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Effects of long-term treatment with human pure follicle-stimulating hormone on semen parameters and sperm-cell ultrastructure in idiopathic oligoteratoasthenozoospermia

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Summary. Ten subfertile men affected by idiopathic oligoteratoasthenozoospermia and exhibiting normal serum hormone levels received a long-term treatment with human pure follicle-stimulating hormone (hp-FSH) (150 IU, intramuscularly, three times per week for 6 months). Semen parameters and ultrastructural features of spermatozoa were evaluated before and after therapy. The results showed an increase in sperm cell concentration and, more interestingly, motility. Electron microscopic examination revealed an improved fine architectural pattern, mainly involving acrosome, head and chromatin and middle-piece, in accordance with the positive changes of functional data. No significant changes of hp-FSH treatment on serum hormone levels were observed, since the latter were found to be substantially unchanged after 6 months of therapy. The present data suggest: (i) the benefit of hp-FSH administration in idiopathic oligoteratoasthenozoospermia, when hormone parameters support a substantial integrity of spermatogenetic microenvironment and (ii) an optimal effect after long-term (6 months) therapy.

Introduction

Idiopathic oligoteratoasthenozoospermia (OTA), without evidence of physical, metabolic or biochemical alteration, is the most common clinical feature in infertile man (Nieschlag, 1993). The choice of therapeutic approach is a major problem, because idiopathic OTA is not a unique clinical entity, and its pathogenetic mechanisms remain unknown. A wide variety of drugs (i.e. gonadotrophin-releasing hormone, gonadotrophins, androgens, antioestrogens, aromatase-inhibitors, antioxidants) have been used, but following empirical rather than causal criteria and, consequently, with unpredictable results (Isidori, 1988; Haidl & Schill, 1991; Nieschlag, 1993; Skakkebaek *et al.*, 1994; Howard, 1995; Schill, 1995; Forti & Krausz, 1998). The lack of controlled clinical studies makes the effect of these treatments hard to evaluate.

It is generally accepted that follicle-stimulating hormone (FSH) plays a determinant role in normal spermatogenesis, being involved in both its early and late stages (Alphen *et al.*, 1988; Kula, 1991). An adequate intratubular level of testosterone, maintained by luteinizing hormone (LH) through the stimulation of Leydig cells, is also critical in normal spermatogenesis (Skinner, 1991; Sharpe, 1994).

Such a physiological role makes gonadotrophins an elective and effective pharmacological tool for the treatment of hypogonadotrophic hypogonadism (Finkel *et al.*, 1985). The role of gonadotrophins in idiopathic OTA is more controversial (Skakkebaek *et al.*, 1994; Howard, 1995), and the variety of therapeutic protocols reflects such uncertainty. The use of FSH alone, avoiding any combination with LH, has been also proposed in the treatment of OTA (Jockenhovel *et al.*, 1990; Acosta *et al.*, 1991; 1992; Glander & Kratzsch, 1997; Foresta *et al.*, 1998).

Electron microscopy has revealed several structural abnormalities in spermatozoa of infertile men involving mainly the nucleus and acrosome (Bartoov & Fisher, 1982; Zamboni, 1987; Lipitz *et al.*, 1992; Mundy *et al.*, 1994). Such

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abnormalities have been correlated with the fertilizing potential of spermatozoa, both *in vitro* and *in vivo* (Mashiach *et al.*, 1992; Glezerman & Bartoov, 1993). Treatment with FSH has proved to be effective in improving the ultrastructural features of semen (Bartoov *et al.*, 1994; Baccetti *et al.*, 1997).

On the basis of the crucial role of FSH in spermatogenesis, the present study investigated long-term treatment with human pure (hp) FSH in a selected group of subfertile men affected by idiopathic OTA and exhibiting normal hormone serum levels, in order to evaluate the modifications of seminal parameters and sperm cells fine architecture.

