Suppression of the high endogenous levels of plasma FSH in infertile men are associated with improved Sertoli cell function as reflected by elevated levels of plasma inhibin B

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BACKGROUND. In vitro continuous stimulation of Sertoli cells with FSH leads to a desensitization of these cells to FSH action. To evaluate the presence of a desensitization of FSH receptor on Sertoli cells in vivo, we performed a controlled clinical study in 97 men affected by severe oligozoospermia. METHODS. On the basis of FSH and inhibin B plasma concentrations, these subjects were divided into three groups: group A, 33 subjects with high FSH and low inhibin B plasma levels; group B, 32 subjects with high FSH plasma levels and inhibin B concentrations at the lower limit of the normal range; and group C, 32 subjects with normal FSH and inhibin B plasma levels. Patients with high FSH plasma levels (groups A and B) were prospectively randomized into two subgroups, called A1, A2, B1 and B2. Patients of groups A1 and B1 were treated with a GnRH agonist, leuprolide acetate, to induce a hypogonadotrophic state and then were treated with recombinant human FSH (r-hFSH; 100 IU/day) and hCG (2000 IU/twice a week) for 2 months. Subjects of groups A2, B2 and C were treated only with r-hFSH for the same period. RESULTS. In patients of group A1, inhibin B remained unmodified during the whole period of study, whereas in subjects of group B1, we observed a significant reduction of this hormone during the hypogonadotrophic period and then an increase of inhibin B plasma levels that were higher that those observed before therapy. In patients of groups A2 and B2, FSH treatment did not induce a significant increase in inhibin B concentrations. In patients of group C, FSH induced a significant increase in inhibin B plasma levels. CONCLUSIONS. In infertile men, suppression of the high endogenous levels of plasma FSH associated with much lower exogenous FSH levels is able to evoke higher inhibin B production, which may indicate improved Sertoli cell function and the possibility that this could have a positive effect on spermatogenesis.

Key words: desensitization/FSH treatment/inhibin B/male infertility/Sertoli cell function

Introduction

FSH plays a crucial role in the induction and maintenance of spermatogenesis in humans. This hormone acts by binding to specific receptors (FSH-Rs) confined to the gonads (Matsumoto, 1989; Sharpe, 1989), and Sertoli cells are the only cell type expressing the FSH-R (Kangasniemi et al., 1990; Heckert and Griswold, 1991; Kliesch et al., 1992; Bockers et al., 1994; Ronniki et al., 1995). Therefore, the action of FSH on germ cells has to be indirect and mediated by paracrine signals from Sertoli cells, and a close Sertoli–germ cell interaction is required to allow a normal spermatogencic process.

In man, the feedback control system regulating FSH secretion in a physiological setting appears to involve mainly inhibin B secreted by Sertoli cells (Anderson and Sharpe, 2000; Hayes et al., 2001; Ramaswamy and Plant, 2001). The contribution of testicular steroids to the regulation of FSH secretion is noticeably less important than that exerted by inhibin B signalling (Tilbrook and Clarke, 2001). The production of inhibin B by Sertoli cells is stimulated by FSH (Foresta et al., 1999b, 2000, 2002; Hayes et al., 2001), but it also reflects interactions between these and the neighbouring germ cells (Pineau et al., 1990; Allenby et al., 1991). In men affected by primary testicular disorders, inhibin B falls in parallel with an increase in FSH plasma levels (Anawalt et al., 1996; Foresta et al., 1999a; de Kretser et al., 2000). Therefore, the presence of high FSH concentrations in infertile patients is considered a marker of spermatogenic failure.

