Seminal anti-Müllerian hormone level is a marker of spermatogenic response during long-term gonadotropin therapy in male hypogonadotropic hypogonadism

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BACKGROUND: In adult men, anti-Müllerian hormone (AMH) levels are higher in semen than in serum, but the significance and control of its seminal secretion are still unknown. This study evaluated seminal and serum AMH levels during long-term gonadotropin therapy in men with hypogonadotropic hypogonadism (HH). METHODS: A total of 20 men with never treated prepubertal-onset HH received i.m. hCG to normalize testosterone (T) and induce puberty. Afterwards, 11 of them, requiring fertility, were treated with HCG plus recombinant FSH (rFSH) (75 IU) twice a week, whereas 9 continued to receive hCG alone for 12 months. Before and during therapy, serum AMH, inhibin B and T levels were assessed. Semen samples were also collected during therapy for sperm count and seminal AMH assay. RESULTS: HCG alone decreased basal high serum AMH and stimulated T and inhibin B levels. rFSH plus hCG increased seminal AMH levels, which were consequently significantly higher than with hCG alone, and positively correlated to sperm densities and testicular volumes at 3 and 12 months (P<0.001). CONCLUSIONS: Our data demonstrate that rFSH, added to hCG, stimulates seminal AMH and spermatogenesis in HH. Thus, seminal AMH levels are under T and FSH control and are closely related to progression of spermatogenesis. Our results also suggest that an early seminal AMH increase may be a marker of good future response to gonadotropin therapy in HH.

Keywords: AMH; hypogonadotropic hypogonadism; spermatogenesis; recombinant FSH; Sertoli cells

Introduction

Anti-Müllerian hormone (AMH), a member of the transforming growth factor-β family expressed in the Sertoli cells, exerts paracrine inhibition of Müllerian derivatives during fetal life (Teixeira et al., 2001). Circulating AMH levels in males are low on the second day of life, but rapidly rise and remain high until the onset of puberty, when they are decreased to their lowest values in normal adults (Josso et al., 1990; Rey et al., 1993; Lee et al., 1996; Bergadà et al., 2006). AMH expression and secretion by Sertoli cells is regulated by inhibitory paracrine actions of intratesticular testosterone (T) and neighbouring meiotic germ cells and by a stimulating hormonal effect of FSH (Josso et al., 1990; Rey et al., 1993; Lee et al., 1996; Al-Attar et al., 1997; Rajpert-De Meyts et al., 1999; Rey, 2000; Lukas-Croisier et al., 2003). Serum AMH is a marker of Sertoli cell activity in boys, and its decline during puberty may be interpreted as an early sign of local T activity and spermatogenetic activation in the testis (Rey, 2000). The role and the clinical significance of AMH secretion in adult men are still not completely clarified. AMH in the testis is secreted by Sertoli cells both apically into seminiferous tubules and basally towards the interstitium and the circulation (Josso et al., 1979; Vigier et al., 1983; Cazorla et al., 1998). After puberty, AMH is released preferentially by the apical pole of the Sertoli cell towards the lumen of the seminiferous tubules, resulting in higher concentrations in the seminal plasma than in the serum (Fallat et al., 1996; Fénelich et al., 1999; Fujisawa et al., 2002; Al-Qahtani et al., 2005). Although this evidence suggests that seminal AMH might be a marker for Sertoli cell functional maturation and spermatogenesis progression, the significance and the control of AMH seminal secretion in adults remain uncertain. Treatment with exogenous gonadotropins in males with hypogonadotropic hypogonadism (HH) is effective in inducing testis development, puberty and spermatogenesis (Matsumoto, 1994). Recent reports showed that high serum AMH levels in HH men are...
inhibited by T or hCG administration and stimulated by short-term administration of recombinant FSH (rFSH) alone (Young et al., 1999, 2003, 2005). Whether gonadotropin chronic administration, aimed at inducing puberty and spermatogenesis in men with HH, is associated with changes of seminal AMH levels has so far not been addressed. In this study, we report the dynamics of seminal and serum AMH levels in a group of males with prepubertal onset HH treated chronically with hCG alone or hCG plus rFSH to induce puberty and spermatogenesis.

