ISSN: 2047-2919

ORIGINAL ARTICLE

Correspondence:
Francesca Caprio, Via Duomo, 319-80133 Naples, Italy.
E-mail: francescacaprio@infinito.it

Keywords:
anti-Müllerian hormone, assisted reproduction, male idiopathic infertility, recombinant follicle-stimulating hormone

Received: 4-Feb-2015
Revised: 22-Apr-2015
Accepted: 18-May-2015

doi: 10.1111/andr.12065

Seminal anti-Müllerian hormone levels during recombinant human follicle-stimulating hormone treatment in men with idiopathic infertility undergoing assisted reproduction cycles

F. Caprio, P. De Franciscis, C. Trotta, R. Ianniello, D. Mele and N. Colacurci

Outpatient Fertility Unit, Second University of Naples, Naples, Italy

SUMMARY

A prospective study was designed to investigate the effects of recombinant human follicle-stimulating hormone (rhFSH) on seminal anti-Müllerian hormone (AMH) levels in men with idiopathic oligoasthenoteratozoospermia (iOAT), researching possible relationships between the seminal AMH behavior and the response to the treatment. Thirty-nine men who were candidates for intracytoplasmic sperm injection (ICSI) because of iOAT were enrolled. Patients were treated on alternately days with 150 IU of rhFSH for at least 3 months before assisted reproduction cycles. Main outcome measures were seminal AMH concentrations before and after rhFSH therapy. After treatment, 16 subjects (responders) showed an improvement in their sperm count compared to baseline (7.6 ± 2.9 vs. 19.3 ± 7.7, $p < 0.01$) whereas 23 men (non-responders) experienced no sperm modifications. Baseline seminal AMH concentrations were significantly higher in responders than in non-responders (53.0 ± 30.6 vs. 34.6 ± 18.5, $p < 0.025$). Following therapy, a greater increase in AMH levels was observed in responders compared to non-responders ($\Delta = 24.8 \pm 36.4$ vs. $\Delta = 6.4 \pm 11.2$, $p < 0.028$). Seminal AMH levels significantly and positively correlated with sperm count (after rhFSH treatment rho = 0.647, $p < 0.001$). Our study suggests that rhFSH improves sperm count in a quota of iOAT men, and the subjects who respond to the treatment have higher baseline seminal AMH concentrations than the patients who are not responsive. Seminal AMH could be helpful to select those infertile men who may benefit from rhFSH treatment.

INTRODUCTION

Idiopathic iOAT affects approximately one-third of all infertile men and dramatically reduces their reproductive capacity (Cavallini, 2006). ICSI is actually the treatment of choice for men affected by severe iOAT. Nevertheless, the poor semen quality of these subjects may impair ICSI outcomes because of morphological and functional alterations of their spermatozoa (Ben-Rafael *et al.*, 2000; Liu *et al.*, 2004).

Several therapies have been proposed with the aim to improve fertilizing potential of spermatozoa in iOAT conditions, but their effectiveness is still a matter of dispute (Cavallini, 2006). Since follicle-stimulating hormone (FSH) plays a pivotal role in regulating spermatogenesis, iOAT has been empirically treated with gonadotropins (Attia *et al.*, 2007).

It has been reported that recombinant human FSH (rhFSH) may be a specific pre-treatment for infertile male partners of couples undergoing assisted reproductive technique (ART) procedures, rather than a global strategy for subfertile male patients in general (Caroppo *et al.*, 2003). Furthermore, it has been suggested that subgroups of iOAT males may benefit from rhFSH administration (Foresta *et al.*, 2005) and predictive parameters able to identify responder subjects to rhFSH have been proposed (Foresta *et al.*, 2007).

Recently, research attention has been directed towards the study on semen components and its biochemical characteristics, that could provide valuable non-invasive biomarkers for evaluating the function of different accessory sex glands and seminiferous tubules involved in semen production. Anti-Müllerian hormone (AMH), a dimeric glycoprotein belonging to the transforming growth factor beta family, is one of the most promising molecules (Teixeira *et al.*, 2001). It is secreted by the Sertoli cells and induces the regression of the Müller ducts during fetal life (Teixeira *et al.*, 2001).