Patients and methods

Selection of patients

The present study included 10 patients (mean age 37.0 ± 1.9 years, range 33–40 years) selected from a cohort of over 400 patients followed at the Endocrinology Department, Andrology Unit, during the past 2 years. The patients underwent medical evaluation which included history and clinical examination (testicular volume was determined using Prader's orchidometer), as well as semen analysis and serum sampling for measurement of FSH, LH, testosterone (T), oestradiol (E_2) and prolactin (PRL) levels (determined by standard radioimmunoassays). Furthermore, in order to reach a definite diagnosis of OTA essential for the patient's entry in the study, the following additional examinations were performed.

(i) Semen analysis and swim-up: these tests were performed by the same investigator according to the WHO criteria (World Health Organization, 1992). Briefly, seminal fluid was obtained at the Hospital by masturbation, after 3–5 days of sexual abstinence. The sperm count was determined with a Makler chamber. Sperm motility was assessed by phase-contrast microscopy and graded as follows: class 1 and 2, including fast and weak forward motility; class 3, non-progressive motility; class 4, immotile spermatozoa. Smears of seminal fluid were stained with the Giemsa method and sperm morphology was evaluated by oil immersion light microscopy. For the swim-up technique seminal fluid was diluted in HAMS 10 culture medium (1 : 2), centrifuged at 500 ***g*** for 10 min and incubated at 37 °C for 1 h after further addition of 0.5 ml of culture medium.
(ii) MAR-test (SperMar test, Diasint, Florence, Italy), for the presence of antisperm antibodies;
(iii) Sperm culture, urethral specimen collection for *Chlamydiae* and *Mycoplasma* assays, secretory anti-*Chlamydiae* IgA and serum anti-*Chlamydiae* antibodies;
(iv) Testicular, prostatic and seminal vesicles ultrasonography, and eco-color Doppler of the venous spermatic plexus, for anatomical abnormalities and varicocele detection.

The following inclusion criteria were adopted: (i) sperm count < 20 millions ml^{-1} and sperm motility (grade $1 + 2) < 35\%$ at two baseline sperm analyses; (ii) normal baseline serum levels of gonadotrophins and other measured hormones; (iii) absence of genital infectious diseases, of anatomical abnormalities of the genital tract, of varicocele and antisperm antibodies, as well as of other systemic diseases.

Electron microscopy

Ejaculated spermatozoa of the enrolled patients were also collected for ultrastructural examination at baseline and after 6 months of therapy. After liquification, a sample of semen (0.5–1 ml) was washed with saline and a pellet of spermatozoa was collected after gentle centrifugation ($600 \times \boldsymbol{g}$). The pellet was resuspended in saline and fixed in 2% glutaraldehyde in cacodylate buffer 0.15 mol l^{-1}, postfixed in 1% osmium tetroxide and embedded in an epon–araldite mixture. Semithin sections (2 μm thick) were stained with toluidine blue. Thin sections (0.6–0.8 μm thick), stained with lead citrate, were studied with a Zeiss 902 or Philips CM10 electron microscope. Longitudinal- and cross-sections of 100 spermatozoa were examined using different grids, in order to avoid multiple observations of the same cell. All cell compartments were investigated (acrosome, head and chromatin, middle piece, tail). The following abnormalities were standardized and quantitated using longitudinal sections: acrosomal shape (irregular, small, absent, lifted) and acrosomal matrix (A type); chromatin alterations (hypo- or decondensation, intranuclear vacuoles) (C type); dismorphic (D type) or round-shaped (R type); mixed alterations resulting from combination of A, C and R alterations (A–C–R type); middle piece alterations (baseplate, mitochondria, fibrous sheath) (MP type); features suggestive of necrotic spermatozoa (NE type abnormalities) were also estimated. Furthermore, an analysis of submicroscopic organization of the flagellum was performed by a high magnification study of the cross-sections.