Materials and Methods

Subjects
This observational study was performed on a cohort of 22 outpatients with prepubertal onset HH, requiring induction of puberty and spermatogenesis, and followed at the Endocrine and Medical Andrology Section, Department of Clinical and Experimental Medicine and Surgery, Second University of Napoli (Italy). All patients gave informed written consent to participate to the study, which was approved by the institutional review board and conducted in accordance with Helsinki II Declaration on human experimentation. Clinical and hormonal data are reported in Table I. The diagnosis of HH had been made by the absence of secondary sexual characteristics after a chronological age of 16–18 years; low plasma T levels in the presence of low/undetectable plasma LH and FSH levels or with a lacking or subnormal response to a GnRH stimulation test. Five patients had Kallmann’s syndrome on the basis of the presence of ipo-/anosmia and olfactory bulbs and/or sulci abnormality at magnetic resonance imaging. None of them had received prior hormonal treatment for induction of puberty. Patients with panhypopituitarism had normalized the other pituitary deficiencies with appropriate replacement therapy. All patients received HCG (Profasi HP, Serono or Gonasi HP, AMSA) 2000 IU i.m. two times weekly to induce pubertal maturation and adult T levels. Two patients were excluded from the study (one lost during the follow-up, another because of the need for T substitution therapy). After 6–12 months (phase 1), 19 subjects reached Tanner stage 3, one reached Tanner stage 2 and all T levels were in the adult range (Table I). Patients entered treatment phase 2 when they reached Tanner stage 3. During this phase, 9 patients continued to receive hCG alone (group A), whereas 11 requiring fertility received rFSH (Gonal F, Serono), 75 IU s.c. two times weekly in combination with hCG (group B) for a further 12 months. The single man who did not reach Tanner stage 3 was treated with hCG alone and included in the group A, because he had a serum T level overlapping that of the other patients on Tanner stage 3 and referred ejaculations. Clinical evaluation performed at 3 month intervals was assessed through testicular volume with an orchidometer and by Tanner stage secondary sexual characteristics.

Samples and assays
Blood samples were obtained at enrollment for baseline evaluation and every three months during treatment (2–3 days after the previous gonadotropin injection). Serum obtained by centrifugation of clotted samples was stored at −20°C for T and inhibin B assays, and at −80°C for the AMH assay. Semen samples were collected during visits in patients who achieved at least Tanner stage 3 during hCG treatment (T0) and every three months during the following phase. Semen samples were obtained by masturbation, after 3–5 days of abstinence, and analysed according to the WHO guidelines (World Health Organization, 1992). A semen fraction was centrifuged immediately after liquefaction and stored at −80°C until the AMH assay.

Serum T levels were assayed by RIA (Radim, Pomezia, Italy), and Inhibin B was assayed by ELISA (DSL, Oxon, UK). Serum and seminal AMH levels were assayed using the AMH/MIS kit by Immunotech (Beckman, Marseille, France); the assay sensitivity was 0.1 ng/ml; the inter-assay and intra-assay coefficients of variation were 8–10 and 6–8%, respectively.

To validate the AMH assay for detecting AMH in seminal samples, recovery and parallelism were checked. When a known amount of human AMH (0–21 ng) was added to a fixed amount of seminal plasma collected on T0, the data (not shown) were additive indicating a good recovery. Moreover, dilution curves of seminal plasma collected at the end of the study paralleled human AMH standards (data not shown).

Statistical analysis
All data are expressed as median, ± standard error and range. Non-parametric tests were used to compare the data because of their

| Table I. Clinical and hormonal data of the hypogonadotropic hypogonadism patients at baseline and after treatment with hCG alone (T0). |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | All patients    | Group A (n = 9) | Group B (n = 11) | Adult normal range |
| At baseline     |                 |                 |                 |                  |
| Age at diagnosis, years | 20.0 ± 2.5 | 19.4 ± 1.87 | 20.5 ± 2.9       |
| Age at start of therapy, years | 20.4 ± 2.1 | 19.7 ± 1.5 | 21.0 ± 2.4       |
| BMI at diagnosis | 25.1 ± 1.9 | 25.0 ± 1.9 | 25.2 ± 2.1       |
| Diagnosis       |                 |                 |                 |                  |
| IHH normosmic   | 13              | 6               | 7               |
| Kallmann's syndrome | 5            | 2               | 3               |
| Panhypopituitarism | 2             | 1               | 1               |
| Testicular volume (ml) | 3.35 ± 1.4 | 3.33 ± 1.0 | 3.36 ± 1.7       |
| Tanner stage puberty | 1–2           | 1–2             | 1–2             |
| LH (UI/l)       | 0.38 ± 0.2     | 0.40 ± 0.2     | 0.37 ± 0.3      |
| FSH (UI/l)      | 0.61 ± 0.5     | 0.59 ± 0.3     | 0.63 ± 0.6      |
| Testosterone (ng/ml) | 0.22 ± 0.15 | 0.23 ± 0.2     | 0.22 ± 0.1      |
| Inhibin B (pg/ml) | 62.85 ± 9.5  | 61.89 ± 8.5     | 63.6 ± 10.6     |
| At T0           |                 |                 |                 | (50.0–250.0)     |
| Testicular volume (ml) | 4.6 ± 1.22 | 4.7 ± 1.7       |
| Tanner stage puberty | 3 (n = 8)–2 (n = 1) | 3 (n = 11) |
| Testosterone (ng/ml) | 3.9 ± 1.29 | 4.03 ± 1.9      |
| Inhibin B (pg/ml) | 103.2 ± 25.14 | 114.9 ± 27.2    |

Group A, treated with hCG alone; Group B, treated with hCG followed by hCG plus rFSH; BMI, body mass index.
non-Gaussian distribution. Differences were evaluated using Mann–Whitney U-test and Wilcoxon as appropriate. Bivariate correlations were evaluated using Spearman’s rho. A P-value < 0.05 was considered statistically significant.