Seminal AMH concentrations are diminished in the case of impaired spermatogenesis (Muttukrishna *et al.*, 2007). Higher seminal AMH concentrations have been found in normozoospermic than in oligozoospermic men (Fénichel *et al.*, 1999; Fujisawa *et al.*, 2002; Mostafa *et al.*, 2007; Duvilla *et al.*, 2008). Moreover in men affected by non-obstructive azoospermia the seminal AMH concentration has been found lower than in subjects with obstructive azoospermia (Fénichel *et al.*, 1999; Duvilla *et al.*, 2008).

Therefore, AMH is a sensitive biomarker in semen and could reflect the functional state of the seminiferous epithelium (Sabetian, 2010).

Testicular AMH secretion is stimulated by rhFSH (Young *et al.*, 2005) and correlates with spermatogenesis in men with hypogonadotropic hypogonadism (Sinisi *et al.*, 2008).

These findings suggest that seminal AMH may be a marker of spermatogenesis and could be predictive of the possible variations in sperm parameters during rhFSH treatment.

The aim of the present study was to investigate the effects of rhFSH on seminal AMH levels in iOAT men, researching possible relationships between seminal AMH behavior and response to the treatment.

MATERIALS AND METHODS

Subjects and study protocol

Thirty-nine infertile couples, who were candidates for ICSI because the male partners suffered from iOAT, were enrolled as eligible for the study.

The experimental study was conducted in accordance with principles of the Helsinki Declaration of 1975, using routine clinical practice procedures usually performed during in vitro fertilization (IVF) cycles; such procedures did not involve additional risks to the patients and all the medical decisions concerning individual patients were not affected by the study. Moreover, written consent was obtained from the patients for the collection and analysis of data. Therefore, the study did not require permission from the ethics committee.

Inclusion criteria were as follows: history of unexplained infertility of >2 years duration, sperm count ranging from 1 to 10×10^6 spermatozoa/mL; normal plasma FSH, luteinizing hormone, prolactin, testosterone and inhibin B levels; absence of cryptorchidism, varicocoele, testicular trauma, genital infections, antisperm antibodies, Y chromosome microdeletions, karyotypic abnormalities, CFTR gene mutations, systemic chronic diseases.

Female infertility factors were excluded in order to try to eliminate any contribution the female partner had to the couple's infertility.

Each subject underwent a careful physical examination to exclude abnormalities of the external genitalia, and his testicular size was recorded using a Prader orchidometer. Two semen samples were obtained at a first visit (screening) and at enrollment within 1 month for baseline evaluation. Then, men started 150 IU of subcutaneous rhFSH (Gonal-f; Merck Serono S.p.A., Rome, Italy) on alternately days for at least the 3 months preceding the scheduled ART cycles. Semen analysis and measure of seminal AMH concentrations were performed at baseline and at the time of the ART.

Assisted reproduction procedures

Female partners underwent downregulation with a Gonadotropin Releasing Hormone (GnRH) agonist and ovarian stimulation with rhFSH. Follicular growth was monitored according to estradiol plasma levels and ultrasound (US) assessments. Oocyte retrieval was performed under US guidance approximately 35 h after the injection of human Chorionic Gonadotropin (hCG).

In the presence of a sperm count $<10 \times 10^6$ spermatozoa/mL and a percentage of spermatozoa with forward motility <20%, ICSI was performed. In the case of achievement of semen with characteristics above such values after rhFSH treatment, conventional IVF was carried out (van Rumste *et al.*, 2000).

Embryo development was evaluated 2 days after IVF/ICSI by determining the number of blastomeres and the relative proportion of embryo volume occupied by anucleate cell fragments. Embryos with <10% fragments, with 10–20% fragments, with 20–30% fragments, and with >30% fragments were referred to as grade 1, 2, 3, and 4, respectively.