Treatment and follow-up

The enrolled patients underwent intramuscular administration of 150 IU hp-FSH (Metrodin HP,

Table 1. Effects of hpFSH administration on semen parameters (concentration, motility and light microscopy morphology)

		hpFSH	
	Baseline	3 months	6 months
Sperm cell concentration (million ml^{-1})	18.3 ± 10.4	24.8 ± 16.6 ns	46.2 ± 23.3*
Sperm cell motility (%)			
Grade 1 (fast forward)	7.3 ± 7.7	9.3 ± 6.7 ns	14.0 ± 9.2*
Grade 2 (fast + weak forward)	27.6 ± 11.2	30.1 ± 10.6 ns	43.2 ± 11.8*
Sperm cell motility after swim-up (%)			
Grade 1 (fast forward)	8.2 ± 8.8	29.0 ± 14.4*	28.3 ± 12.5*
Grade 2 (fast + weak forward)	27.2 ± 15.9	59.1 ± 29.3*	64.6 ± 21.8*
Atypical spermatozoa (%)	58.9 ± 4.9	57.3 ± 4.2 ns	55.7 ± 5.1 ns

ns = not significant; $P < 0.05$.

Serono, Milan, Italy) three times per week for 6 months. No interruptions or delays in the administration were reported. Clinical examination, semen analysis, swim-up, and serum hormone assays were performed at baseline and after 3 and 6 months of therapy (blood samples were collected at least 12 h after the injection of hp-FSH). Electron microscopy of spermatozoa was performed at baseline and after 6 months of therapy.

Statistical analysis

The results obtained before and after treatment were statistically analysed using the Student's *t*-test for paired data.

Results

No clinical changes were observed during the therapy, including the absence of variation of the testicular volume and of gynaecomastia. Serum levels of gonadotrophins and other measured hormones did not change significantly during treatment (data not shown).

Ejaculated sperm modifications and swim-up are illustrated in Tables 1 and 2.

Sperm cell concentration

The mean concentration of spermatozoa at baseline $(18.3 \pm 10.4) \times 10^6\ ml^{-1}$ increased slightly but not significantly after 3 months of hpFSH treatment to $(24.8 \pm 16.6) \times 10^6\ ml^{-1}$; and rose significantly $(46.2 \pm 23.3) \times 10^6\ ml^{-1}$; $P < 0.05$ after 6 months of therapy (Table 1).

Sperm cell motility

The mean baseline percentage of spermatozoa with grade 1 motility increased from baseline (7.3 ± 7.7%) to 14.0 ± 9.2% after 6 months of FSH treatment ($P < 0.05$) (Table 1). Furthermore, an increase from baseline values of 27.6 ± 11.2% to 43.2 ± 11.8% was detected at the same time point when grade 1 + 2 forward motility was considered.

Sperm cell motility after swim-up

An increase of sperm motility after FSH treatment was also observed after swim-up, already becoming significant after 3 months of treatment (Table 1). In particular, the mean percentage of grade 1 motile spermatozoa increased from a baseline value of 8.2 ± 8.8% to 29.0 ± 14.4% ($P < 0.05$) after 3 months of treatment and did not show any further increase after 6 months. Similarly, the mean percentage of grade 1 + 2 forward motility displayed a significant increase (from 27.2 ± 15.9% to 59.1 ± 29.3%; $P < 0.05$) after 3 months of treatment and a slight further increase (up to

Table 2. Effects of hpFSH administration on the main ultrastructural parameters of spermatozoa

Electron microscopy features	Baseline (%)	hpFSH, 6 months (%)
Normal spermatozoa	5.8 ± 2.4	14.8 ± 3.1**
Alterations:		
A-C-R type	34.7 ± 6.0	27.2 ± 4.1**
D type	21.2 ± 3.4	14.5 ± 2.7**
MP type	6.1 ± 0.4	4.6 ± 0.8*
Necrotic spermatozoa	37.0 ± 8.1	30.9 ± 7.7*

**$P < 0.001$; *$P < 0.05$.

64.6 ± 21.8%) after 6 months. After 3 and 6 months of therapy, a significant reduction ($P < 0.05$) of non-progressive motility (grade 3) and immotile spermatozoa (grade 4) was also detected.