Results

Pubertal changes, testicular volume and spermatogenesis during gonadotropin therapy

After 6–12 months of hCG treatment, 19 out 20 patients achieved Tanner stage 3 puberty. Pubertal maturation advanced further in the following treatment period with hCG alone (group A) or with hCG combined with rFSH (group B). Testicular volume, depicted in Fig. 1, was higher in group B, reaching a significant difference after 9 and 12 months (P < 0.05 and P < 0.001, respectively, compared with group A). At the end of phase 1 therapy, semen analysis, performed in the 19 patients achieving Tanner stage 3, showed azoospermia. Throughout treatment phase 2, 2 out of 9 patients treated with hCG alone (group A) showed severe oligozoospermia (0.1 and 0.3 million/ml). Among the 11 patients in group B, spermatozoa were found in the semen of 3 patients after 3 months, and in another 6 after 12 months from the start of rFSH administration; one patient was unable to provide semen samples during control visits, and another who was azoospermic after 12 months of combined treatment, underwent testicular biopsy revealing Sertoli cells only syndrome. Sperm density was significantly different at 6, 9 (P < 0.05) and 12 (P < 0.001) months in group B versus group A (Fig. 2).

T and inhibin B levels

Low baseline T levels (Table I) increased in all patients during treatment with HCG alone. A further increment was observed throughout phase 2, without difference between the two groups (Fig. 1). Inhibin B levels increased progressively during therapy reaching higher levels in the group B than in group A from the third month onwards (P < 0.05–0.001) (Fig. 1).

Serum and seminal AMH levels

Baseline elevated serum AMH levels progressively decreased during hCG treatment alone, attaining very low values by the end of phase 1 (Fig. 1). rFSH together with hCG administration induced a significant (P < 0.05) increase of circulating AMH after 3 months, that later disappeared. Seminal AMH levels were low and did not show significant variations during hCG alone, but markedly increased in patients under combined therapy, resulting in levels which were significantly higher than in patients treated with hCG alone (P < 0.001) (Fig. 2). The correlations between seminal AMH levels, testicular volumes and sperm densities at different time points of phase 2 are summarized in Table II. As shown, seminal AMH levels were positively correlated to sperm densities by 6 and also to testicular volumes by 9 months. Moreover, seminal AMH levels after 3 months of phase 2 gonadotropin treatment (with or without rFSH) were positively correlated to the final (12 months) sperm densities and testicular volumes (Fig. 3).
On the contrary, serum AMH levels were not correlated to sperm densities or testicular volumes.

Discussion
In this study we investigated spermatogenesis, serum and seminal AMH levels during substitutive therapy with hCG alone or hCG plus rFSH in men with previously untreated prepubertal-onset HH. Our results show that, during long-term hCG administration, serum AMH levels decrease along with a parallel increase of serum T. Moreover, they showed a marked increase of seminal AMH concentrations, positively correlated to sperm output, during gonadotropin administration. This study first evaluated seminal AMH dynamics during long-term replacement therapy with gonadotropins in men with HH, adding new information concerning the relative role of T and FSH on the regulation of testicular AMH secretion. In men with untreated congenital and acquired HH, serum AMH levels have been found to be elevated basally, and inhibited to undetectable concentrations by T or hCG and stimulated by short-term rFSH administration (Young et al., 1999, 2005). Our results confirm that hCG decreases serum AMH levels in HH, supporting the hypothesis that androgen production by Leydig cells driven by hCG/LH exerts a paracrine negative effect on AMH secretion by Sertoli cells (Josso et al., 1979; Rajpert-De Meyts et al., 1999; Young et al., 1999, 2003, 2005). The transient slight increase of serum AMH observed after 3 months of rFSH addition might be due to proliferation of Sertoli cells induced by rFSH or/and lack of the androgen inhibiting action. Sertoli cell proliferation occurs in two well-defined temporal windows, i.e. in the fetal-neonatal period and at the onset of puberty (Sharpe et al., 2003). Immature Sertoli cells may acquire terminal differentiation at puberty under the influence of both FSH and intratesticular T action and in presence of advanced germ cell development stages (Baarends et al., 1995; Rajpert-De Meyts et al., 1999; Sharpe et al., 2003). High AMH concentrations have been found in the rete testis in several species (Josso et al., 1979; Vigier et al., 1983; Cazorla et al., 1998) and in the seminal plasma in humans (Fallat et al., 1996; Fénichel et al., 1999; Fujisawa et al., 2002; Al-Qahtani et al., 2005). These studies demonstrated that AMH levels are higher in semen than in blood in adults, suggesting that this hormone is preferentially secreted by apical pole of the Sertoli cells towards seminiferous tubule lumen after puberty. In prepuberty, immature Sertoli cells secrete AMH through the basal layer determining a high serum concentration. At puberty, both increased androgen levels and spermatogenesis progression down-regulate testicular AMH expression and direct AMH secretion towards the apex (Baarends et al., 1995; Sharpe et al., 2003). A secretory change during puberty has week to restore spermatogenesis in hypogonadal men (Liu et al., 1999; Bouloux et al., 2002). The present study suggests that a low dose rFSH regimen is able to achieve a minimal fertilizing sperm concentration in the majority of men with prepubertal-onset HH. Our results demonstrate that, during long-term hCG administration, serum AMH levels decrease along with a parallel increase of serum T. Moreover, they showed a marked increase of seminal AMH concentrations, positively correlated to sperm output, during gonadotropin administration. This study first evaluated seminal AMH dynamics during long-term replacement therapy with gonadotropins in men with HH, adding new information concerning the relative role of T and FSH on the regulation of testicular AMH secretion. In men with untreated congenital and acquired HH, serum AMH levels have been found to be elevated basally, and inhibited to undetectable concentrations by T or hCG and stimulated by short-term rFSH administration (Young et al., 1999, 2005). 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Table II. Correlations between seminal AMH, sperm density and testicular volume.

