroidal contraception. *Clin. Endocrinol. (Oxf.)*, **55**, 331–339.
- Seminara, S.B., Boepple, P.A., Nachtigall, L.B., Pralong, F.P., Khoury, R.H., Sluss, P.M., Lecain, A.E. and Crowley, W.F., Jr (1996) Inhibin B in males with gonadotropin-releasing hormone (GnRH) deficiency: changes in serum concentration after short term physiologic GnRH replacement—a clinical research center study. *Clin. Endocrinol. Metab.*, **81**, 3692–3696.
- Vale, W., Rivier, C., Hsueh, A., Campen, C., Meunier, H., Bicsak, T., Vaughan, J., Corrigan, A., Bardin, W., Sawchenko, P. *et al.* (1988) Chemical and biological characterization of the inhibin family of protein hormones. *Recent Prog. Horm. Res.*, **44**, 1–34.
- Vannelli, G.B., Barni, T., Forti, G., Negro-Vilar, A., Vale, W., Serio, M. and Balboni, G.C. (1992) Immunolocalization of inhibin alpha-subunit in the human testis. A light- and electron-microscopy study. *Cell Tissue Res.*, **269**, 221–227.
- Wallace, E.M., Groome, N.P., Riley, S.C., Parker, A.C. and Wu, F.C. (1997) Effects of chemotherapy-induced testicular damage on inhibin, gonadotropin, and testosterone secretion: a prospective longitudinal study. *J. Clin. Endocrinol. Metab.*, **82**, 3111–3115.
- World Health Organization (1999) *Laboratory manual for the examination of human semen and sperm–cervical mucus interaction*. 4th edn, Cambridge University Press, Cambridge.
- Young, J., Couzinet, B., Chanson, P., Brailly, S., Loumaye, E. and Schaison, G. (2000) Effects of human recombinant luteinizing hormone and follicle-stimulating hormone in patients with acquired hypogonadotropic hypogonadism: study of Sertoli and Leydig cell secretions and interactions. *J. Clin. Endocrinol. Metab.*, **85**, 3239–3244.
- Zhengwei, Y., Wreford, N.G., Royce, P., de Kretser, D.M. and McLachlan, R.I. (1998) Stereological evaluation of human spermatogenesis after suppression by testosterone treatment: heterogeneous pattern of spermatogenic impairment. *J. Clin. Endocrinol. Metab.*, **83**, 1284–1291.
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