Sperm cell morphology, light microscopy

No significant variations in sperm morphology were detected after FSH administration (Table 1). The mean baseline percentage values of atypical spermatozoa (59.8 ± 4.9%) remained almost unchanged after 3 and 6 months of treatment (57.3 ± 4.2% and 55.7 ± 5.1%, respectively).

Sperm cell morphology, electron microscopy

The ultrastructural analysis before FSH administration revealed a low number of normal spermatozoa (5.8 ± 2.4%), with a large spectrum of teratospermia involving mainly multiple-associated alterations of acrosome, head and chromatin (associated A–C–R type, 34.7 ± 6.0%) or isolated head dysmorphic pattern (D type 21.2 ± 3.4%). Middle-piece alterations were evident in 6.1 ± 0.4% of examined spermatozoa and necrospermia features were observed in 37.0 ± 8.1% of the examined population. High magnification of sperm tails revealed axonal abnormalities (absence or disorganization of the axonemal complex) in 41.6 ± 6.9% of spermatozoa. An overview of the main features found in idiopathic OTA men is presented in Fig. 1.

Effects of hpFSH administration on sperm cells ultrastructure

An improvement of the ultrastructural pattern was detected after hpFSH treatment (normal spermatozoa, 5.8 ± 2.4% at baseline; 14.8 ± 3.1% after 6 months of therapy, $P < 0.001$) (Table 2). A marked reduction of A–C–R and D type abnormalities was clearly evident (from 34.7 ± 6.0% to 27.2 ± 4.1% and from 21.2 ± 3.4% to 14.5 ± 2.7%, respectively; $P < 0.001$). Middle-piece alterations were also quantitatively reduced, as well as the features of necrosis, although in a less prominent way (from 6.1 ± 0.4% to 4.6 ± 0.8% and from 37.0 ± 8.1% to 30.9 ± 7.7%, respectively; $P < 0.05$). On the contrary, axonemal alterations in the sperm tail remained almost unchanged after 6 months of therapy (from 41.6 ± 6.9% to 40.5 ± 4.6%; not significant) (data not shown).

Discussion

The therapeutic use of gonadotrophins has been restricted in the past to the classic hypogonadotrophic hypogonadism (Finkel *et al.*, 1985), but more extensive indications have also been suggested (Hommonai *et al.*, 1978; Chehval & Mehan, 1979; Margalioth *et al.*, 1983; Pusch *et al.*, 1986; Jockenhovel *et al.*, 1990; Acosta *et al.*, 1991; 1992; Glander & Kratzsch, 1997; Foresta *et al.*, 1998). Current knowledge of FSH biology and of its peripheral targets (Alphen *et al.*, 1988; Kula, 1991) makes the administration of hp-FSH a rational therapeutic approach in idiopathic OTA when hormone (FSH, LH, T, E_2 and PRL) serum levels are normal. The administration of exogenous FSH can activate Sertoli cells which are responsible for spermatogenesis support, without interfering with Leydig cell function, and also avoiding the increase in local oestrogens.

However, recent data suggest that the FSH pathophysiology is more complex than was believed until a few years ago. The well-known discrepancies between the biological activity of FSH and its immunoreactivity suggest that a qualitative hormone defect could be somehow involved in these clinical alterations (Schill, 1995). Furthermore, it has been shown that serum FSH is a mixture of microheterogeneous isoforms which vary in different physiological and pathological conditions (Huhtaniemi & Aittomaki, 1998). Finally, several gain- and loss-of-function mutations of FSH and FSH-receptor genes (i.e. FSH β-subunit gene) have been discovered in man (Huhtaniemi *et al.*, 1998). Although extremely rare, such mutations could be involved in some cases of OTA.