The FSH-R is a seven transmembrane receptor belonging to the large family of GTP-binding protein (G-protein)-coupled receptors. The binding of FSH to its receptor results in a stimulation of the Gs protein, which in turn activates the membrane-associated adenylate cyclase causing an elevation of intracellular camp (Simoni et al., 1997). In addition, the signal transduction triggered by FSH may involve the entry of extracellular calcium through voltage-dependent and -independent calcium channels and through the PKG/PI signalling pathway (Simoni et al., 1997)
Both in intact animals and in cultured Sertoli cells, chronic stimulation by FSH induces a desensitization and down-regulation of FSH signaling (O’Shaughnessy and Brown, 1978; Gnanaprakasam et al., 1979; Themmen et al., 1991). This phenomenon is evidenced by a depression of CAMP production (Themmen et al., 1991) and involves multiple identified steps in the FSH signal transduction pathway including: (i) uncoupling of FSH-R from the effector system induced by phosphorylation of serine or threonine residues of the C-terminal, intracellular domain of G protein-coupled receptors (Conti et al., 1983; Ford and LaBarbera, 1988; Grasso and Reichert, 1989; Sanchez-Yague et al., 1993); and (ii) a decrease of the number of FSH-Rs mediated, at least in part, by extensive clustering and internalization of the hormone–receptor complex and by reduced receptor protein synthesis as a result of both decreased transcription and reduced mRNA half-life (Fletcher and Reichert, 1984; Saez and Jaillard, 1986; Shimizu and Kawashima, 1989; Themmen et al., 1991). It is not known if in humans a down-regulation of FSH receptors exists in vivo and if this condition may contribute to a further alteration of the tubular function.

To examine the presence of possible desensitization of FSH-R on Sertoli cells in male infertility, we performed a controlled clinical study in well-selected groups of infertile patients affected by different degrees of testicular damage and characterized by high FSH plasma concentrations. In these subjects, the inhibin B behaviour, considered a marker of Sertoli cell function, has been evaluated during FSH treatment in the presence and absence of a GnRH agonist to suppress endogenous high levels of gonadotrophin secretions.

Materials and methods

Subjects

The local hospital ethical committee approved the study protocol, and written informed consent was obtained from each patient. Ninety-seven men affected by oligozoospermia, sperm count <10^9/ml, due to different causes and a history of infertility for at least 2 years were enrolled in the present study. The baseline characteristics and causes of testicular alteration in the different groups are reported in Table I. Each subject was evaluated with a clinical examination pointing to clinical andrological characteristics. All semen samples were obtained by masturbation after 3 days of sexual abstinence and were evaluated on at least three separate occasions, separated by a 3 week interval. After liquefaction at room temperature, semen volume, pH, sperm concentration, motility and morphology were determined following WHO guidelines for semen analysis (World Health Organization, 1999). The presence of antisperm antibodies was evaluated utilizing the immunobead and the Sperm-Mar test (Ortho Diagnostic System, Milan, Italy). To exclude infections, a microbiological culture was performed in each subject before starting the treatment.

The spermatogenic activity was studied by means of bilateral testicular fine needle aspiration cytology (FNAC), performed as previously described (Foresta and Varotto, 1992; Foresta et al., 1992, 1995). This method allows the identification of all germinal cells at their different stages of maturation as well as of Sertoli cells; furthermore, it permits the characterization of specific tubular damage. The Sertoli index (the ratio of Sertoli to spermatogenic cells) constitutes an attempt to simplify the understanding of cytological results: because the number of Sertoli cells in adults is constant per unit of tubular length, an increase in the Sertoli index may reveal the existence of tubular germ cell depopulation and thus hypospermogenesis (Foresta and Varotto, 1992; Foresta et al., 1992, 1995). Before FNAC, testicular ultrasound was performed in each subject to analyse testicular volume using the approximation for an ellipsoid (volume = length \times width \times depth \times 0.523), and morphology. Hormonal parameters including basal LH, FSH, testosterone and inhibin B plasma levels were evaluated in each subject (see below). Y chromosome microdeletions were excluded in all subjects, and karyotype analysis showed in all cases a normal 46,XY constitution.

Patients were subgrouped into three groups on the basis of FSH and inhibin B plasma concentrations: group A, 33 subjects with high FSH (at least 2 SD higher than mean normal values, >8 IU/l) and low inhibin B plasma levels (at least 2 SD lower than mean normal values, <100 pg/ml); group B, 32 subjects with high FSH plasma levels and inhibin B concentrations at the lower limit of the normal range (>100 pg/ml); and group C, 32 subjects with normal FSH and inhibin B plasma levels.