The luteal phase was supported with the administration of 33 mg/day of natural progesterone starting from pick-up day and than 50 mg/day from embryo transfer day.

Serum levels of hCG were measured 14 days after embryo transfer and, if positive, were obtained every 3–6 days until an intrauterine gestational sac was demonstrated by US examination that was repeated every 2 weeks until the 12th week of gestation.

Samples and assays

Semen samples were obtained by masturbation, after 3–5 days of abstinence, and analyzed according to the World Health Organization (2010). A semen fraction was centrifuged immediately after liquefaction at room temperature and stored at −80 °C until assay. An antiprotease was added (Complete Mini; Roche Diagnosis, Indianapolis, IN, USA) to the seminal plasma, immediately after thawing, before the assay in order to avoid an enzymatic reaction capable of interfering with the quantification of the assay (Fénichel *et al.*, 1999).

Seminal AMH dosage was carried out using an ELISA sandwich technique (AMH/MIS kit, reference A16507; Immunotech, Beckman Coulter, Marseilles, France). Analytical sensitivity, defined as the lowest AMH concentration significantly different from the zero calibrator, was 0.7 pmol/L (1 ng/mL = 7.14 pmol/L). Intra- and inter-assay coefficients of variation were <10 and 12%, respectively.

Statistical analysis

In order to test the hypothesis that iOAT men who respond to rhFSH have a different seminal AMH behavior compared to treatment-refractory patients, on the basis of our preliminary results (unpublished data), it has been calculated that a sample size of 15 subjects in each group has 80% (1-β) power to detect a difference in means of 7.3 pmol/L in seminal AMH levels with a Student's *t*-test with two-sided significance level of 0.05.

One-way analysis of variance and Mann–Whitney *U*-test were used to evaluate differences between groups for normally and not normally distributed data, respectively.

Table 1 Seminal characteristics and AMH levels before and after rhFSH treatment

	Responder group (n = 16)		Non-responder group (n = 23)	
	Baseline	After rhFSH treatment	Baseline	After rhFSH treatment
Sperm concentration ($\times 10^6$/mL)	7.6 ± 2.9	19.3 ± 7.7[a]	7.3 ± 2.4	6.4 ± 4.1
Total sperm count ($\times 10^6$/mL)	19.4 ± 7.2	46.7 ± 8.6[a]	18.1 ± 5.7	16.6 ± 4.5
Forward motility (%)	22.7 ± 5.2	25.1 ± 5.5	22.4 ± 4.6	23.0 ± 4.7
Normal morphology (%)	28.3 ± 5.5	29.7 ± 7.9	27.1 ± 6.2	26.8 ± 8.3
Seminal AMH levels (pmol/L)	53.0 ± 30.6[b]	77.8 ± 59.7[c,d]	34.6 ± 18.5	41.0 ± 23.8[c]

Results are reported as means ± SD. AMH, anti-Müllerian hormone; rhFSH, recombinant human follicle-stimulating hormone. [a]$p < 0.01$ vs. baseline and vs. non-responder group at the same time point. [b]$p = 0.025$ vs. non-responder group at the same time point. [c]$p < 0.05$ vs. baseline. [d]$p = 0.01$ vs. non-responder group at the same time point.

Table 2 Baseline clinical and hormonal characteristics of the male patients

	Group A (rhFSH responders) (n = 16)	Group B (rhFSH non-responders) (n = 23)	p
Age (years)	31.8 ± 2.9	33.6 ± 3.5	0.09
Time of infertility (years)	3.1 ± 2.3	3.7 ± 3.2	0.9
Body mass index (kg/m^2)	24.3 ± 1.9	25.1 ± 2.2	0.2
Testicular volume (mL)	12.9 ± 2.8	12.6 ± 3.0	0.7
Serum FSH (IU/L)	5.4 ± 1.3	5.7 ± 1.6	0.5
Serum LH (IU/L)	3.1 ± 1.2	2.8 ± 1.4	0.4
Serum testosterone (ng/mL)	5.3 ± 1.9	4.9 ± 2.1	0.5
Serum inhibin B (pg/mL)	161.8 ± 12.1	155.9 ± 9.7	0.09

Results are reported as means ± SD. rhFSH, recombinant human follicle-stimulating hormone; LH, luteinizing hormone.