Previous studies have shown an improvement of the fine structural morphology of spermatozoa after short-term (1–3 months) therapy with FSH (Bartoov *et al.*, 1994; Baccetti *et al.*, 1997). The striking feature of our results is the positive effect of long-term (6 months) FSH therapy on sperm concentration, ultrastructure and, more interestingly, sperm motility in idiopathic oligoasthenozoospermic patients. Such data may have practical implications both in natural and medically assisted fertilization, due to the long-term latency before an optimal effectiveness of the therapy is reached.

No effect of FSH treatment on serum hormonal concentrations was observed in our patients. Sperm-cell motility was significantly increased after hpFSH treatment; both fast and weak forward motility were positively affected, reaching an increase that was more than double the baseline value. Such an improvement was statistically significant after 6 months of hp-FSH administration. A significant variation was detected even after 3 months of treatment, when the sperm motility evaluated after swim up confirmed the sensitivity of this latter approach.

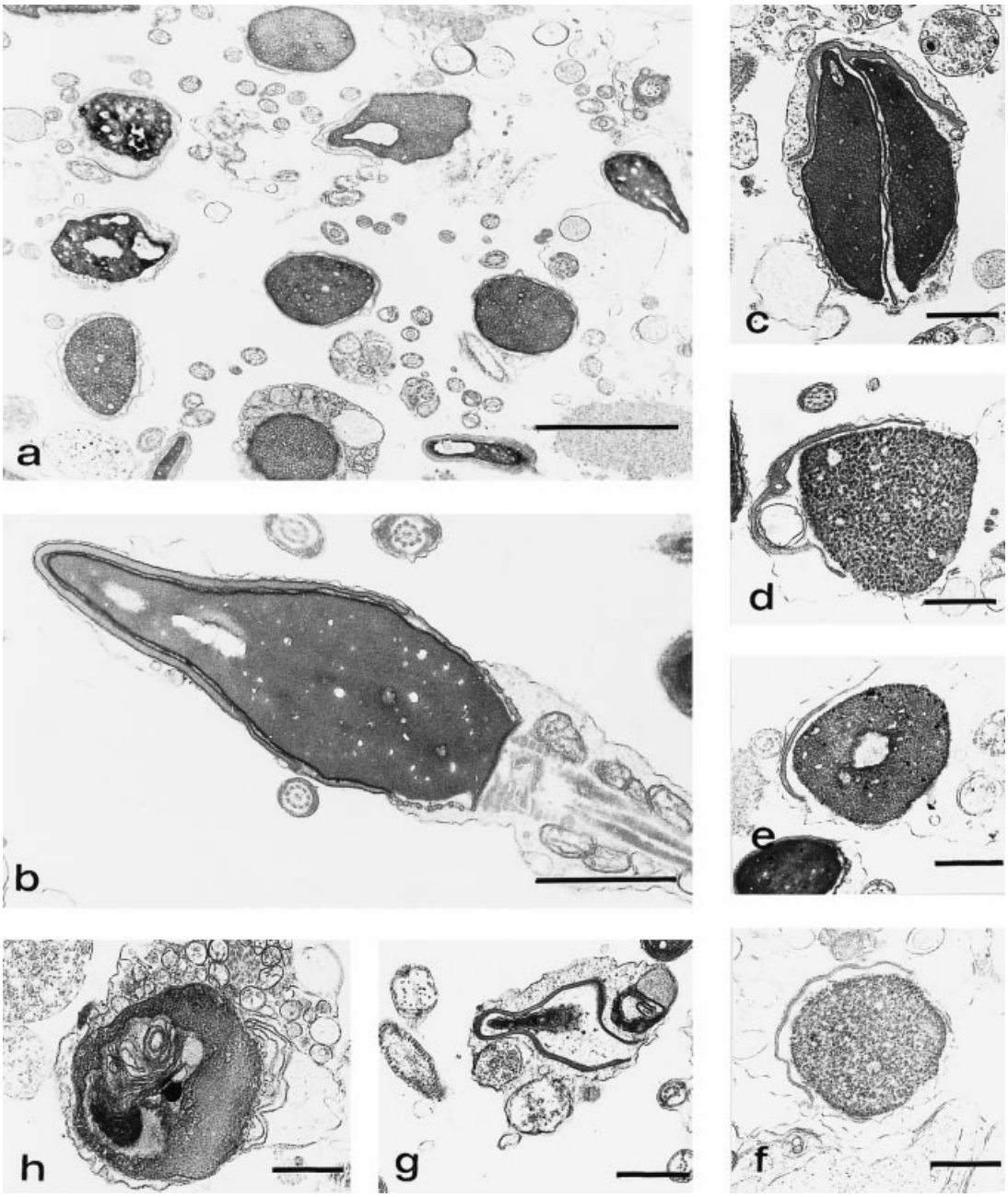

Figure 1. Overview of ultrastructural abnormalities detected in spermatozoa obtained from patients affected by idiopathic oligoasthenozoospermia. (a) Survey of teratospermic heads, scale bar, 2.8 μm; (b) head and middle piece of a normal sperm cell, scale bar, 1 μm; (c) double heads joined by a unique acrosome (D type alteration), scale bar, 1 μm; (d–f), round or dismorphic (hypo-elongated) heads with small or lifted acrosome and chromatin hypocondensation (combined A–C–R type alteration), scale bar, 1 μm; (g,h) abnormal heads, with heavy chromatin alterations and large intranuclear vacuoles, respectively; middle-piece abnormalities (MP type) are also evident. Scale bar, 1 μm.

Kinetic changes were accompanied by structural modifications of the spermatozoa, which were not evident in conventional light microscopy, but were revealed by ultrastructural analysis. In particular, the experimental model used in the present study showed a relevant reduction of modifications of subcellular organelles, mainly in the acrosome, head and chromatin, and, less prominently, in the middle-piece. Necrotic spermatozoa were also reduced. These features are well in accordance with the improvement of functional data. These could seem inconsistent with the relatively high aliquots of teratospermic features which persist after 6 months of FSH administration (see Table 1), but it should be kept in mind that the biological programme of spermatogenesis implicates a high degree of teratospermia as a normal condition (World Health Organization, 1992). Furthermore, the same ultrastructural alterations described here can be found, in our personal