Forty age-matched fertile subjects (proven fathers recruited consecutively at the time of delivery) were considered as controls for seminal and hormonal parameters. Forty normozoospermic infertile subjects (mean sperm count 67.5 ± 26.6 \times 10^6/ml), whose characteristics have been reported in previous studies (16 autoimmune and 24 idiopathic), were considered as controls for testicular cytological analysis (Foresta and Varotto, 1992; Foresta et al., 1992).

Study design

The overall experimental design is presented in Figure 1.

Treatment protocol 1: gonadotrophin suppression followed by stimulation with r-hFSH

In order to investigate the effects of FSH treatment in infertile subjects with high FSH plasma concentrations, our patients with high FSH plasma levels (groups A and B) were prospectively randomized into two subgroups, called A1, A2, B1 and B2.

Patients of group A1 (high FSH plasma levels and low inhibin B plasma concentrations, 17 subjects), and patients of group B1 (high FSH plasma levels and inhibin B concentrations at the lower limit of the normal range, 16 subjects), were treated with a GnRH agonist (leuprolide acetate, 3.75 mg i.m. every 28 days for 4 months) to induce a hypogonadotropic state. After 60 days from the first leuprolide injection, all subjects were treated with recombinant human FSH (r-hFSH) at a dose of 100 IU each day and with hCG at a dose of 2000 IU twice a week for 2 months. All patients were evaluated for LH, FSH, testosterone and inhibin B plasma levels before the treatment and every 30 days during the period of study. The subjects

Table I. Baseline characteristics and causes of testicular alteration in oligozoospermic subjects

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Study design

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<th>Treatment protocol 2 (Groups A2, B2 and C)</th>
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<td>Stimulation period</td>
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**Figure 1.** Study protocol: 97 infertile men were enrolled in this trial. Subjects were divided into three groups on the basis of FSH and inhibin B plasma concentrations (group A, high FSH and low inhibin B plasma levels; group B, high FSH and inhibin B at the lower limit of normal range; and group C, normal FSH and inhibin B plasma levels). Subjects with high FSH plasma levels (groups A and B) were prospectively randomized into two subgroups (A1, A2, B1 and B2). Treatment protocol 1: patients of groups A1 and B1 were treated with a GnRH agonist, leuprolide acetate, for the duration of the study; after 60 days of leuprolide treatment, these subjects were treated for a further 2 months with r-hFSH plus hCG (stimulation period). Treatment protocol 2: subjects of groups A2, B2 and C were treated only with r-hFSH for 2 months.

were informed about the possible side effects related to androgen withdrawal caused by GnRH agonist administration.

**Treatment protocol 2: FSH therapy alone**

Patients of groups A2, 16 subjects, and B2, 16 subjects, were treated only with r-hFSH at a dose of 100 IU each day for 2 months.

Patients of group C (normal FSH and inhibin B plasma levels) were considered as the infertile control group for treatment with FSH. Subjects of this group also were treated with r-hFSH at a dose of 100 IU each day for 2 months.

Before the FSH treatment and every 30 days during the period of treatment, all patients of these three groups were evaluated for LH, FSH, testosterone and inhibin B plasma levels.

**Clinical monitoring**

All subjects were advised of the possible side effects during the period of treatment. For evaluation of possible psychosexual effects of treatment, patients were studied monthly during the period of treatment pointing to side effects and sexual function. On these occasions, body weight, pulse and blood pressure were measured and a clinical examination, with attention to testicular size (as evaluated by orchidometer) and mammary gland examination, was carried out. Blood samples were obtained at every visit for endocrine measurements and, in patients treated with GnRH agonist, a haematological (haemoglobin, hematocrit, platelets and white count), biochemical (liver enzymes, glucose, electrolytes) and lipid profile (total cholesterol and triglycerides) was carried out on the same occasions.