Relationship between variables was evaluated by the Spearman rank test. Comparison of frequencies was performed by chi-squared test or Fisher exact test.

The paired Student's t-test or the Wilcoxon signed-rank test was utilized to analyze paired data within a group in case of data with normal or non-normal distribution, respectively. A two-tailed p value <0.05 was considered to be statistically significant. Results are reported as means ± SD. Data were analyzed using the Statistical Package for the Social Sciences (SPSS release 16.0.2; SPSS Inc., Chicago, IL, USA).

RESULTS

The rhFSH treatment lasted 98.4 ± 16.9 days, was correctly executed and completed by all the participants. In all cases, a semen sample was obtained both at baseline and after the end of treatment.

When considering all patients on the whole, no modifications in sperm parameters were observed after therapy: sperm concentration passed from the basal 7.5 ± 2.6 to 11.6 ± 8.6 × 10^6/mL at the end of therapy. Nevertheless, when analyzed individually, two subgroups of men were identified: after rhFSH, 16 out of the 39 subjects experienced an improvement in their sperm count with a doubling of concentration vs. baseline (responder group), whereas 23 men showed no significant sperm modifications (non-responder group); no significant variation was observed in other seminal parameters, such as forward motility and normal morphology in both groups (Table 1). Baseline clinical and hormonal characteristics were similar between the two groups (Table 2).

The seminal AMH levels of the whole study population increased significantly after rhFSH (from 42.2 ± 25.5 to 56.1 ± 45.5 pmol/L; $p < 0.01$ at the Wilcoxon signed-rank test). The analysis of the two groups of patients showed that the baseline seminal concentrations of AMH in the responder group was significantly higher ($p = 0.025$ by the Mann–Whitney U-test) than the non-responder group. Responder subjects experienced a more evident increase in AMH levels compared to non-responders and after treatment AMH values were significantly higher ($p = 0.01$ at the Mann–Whitney U-test) in the former than in the latter group (Table 1). The increase in the seminal AMH concentrations, expressed as the difference between post- and pre-treatment AMH levels (Δ), was significantly greater ($p = 0.028$ by the Mann–Whitney U-test) in the responder group (Δ = 24.8 ± 36.4) than in the non-responder group (Δ = 6.4 ± 11.2).

A statistically significant and positive correlation between seminal AMH concentrations and sperm count was found, with a stronger association after the hormonal treatment compared to baseline (Fig. 1).

Two women whose partners belonged to the responder group experienced a spontaneous pregnancy (both at the end of the

Figure 1 Correlations between seminal anti-Müllerian hormone (AMH) concentrations and sperm count before (a) and after (b) recombinant human follicle-stimulating hormone treatment.

