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Human chorionic gonadotrophin treatment prior to microdissection testicular sperm extraction in non-obstructive azoospermia

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BACKGROUND: Despite the improved success rate of sperm retrieval by microdissection testicular sperm extraction (micro-TESE), methods to stimulate spermatogenesis in men with non-obstructive azoospermia (NOA) remain unexplored. The aim of this study was to evaluate the effects of hCG-based hormonal stimulation in men with NOA on the success of sperm retrieval by micro-TESE.

METHODS: Forty-eight men with NOA who had negative sperm retrieval results by the micro-TESE procedure were included. A second micro-TESE was subsequently performed on these men: 20 were not treated by any hormonal therapy, and 28 subjects received daily subcutaneous injections of hCG for 4–5 months prior to the second micro-TESE. Recombinant FSH was added if endogenous gonadotrophin levels decreased during the hCG stimulation. The sperm retrieval rate at the second micro-TESE; the levels of gonadotrophins, testosterone and estradiol; and the effects of hormonal therapy on testicular histology were evaluated.

RESULTS: Among the 28 men with hCG stimulation, 15 (54%) showed decreased LH and FSH levels (0.67 ± 0.10 and 0.96 ± 0.14 mIU, mean $\pm$ SEM, respectively) due to elevated serum testosterone (9.5 ng/dl). Sperm were obtained at the second micro-TESE from six men who had received hormonal therapy (21%), whereas no sperm were retrieved from untreated men ($P < 0.05$). Success at the second micro-TESE was more likely if histology at the first micro-TESE showed hypospermatogenesis.

CONCLUSIONS: The Leydig cells of the testis can respond positively to exogenous hCG even under hypergonadotropic conditions. HCG-based hormonal therapy prior to a second micro-TESE attempt is effective in men with hypospermatogenesis.

Key words: non-obstructive azoospermia / microdissection testicular sperm extraction / hormonal therapy

Introduction

The positive clinical outcome of gonadotrophin treatment for infertile men whose azoospermia is due to hypogonadotropic hypogonadism has been well documented. However, no established hormonal therapy is available for treatment of men with non-obstructive azoospermia (NOA), who usually present with high plasma gonadotrophin levels. Microdissection testicular sperm extraction (micro-TESE), which allows detection of small regions of sperm production in the testes, combined with ICSI plays a pivotal role in the treatment of NOA. The typical success rate of sperm retrieval by these procedures is 40–50%. Recent studies suggest that almost 60% of men with NOA have some sperm production in the testes (Schlegel, 2009); however, a number of patients remain surgically unmanageable because of maturation arrest and/or Sertoli cells only.

The actions of FSH and LH on germ cells are indirect and mediated by paracrine signals from Sertoli and Leydig cells, and close cell–cell interactions are required to maintain normal spermatogenesis (Plant and Marshall, 2001). It was generally believed that gonadotrophin treatment would be ineffective in the presence of high plasma levels of endogenous gonadotrophin. Plasma gonadotrophin causes down-regulation of FSH and LH receptors in Sertoli and Leydig cells, and a 'gonadotrophin reset' is the only strategy to prevent this gonadotrophin hyperstimulation. Foresta *et al.* (2004) have demonstrated an improvement in Sertoli cell function in severe testicular damage after reduction of high FSH plasma concentrations by administration of a

GnRH agonist (GnRHa). Previous attempts to improve spermatogenesis in men with NOA by treatment with either recombinant human (rh)-FSH alone or in combination with hCG have been successful (Selman et al., 2004; Efesoy et al., 2009).

We investigated the effects of high doses of hCG on serum testosterone and gonadotrophin levels and on sperm retrieval success rate by a second micro-TESE procedure, after a failed first attempt. To explain the effectiveness of high-dose hCG in stimulating Leydig cell function, we also examined the overnight secretion of gonadotrophins in men with NOA to assess the pulse frequency and amplitude of gonadotrophin release.

Materials and Methods

Subjects and treatment protocol

We retrospectively reviewed the records of consecutive NOA patients who underwent micro-TESE, performed by the same surgeon (K.S.), between April 2002 and March 2011. Of 139 micro-TESE procedures at the first attempt, 59 (42%) were successful in retrieving sperms or late spermatids. Of 80 patients with negative micro-TESE retrieval results, 62 men (78%) returned to our clinic to receive further therapy. After exclusion of cases of chromosomal abnormality (e.g. Klinefelter syndrome)—because sperm retrieval rate of these abnormalities vary widely, extremely small testes (<4 ml each; assessed by punched-out orchidometer), low levels of serum testosterone (<200 ng/dl) or any history of reproductive disorders (e.g. varicocele and cryptorchidism) and life-threatening diseases, 48 subjects were enrolled in this study.