**Hormone assays**

Gonadotrophins and inhibin B. Serum FSH and LH concentrations were measured in each subject by radioimmunoassay using $^{125}$I-labelled FSH and LH (Ares-Serono, Milan, Italy). The sensitivity of the FSH assay was 0.05 IU/l, as defined by a mean +2 SD multiple zero-sample measurements. Inter- and intra-assay coefficients of variation were 2.8 and 3.7%, respectively, for LH, and 2.6 and 3.6%, respectively, for FSH. Inhibin B plasma concentrations were measured by a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) specific for the dimeric inhibin B form (Serotec, Oxford, UK) (Groome et al., 1996; Illingworth et al., 1996). The first antibody is directed to the BB-subunit, and the second antibody to the $\alpha$-subunit, and conjugated to alkaline phosphatase. The assay has a cross-reactivity of <0.1% with activins and ~1% with inhibin A. Assay sensitivity was 15 pg/ml and the inter- and intra-plate coefficients of variation were 6.4 and 6.8%, respectively.

**Sex steroids.** Testosterone plasma concentrations were determined in all subjects using the double antibody radioimmunoassay utilizing commercial kits (Radim, Rome, Italy). The assay has a sensitivity of 0.1 nmol/l and cross-reacts minimally with other steroid hormones. All specimens were measured in duplicate in the same assay. Intra- and inter-assay coefficients of variation were 7.8 and 7.0%, respectively.

**Statistical analysis**

The results are expressed as mean ± SD. Statistical analysis was performed using the Statview statistical package (Abacus, Berkeley, CA). Differences among the means in the group values at baseline were calculated by Student’s t-test. Differences from baseline values during the treatment period were calculated for each group using the Student’s t-test. For statistical purposes, baseline values were calculated as the mean of two measurements, one at the time of the first evaluation (pre-treatment data) and the other at the time of GnRH agonist administration (time 0). P-values <0.05 were regarded as statistically significant.

**Results**

Table II summarizes the clinical, hormonal, seminal and cytological parameters before hormonal treatment of all 97 oligozoospermic subjects compared with the controls. Patients of groups A and B showed a comparable severe oligozoospermia (sperm count <3.0 × 10⁶/ml), but the testicular cytological analysis showed a worse testicular pattern in patients of group A. In fact, although subjects of both groups had a severe hypospermatogenesis without maturation disturbances, those of group A showed a more severe phenotype (Sertoli index 634 ± 122.7, with respect to 428 ± 136.5 and 30.0 ± 19.5 of controls, P < 0.001, respectively). Patients of group C showed a moderate oligozoospermia (sperm count between 5 and 10 × 10⁶/ml) and a testicular picture of moderate hypospermatogenesis (Sertoli index 186.4 ± 64.3, P < 0.001 versus controls). The baseline inhibin B plasma levels in group A were lower than that in groups B and C (42.3 ± 19.1 versus 124.8 ± 44.8 and 188.7 ± 70.3 pg/ml, respectively, P < 0.01), while FSH concentrations were higher in groups A and B with respect to group C (22.6 ± 10.5 and 14.9 ± 5.8 versus 4.4 ± 1.8 IU/l, respectively, P < 0.01).

Seventeen patients of group A (group A1) and 16 of group B (B1) were treated with r-hFSH after a period of suppression with GnRH agonist. All other subjects (group A2, 16 subjects; group B2, 16 subjects; and group C, 32 subjects) were treated only with r-hFSH. Clinical characteristics, and in particular
testicular cytological pattern, were similar in subjects of groups A1 and A2, as well as in subjects of groups B1 and B2.

**Treatment protocol 1: gonadotrophin suppression followed by stimulation with FSH**

In patients of groups A1 and B1, the treatment with leuprolide induced a significant reduction in gonadotrophin and testosterone plasma levels after 1 month in all subjects studied ($P < 0.001$ versus pre-treatment), and these hormones remained at the lower limit of detection after 60 days from the first leuprolide injection (Figure 2). The fall of gonadotrophins and testosterone below the sensitivity of the assay confirmed that a state of hypogonadism was achieved.

In patients of group A1 (high FSH plasma levels and low inhibin B plasma concentrations), circulating concentrations of inhibin B remained unmodified during GnRH agonist treatment, while in subjects of group B1 (high FSH plasma levels and inhibin B concentration at the lower limit of the normal range), gonadotrophin suppression induced a significant reduction of inhibin B plasma levels after 1 and 2 months of treatment (101.1 ± 60.0 and 100.8 ± 18.6 pg/ml, respectively, $P < 0.05$ versus pre-treatment) corresponding to a 21.25% reduction with respect to baseline values.