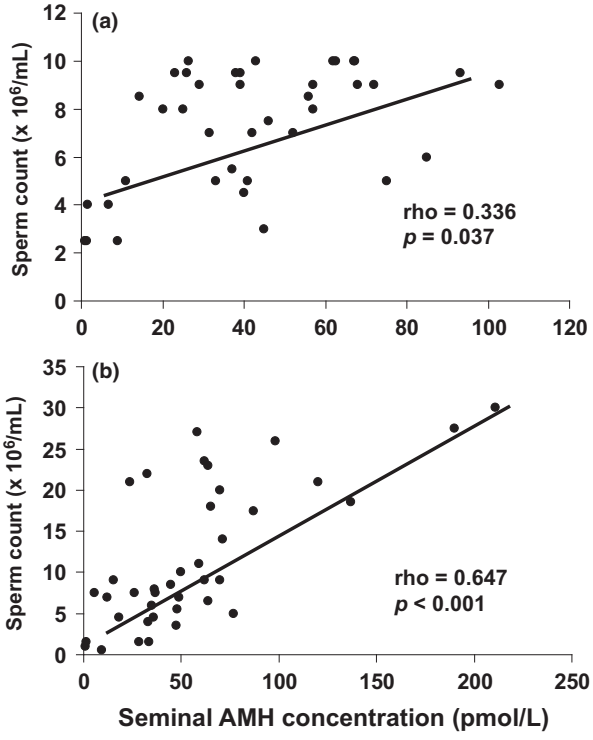

Table 3 Baseline clinical characteristics of the female partners and ART outcome parameters

	Group A (rhFSH responders)	Group B (rhFSH non-responders)	*p*
Age (years)	31.2 ± 2.8	31.9 ± 2.9	0.4
Body mass index (kg/m^2)	23.3 ± 2.3	23.7 ± 2.0	0.5
Day-3 serum FSH (IU/mL)	7.4 ± 1.9	6.9 ± 2.3	0.4
Total dose of rFSH used (IU)	2098.9 ± 837.6	2122.5 ± 786.5	0.9
Estradiol on day of hCG (pg/mL)	1872 ± 684.6	1928.2 ± 708.1	0.8
Retrieved oocytes (*n*)	9.7 ± 4.5	10.2 ± 3.9	0.7
Metaphase II oocytes (*n*)	6.5 ± 1.7	6.7 ± 1.6	0.7
Fertilization rate (%)	85.7	76.8	0.1
No. of grade 1 embryos	0.8 ± 0.6	0.6 ± 0.7	0.35
No. of pregnancies (%)	3 (21.4)	4 (17.4)	0.75

Results are reported as means ± SD. ART, assisted reproductive technique; rhFSH, recombinant human follicle-stimulating hormone.

3rd month of rhFSH therapy). The improvement in seminal parameters allowed standard IVF-ET procedures in eight of the remaining fourteen cases belonging to the responder group. The other six couples in the responder group and all the subjects in the non-responder group underwent ICSI.

No significant difference was found in the ART outcome parameters between the two study groups (Table 3), although a larger sample size is necessary for a meaningful statistical evaluation of this issue.

Following ART, three pregnancies in patients from the responder group and four pregnancies in the non-responder group were achieved (Table 3). All the three pregnancies obtained in responder group were ongoing at the 12th week of gestation. Two women whose partners belonged to the non-responder group miscarried.

DISCUSSION

The present study suggests that rhFSH improves sperm count in a quota of iOAT men, and subjects who respond to treatment have higher baseline seminal AMH concentrations than patients who are not responsive. Furthermore, responder subjects also experience a greater increase in their seminal AMH levels after rhFSH administration.

A low seminal AMH concentration could reflect both a spermatogenic dysfunction and an immaturity of Sertoli cells (Blagosklonova *et al.*, 2002). The seminal concentration of AMH should be helpful for determining the extent to which Sertoli cell immaturity is associated with defective spermatogenesis or contributes to spermatogenic dysfunction.

In the present study, the finding of higher baseline concentrations of seminal AMH in the responder group led us to hypothesize that a certain range of seminal AMH production is associated to a better intra-testicular environment. In support of our hypothesis, we found that, after rhFSH treatment, the increase in AMH levels was more evident in the responder group. Furthermore, seminal AMH values significantly and positively correlated with sperm count, in agreement with previous data (Fénichel *et al.*, 1999; Fujisawa *et al.*, 2002; Mostafa *et al.*, 2007; Sinisi *et al.*, 2008). Thus, seminal AMH may be considered an index of Sertoli cell functional integrity and FSH responsiveness. Altogether, these data also suggest that seminal AMH could predict the extent of the improvement of the reproductive capacity in infertile men treated with rhFSH.