Twenty of these 48 men did not agree with the hormonal treatment and underwent a second micro-TESE without any additional treatment (Fig. 1). The other 28 subjects were enrolled in the hormonal treatment protocol, starting at least 6 months after the first micro-TESE with subcutaneous self-injections of 5000 IU of hCG (Gonatropin®; ASKA Pharmaceutical, Tokyo, Japan), three times a week for 3 months. As illustrated in Fig. 1, patients who continued to show high plasma FSH values were maintained on the hCG treatment for 1–2 months prior to the second micro-TESE. Patients whose gonadotrophin levels decreased to lower than 3 mIU/l received additional rhFSH (GONAL-F®, Merck Serono, Geneva, Switzerland) at 150 IU, three times a week for 2 months prior to the second micro-TESE. Levels of serum testosterone, LH and FSH were measured every month. Estradiol (E_2) was measured just before the second micro-TESE. The study protocol was approved by the ethics committee of the Ube Industries Central Hospital, and all the patients gave written informed consent before the hormonal treatment.

Initial evaluation and micro-TESE procedure

Azoospermia was confirmed at least two times by analysis of semen specimens concentrated by centrifugation. No patients had received any hormonal therapy before referral to our center. For each patient, hormonal evaluation included measurement of plasma serum FSH, LH, E_2 and testosterone. The micro-TESE was performed according to the procedure of Schlegel (1999) with some modifications. The tunica albuginea of the testis was opened with a wide incision in an equatorial plane around approximately 300° of the circumference of the organ, and the testicular parenchyma was directly examined under an operative microscope. When no spermatozoa were identified in the initial sample, subsequent samples were obtained from the same testis and, as needed, from the contralateral testis. In men with sperms, the region around the sperm-positive site was analyzed histologically. In men without sperms, the region with the largest diameter of the seminiferous tubules in the testis was sent for histological analysis.

Assays

Blood serum was separated by centrifugation at 4°C for 10 min at 3000 rpm immediately after collection and then frozen at −20°C until analysis. LH and FSH levels were determined by enzyme-linked immunosorbent assay and E_2 and testosterone level by radioimmunoassay. Inter-