experience, in large aliquots (over 60%) of sperm cells in the ejaculate of fertile men without any genital tract pathology (Caucci *et al.*, 1997).

The data shown in the present report suggest that long-term hpFSH therapy improves the micro-environmental conditions of spermatogenesis. The integrity of seminiferous tubules, and particularly of Sertoli cell function, could thus become critically responsible for the observed clinical benefits.

A significant increase in sperm concentration has been described after FSH treatment of oligozoospermic patients in whom testicular cytology, evaluated by fine needle aspiration, did not show maturation defects (Foresta *et al.*, 1998). The same therapy did not result in any positive change in spermatogenesis when a maturation arrest was evident (Foresta *et al.*, 1998). These data also strongly support the assumption that micro-environmental integrity is a basic prerequisite for FSH therapeutic effectiveness.

It has been experimentally proven that deprivation of gonadotrophins increases apoptotic loss of germ cells (Sinha Hikim *et al.*, 1997) and that FSH administration prevents apoptosis in a stage-specific fashion (Henriksen *et al.*, 1996). These data can partially support the improvement of spermiogenesis after FSH administration.

The multiplicity of pathogenetic mechanisms supporting the various clinical expressions of idiopatic hypogonadism (oligozoospermia, variously combined with terato- and asthenozoospermia) is probably responsible for the discrepancies in therapeutic responses to gonadotrophins (see Skakkebaek *et al.*, 1994; Howard, 1995; for review). Although the small number of treated patients and the open model reduce the validity of the present study, the present data lead to the following suggestions: (i) that hp-FSH administration can be a rational and useful tool in the treatment of OTA when hormonal serum parameters support a substantial integrity of spermatogenetic micro-environment; and (ii) that an optimal therapeutic effect is reached after long-term (6 months) therapy.

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