Treatment with r-hFSH and hCG restored physiological concentrations of FSH and testosterone in both study groups. During replacement therapy, the concentrations of circulating inhibin B remained unmodified in patients of group A1. In contrast, in group B1, r-hFSH and hCG treatment restored normal concentrations of inhibin B, as determined by an increase in serum inhibin B concentrations, that reached plasma levels higher than those observed before therapy (178.9 ± 46.5 and 210.3 ± 32.1 pg/ml after 1 and 2 months of therapy, respectively, $P < 0.01$ versus pre-treatment) corresponding to a 64.06% increase with respect to baseline values.

**Treatment protocol 2: FSH therapy alone**

In patients of groups A2 and B2, FSH treatment induced a significant increase in FSH plasma levels ($P < 0.01$ versus pre-treatment), while in both groups inhibin B concentrations remained unchanged in the same period. In patients of group C, r-hFSH induced a significant increase after 1 and 2 months of therapy of FSH ($P < 0.01$) and inhibin B plasma levels (264.8 ± 42.6 and 245.0 ± 39.5 pg/ml, respectively, $P < 0.01$, corresponding to a 57.61 and 45.83% increase with respect to baseline values) (Figure 3).

We did not observe any significant variation of LH or testosterone concentrations during the period of treatment in the three groups (data not shown).

**Side effects**

The treatment was well tolerated in all patients and in no case was the study ended because of side effects.

During the GnRH agonist period of therapy, patients of groups A1 and B1 experienced side effects such as hot flushes, occurring in 50% of patients; headache and asthenia were reported in 16 and 3.1% of patients, respectively. These side effects were preventable during the first days of GnRH agonist administration and were completely reversible. During the last days of GnRH agonist treatment, 70% of patients experienced androgen deprivation complications such as a mild loss of libido and erectile dysfunction. The therapy with hCG restored the normal concentration of testosterone and completely abolished these side effects. In no case was the study terminated because of side effects. We did not observe any important adverse events during the treatment with gonadotrophins in any of the subjects.

During the whole period of therapy, we did not observe any significant variation of body weight, blood pressure or testicular volume. No gynaecomastia was seen during the monthly examinations in any of the subjects.

Haematological, biochemical and lipid parameters remained unchanged during the whole period of therapy in patients treated with GnRH agonist (data not shown).

**Discussion**

It has been demonstrated that in experimental models, prolonged exposure of Sertoli cells to FSH induces desensitization and downregulation of FSH signalling, thus preventing overstimulation of these gonadal cells (O’Shaughnessy and Brown, 1978; Gnanaprakasam et al., 1979; Themmen et al., 1991; Sanchez-Yague et al., 1993). In this study, we attempted to verify whether high endogenous levels of plasma FSH induced by severe testiculopathy suggest the occurrence of desensitization of Sertoli cell function.

To address this hypothesis, we studied Sertoli cell function, as reflected by circulating levels of inhibin B, in three different groups of patients characterized by normal and high FSH plasma concentrations. In order to investigate the presence of Sertoli cell desensitization, subjects with high FSH plasma concentrations.
levels were evaluated through the change of reciprocal behaviour of inhibin B and FSH concentrations during the suppression of endogenous high FSH plasma levels with a GnRH agonist and during the following stimulation with physiological concentrations of FSH.