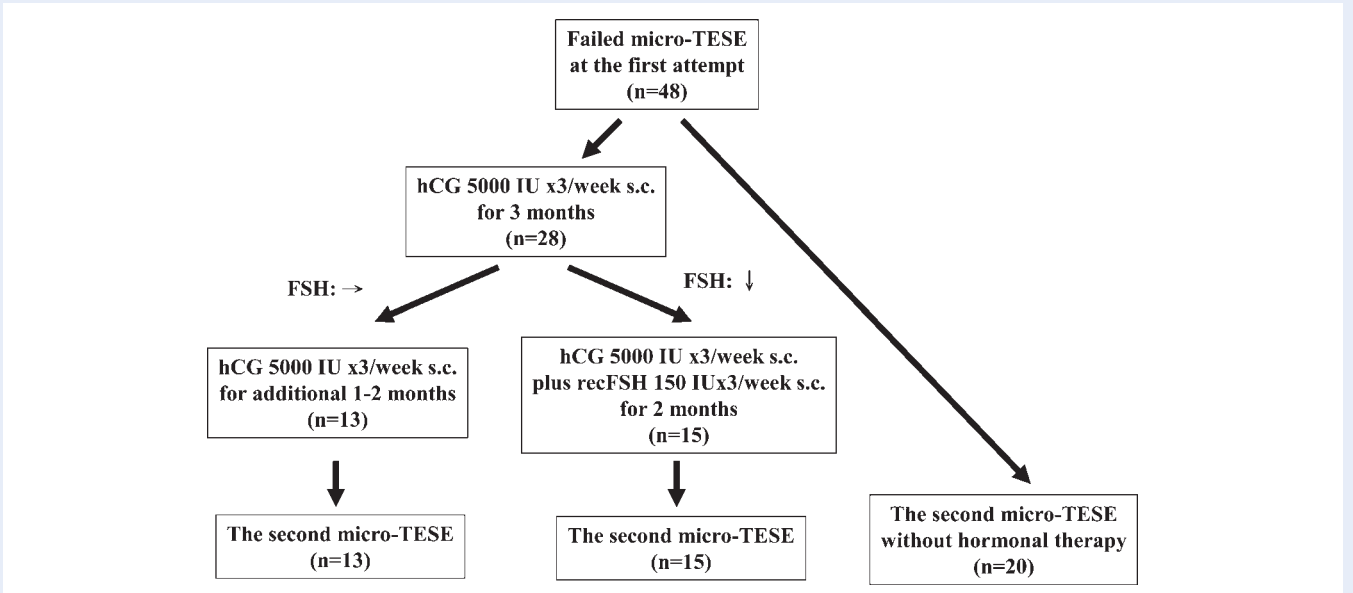

Figure 1 Treatment pathway for men who failed to retrieve sperms or late spermatids at the first micro-TESE.

assay and intra-assay coefficients of variation for all hormonal assays were <5% at the concentrations measured.

Measurement of overnight gonadotrophin secretion

Patients were admitted to the hospital the day before micro-TESE, and their overnight gonadotrophin secretion was analyzed. Of 48 men, 15 (31.5 ± 5.0 years old) agreed to overnight blood sampling. Blood samples were obtained every 20 min for 12 h, from 6:00 p.m. to 6:00 a.m., without disturbing the sleep of subjects. LH and FSH were measured as described already. Ten age-matched fertile men (31.9 ± 5.3 years old) with non-reproductive disorders (e.g. urolithiasis or scrotal hydrocele) were enrolled as normal controls. To evaluate the amplitude of the gonadotrophin secretion adjusted to the basal gonadotrophin levels, the change (Δ) relative to basal secretion was calculated by the following formula:

$$\Delta = (\text{peak value} - \text{lowest value}) \times 100/\text{lowest value}.$$

Testicular biopsies and histological assessment

The testicular biopsies were fixed in Bouin's solution for 12 h and then embedded in paraffin, after which 5-μm sections were cut and stained with hematoxylin and eosin. Histological patterns of the germ cell maturation were categorized as being indicative of (i) normal testicular histology and spermatogenesis, (ii) hypospermatogenesis, (iii) germ cell arrest at the primary spermatocyte stage, (iv) germ cell arrest at the spermatogonia stage, (v) Sertoli cell only syndrome or (vi) complete hyalinization of seminiferous tubules, reported by McLachlan *et al.* (2007). The presence of tubular thickening, Leydig cell hyperplasia and interstitial fibrosis/edema was also recorded.

Statistical analysis

Unpaired Student's *t* and χ^2-tests were used to compare the results between the two groups with or without hormone therapy. Paired Student's *t*-test was used to assess the changes in testosterone, LH and FSH levels during the hormonal therapy relative to pretreatment levels. χ^2 test was used to test histological changes between the first and the

Table I Patients' characteristics, histological patterns and sperm retrieval rate at the second micro-TESE.

	All patients (*n* = 48)	No treatment (*n* = 20)	Hormonal therapy (*n* = 28)	*P*-value
Patients' age	34 ± 5.3 (23–47)[a]	33 ± 4.9	34 ± 5.7	NS[b]
Wives' age	32 ± 5.0 (23–43)[a]	31 ± 4.3	32 ± 4.4	NS[b]
LH (IU/l)	13 ± 5.4 (2.3–33.5)[a]	13 ± 3.6	123 ± 6.4	NS[b]
FSH (IU/l)	28 ± 13.8 (11.0–65.3)[a]	28 ± 12.7	28 ± 14.8	NS[b]
Testosterone (ng/dl)	372 ± 132.6 (210.0–770.0)[a]	368 ± 133.0	375 ± 134.8	NS[b]
Estradiol (pg/ml)	27 ± 11.9 (16.5–54.8)[a]	27 ± 11.5	28 ± 12.3	NS[b]
Bilateral testicular volume (ml)	17 ± 8.9 (8.0–36.0)[a]	16 ± 8.7	17 ± 9.7	NS[b]
Number of patients with T<300 ng/dl	18 (38%)	7 (35%)	11 (39%)	NS[b]
Hypospermatogenesis (at first TESE)	12 (25%)	4 (20%)	8 (29%)	NS[e]
Uniform reduction in late spermatid number	0	0	0	
Moderate reduction in late spermatid number mixed with normal tubules	0	0	0	
Mixed with tubules showing no progression past spermatocyte stage	7 (15%)	3	4	
Mixed with tubules showing no progression past spermatogonia	1 (2%)	1	0	
Mixed with tubules containing no germ cells	0	0	0	
Mixed with hyalinized tubules	4 (8%)	0	4	
Germ cell arrest at primary spermatocyte stage	15 (31%)	7 (35%)	8 (29%)	
Germ cell arrest at spermatogonia stage	7 (15%)	3 (15%)	4 (14%)	
Sertoli cell only syndrome[d]	10 (21%)	4 (20%)	6 (21%)	
Complete hyalinization of seminiferous tubules[d]	4 (8%)	2 (10%)	2 (7%)	
Sperm retrieval rate at the second micro-TESE	6 (13%)	0 (0%)	6 (21%)	$P < 0.05$[c]