The increase in seminal AMH levels and the positive correlation between AMH values and sperm count that we found, particularly after rhFSH treatment, suggest that this hormonal therapy could exert a local priming effect by promoting the expression of FSH-receptors on Sertoli and germ cells' surface, thus producing an increased post-receptor response and changing paracrine testicular microenvironment that could lead to an improvement in spermatogenesis and AMH secretion.

In our study, non-responder patients could suffer from severe alterations in their Sertoli cells that might account for the lack of a sperm improvement. Nevertheless, it cannot be excluded that some of these patients could carry polymorphic changes in FSH receptor leading to an impairment in the cell response to the therapy. Interestingly, a recent study reported a significant increase in seminal AMH levels with Asn/Asn variant of FSHR gene than those with Asn/Ser or Ser/Ser (Zalata *et al.*, 2008). When correlated with seminal AMH values, there was an increase in Asn/Asn in men with high seminal AMH concentrations (Zalata *et al.*, 2008).

Our study indicates that rhFSH treatment may stimulate spermatogenesis and improve sperm count, although sperm motility and morphology were not as well influenced. These results are consistent with those of some authors (Caroppo *et al.*, 2003; Foresta *et al.*, 2005) but not with others (Kamischke *et al.*, 1998). Such discrepancies may be due to the diverse infertile populations examined and to the administration of different protocols of hormonal treatment.

Since the increase in sperm count does not always correspond to improved pregnancy rates, the achievement of spontaneous pregnancies following male treatment with rhFSH, as observed in our study and by other authors (Caroppo *et al.*, 2003; Foresta *et al.*, 2005), implies that other sperm functions are improved by rhFSH. In this regard, it has been reported that infertile men show a high sperm DNA fragmentation index (DFI) and an elevated rate of chromosomal aneuploidy (Liu *et al.*, 2004) that can negatively affect their reproductive outcome (Garrido *et al.*, 2008; Lin *et al.*, 2008). In an in vitro model of cultured segments of human seminiferous tubules, FSH withdrawal increased the incidence of DNA fragmentation in meiotic (primary spermatocytes) and post-meiotic (spermatids) germ cells (Tesarik *et al.*, 2002). Moreover, FSH immunization in men has been found associated to altered chromatin packaging (Krishnamurthy *et al.*, 2000). It is widely demonstrated that FSH stimulates mitotic and meiotic DNA synthesis in spermatogonia and preleptotene spermatocytes through its action on Sertoli cells (Caroppo *et al.*, 2003). Kamischke *et al.* observed that rhFSH treatment increases sperm DNA condensation in idiopathic infertile men (Kamischke *et al.*, 1998). In an our experience, we found that rhFSH improves sperm DNA integrity in iOAT men who show increased sperm DFI values (Colacurci *et al.*, 2012) confirmed by a later study (Ruvolo *et al.*, 2013).

In conclusion, our findings suggest that rhFSH stimulates spermatogenesis together with the production and secretion of AMH which, in turn, appears a marker of response to the treatment in iOAT patients. If our results are confirmed by a larger study, seminal AMH could be helpful to select infertile men who may benefit from rhFSH.

ACKNOWLEDGMENTS

The authors declare that this work was not supported by any source of funding.

FINANCIAL DISCLOSURE STATEMENT

The authors declare no vested interest of a commercial nature. They have no financial affiliation (e.g., employment, direct payments, stock holdings, retainers, consultantship, patient-licensing arrangements, or honoraria) or involvement with any commercial organization with direct financial interest in the subject or material discussed in this manuscript. The authors have no financial interest in any aspect of the work and did not receive any financial support in the form of financial aid, grants, or equipment. Any other potential conflict of interest also is disclosed.

AUTHOR CONTRIBUTIONS

FC PDF NC conceived and designed the experiments. FC CT RI analyzed the data. FC PDF DM wrote the manuscript. NC commented on manuscript.

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