In subjects with high FSH plasma concentrations, independently from inhibin B concentration, r-hFSH administration did not induce any modification of inhibin B production. The same results were obtained previously utilizing highly purified FSH in infertile subjects showing similar basal seminal and hormonal parameters and testicular structure (Foresta et al., 1999a, 2000). The lack of any increase of inhibin B production after FSH administration in subjects with high basal FSH levels reflects incompetence of Sertoli cells in the secretion of this hormone that may be related to an intrinsic alteration or a dysfunctional status of these cells. In particular, this latter condition seems to be present in subjects with high FSH plasma levels and inhibin B concentrations at the lower limit of the normal range (group B). In these subjects, when r-hFSH was administered after the suppression of endogenous gonadotropin production with GnRH agonist (group B1), Sertoli cells responded to FSH treatment with increased inhibin B production to a level exceeding pre-treatment levels. On the contrary, no increment in inhibin B production was observed in subjects with identical clinical characteristics who were not pre-treated with GnRH agonist (group B2). This phenomenon suggests that also in man, prolonged exposure of Sertoli cells to high FSH concentrations is associated with a reduced competence of Sertoli cells to respond to this hormone in terms of inhibin B secretion. A hypothetical explanation of our findings is that high FSH levels induce a desensitization of Sertoli cells and that a reduction of gonadotropin levels for 2 months restores Sertoli cell sensitivity. An alternative hypothesis is that the different responses may be related to a different underlying testicular damage. However, the increase in inhibin levels observed at the end of treatment in group B1 and not in group B2 (that are homogenous with respect to seminal, hormonal and testicular cytological characteristics) strengthens the first hypothesis. In fact, in subjects of group B1, the increase in

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**Figure 2.** Treatment protocol 1: changes in serum hormone levels in patients of groups A1 and B1 during the treatment with leuprolide acetate and r-hFSH and hCG. Serum concentrations of FSH, LH and testosterone significantly decreased in both groups from baseline values after 1 month of treatment with leuprolide ($P < 0.001$); treatment with r-hFSH plus hCG restored physiological concentrations of FSH and testosterone in both groups, while LH concentrations remained unmodified in this second phase of the study. Inhibin B levels remained unmodified throughout the treatment period in subjects of group A1, while in patients of groups B1 we initially observed a significant reduction of serum concentrations of this hormone during the treatment with leuprolide ($P < 0.05$) and then an increase of inhibin B concentrations after therapy with FSH plus hCG ($P < 0.01$ versus pre-treatment). Circles = group A1; squares = group B1. *$P < 0.001$ versus pre-treatment; $^\dagger P < 0.01$ versus pre-treatment; $^\ddagger P < 0.05$ versus pre-treatment.

**Figure 3.** Treatment protocol 2: changes of inhibin B and FSH plasma levels during treatment with r-hFSH alone in patients of groups A2, B2 and C. Circles = group A2; squares = group B2; diamonds = group C. *$P < 0.01$ versus pre-treatment.
inhibin B production after the GnRH agonist treatment is similar to that observed in infertile men of group C (who have normal FSH and inhibin B concentrations before treatment). In our opinion, the different inhibin B production between group B1 and B2 is not due to the hCG used in B1, because there is no evidence of a direct action of this hormone on inhibin B production by Sertoli cells (Anderson and Sharpe, 2000).

In subjects of group A (high FSH and low inhibin B plasma levels), r-hFSH administration did not modify inhibin B concentrations also after the suppression of gonadotrophins with GnRH analogue (group A1). This behaviour is not surprising since the very low inhibin B plasma levels may reflect in this case a complete and irreversible incompetence of Sertoli cells. Furthermore, patients of group A have a worse testicular pathology, as evidenced by testicular cytological analysis. In these subjects, the cause that determined the spermatogenetic defect may have also induced a defect of Sertoli cells.

In this study, our primary end-point was to verify the presence of a desensitization on Sertoli cells in vivo, and thus the seminal parameters of patients have not been evaluated. However, these results seem to open up two perspectives in the treatment of male infertility: (i) in infertile subjects with high FSH plasma levels and inhibin B concentrations at the lower limit of the normal range, the reduction of FSH plasma levels with a GnRH agonist could improve Sertoli function and thus the residual spermatogenic activity; and (ii) the dosage of FSH in the treatment of oligozoospermic subjects with normal FSH plasma concentrations should not determine an elevation of FSH plasma levels above the normal range.

On the basis of the results of the present study, we suggest that infertile patients with high FSH plasma levels and normal inhibin B concentrations should be considered as affected by tubular damage restricted to germinal cells. In these cases, at least in part, Sertoli cell function may be negatively influenced by the chronically elevated concentrations of plasma FSH. The apparent improvement of Sertoli cell function, as reflected by the elevation of inhibin B production after the suppression of FSH levels, is an intriguing phenomenon. Whether it brings about improvement of sperm production is possible and remains to be established.

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