<table>
<thead>
<tr>
<th>Sperm density</th>
<th>T3</th>
<th>T6</th>
<th>T9</th>
<th>T12</th>
<th>Testicular volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seminal AMH T3</td>
<td>0.329</td>
<td>0.543*</td>
<td>0.583**</td>
<td>0.686**</td>
<td>0.314</td>
</tr>
<tr>
<td>Seminal AMH T6</td>
<td>0.286</td>
<td>0.529*</td>
<td>0.681**</td>
<td>0.843**</td>
<td>0.186</td>
</tr>
<tr>
<td>Seminal AMH T9</td>
<td>0.391</td>
<td>0.634**</td>
<td>0.723**</td>
<td>0.804**</td>
<td>0.212</td>
</tr>
<tr>
<td>Seminal AMH T12</td>
<td>0.230</td>
<td>0.471*</td>
<td>0.650**</td>
<td>0.832**</td>
<td>0.174</td>
</tr>
</tbody>
</table>

T3, T6, T9, T12: after 3, 6, 9 and 12 months of hCG or rFSH + hCG administration. *P < 0.05; **P < 0.001.
been demonstrated for other Sertoli cell products, such as transferrin, but its significance remains unknown (Maddocks and Sharpe, 1990; Baarends et al., 1995). Thus, seminal AMH content could be an expression of Sertoli cell maturation at the onset of gonadotropin-induced puberty in HH men. We found that seminal AMH levels correlated with sperm densities and testicular volumes during phase 2 treatment. In particular, seminal AMH levels after 3 months, and at the greatest extent after 6 months, correlated with the final testicular volumes and sperm densities, suggesting that an early seminal AMH increase may be an index of later spermatogenic response to gonadotropin treatment. Several studies on oligo-/azoospermic infertile men have demonstrated a correlation between spermatogenesis and seminal AMH concentrations, confirming that seminal AMH may be an absolute testicular marker (Fénichel et al., 1999; Fujisawa et al., 2002; Mostafa et al., 2007). However, some studies have not found a link between AMH and spermatogenesis status (Al-Qahtani et al., 2005; Duvilla et al., 2007). Measurements of seminal AMH have been evaluated as a tool for prediction of the efficacy of testicular sperm retrieval (TESE) in non-obstructive azoospermia with disappointing results (Duvilla et al., 2007; Mostafa et al., 2007). Problems related to the population studied (limited number of subjects, different causes of the infertility, presence of primary dysfunction in Sertoli cells) or methodological interferences in the seminal assay may account for the contradictory findings in the non gonadotropin deficient infertile subjects and for the poor predictability of seminal AMH in TESE. However, preliminary data combining seminal AMH concentration with other serum markers (inhibin B, FSH) seem to increase the predictive power for TESE outcome (Duvilla et al., 2007).

In conclusion, our results demonstrate that testicular AMH production is regulated by androgens and FSH, and its seminal secretion is closely related to differentiation of Sertoli cells and progression of spermatogenesis in treated HH patients. They also suggest that the assay of seminal AMH may be considered as a tool for prediction of gonadotropin therapy outcome in HH, since its early increase may be a marker of good future spermatogenic response to this therapy.

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