All the data were the ones before the first micro-TESE and expressed as mean ± SEM .
[a]Minimum–maximum values.
[b]Unpaired Student's *t*-test.
[c]χ^2 test.
[d]These cases were enrolled in the hormonal treatment protocol because the presence of germ cells was suspected in the first micro-TESE samples.
[e]After each group was categorized as hypospermatogenesis or not, χ^2 test was performed.

second micro-TESE. Data are expressed as mean ± SEM. A two-sided $P < 0.05$ was considered statistically significant in all analyses. The statistical software InStat (GraphPad Software, La Jolla, CA) was used.

Results

Patients' characteristics, histological patterns and the second micro-TESE results

All the patients were diagnosed as idiopathic NOA and the patient characteristics in this study are listed in Table I. There were no significant differences in age, pretreatment testicular volume or pretreatment serum levels of LH, FSH or testosterone between the groups who received or did not receive hormonal therapy. The number of men with hypospermatogenesis at the first micro-TESE was also similar between the groups (25 and 20%, respectively, Table I). No sperm were detectable in ejaculates of any of the subjects prior to the second micro-TESE attempt. The mean time period between the first and second micro-TESE procedures was 20 months in the group with hormonal therapy versus 17 months in the group without. Of the 20 men without hormonal therapy, no sperm were retrievable at the second micro-TESE. In contrast, sperm were successfully retrieved at the second micro-TESE from 6 of the 28 men (21.4%) treated with hormonal therapy ($P < 0.05$) (Table I). The collected sperm samples were kept frozen until ICSI. No significant differences in testicular volume were observed between the groups just before the second micro-TESE (data not shown).

Changes in gonadotrophins, testosterone and E_2 levels during the hormonal therapy

Hormonal therapy was started by subcutaneous injection of 5000 IU of hCG, three times a week. At 12 weeks, subcutaneous administration of 150 IU rhFSH, three times a week for an additional 8 weeks, was begun in 15 of the 28 subjects in the hormonal therapy group (54%) because of their remarkably low levels of circulating FSH (below 2 mIU/l). The FSH levels did not decrease in 13 of the 28 men (46%), and they continued to receive hCG for an additional 1–2 months prior to the second micro-TESE (Fig. 1). Table II lists the patient characteristics based on the hormonal therapy. There was no significant difference in age, testicular volume or pretreatment serum testosterone level. The number of patients with serum testosterone levels below 300 ng/dl was similar between the groups (Table II). Too few patients were included to determine if the additional administration of rhFSH caused a significant difference in the sperm retrieval rate (Table II). Figure 2 illustrates the changes in serum levels of testosterone, LH and FSH of all subjects who received hormonal therapy, those who received hCG/rhFSH and those who received hCG alone. Elevation of serum testosterone was prominent in subjects who eventually received rhFSH (Fig. 2D), and significant decrease in gonadotrophins rapidly followed the start of treatment (Fig. 2E and F). There was no intermediate level of gonadotrophins between the hCG groups with or without rhFSH, and the results were clearly stratified into the two groups. The serum E_2 concentration (mean ± SD) was significantly ($P < 0.0001$) higher after hormonal treatment than at initial evaluation (50 ± 13 versus 28 ± 12 pg/ml, respectively). The hormonal treatments were well tolerated in all patients, and in no case was the study ended because of adverse effects. Three of the patients (11%) showed symptoms of acne, and two (7%) of gynecomastia, but no specific additional treatment was needed for these cases.

Table II Patients' characteristics and sperm retrieval rate categorized by hormonal therapy regimen.

	hCG alone ($n = 13$)	hCG plus rhFSH ($n = 15$)	*P*-value
Patient age	32 ± 5.8	37 ± 4.8	NS[a]
Bilateral testicular volume (ml)	19 ± 8.3	16 ± 6.4	NS[a]
LH (IU/l)	16 ± 7.6	10 ± 2.9	<0.05[a]
FSH (IU/l)	38 ± 16.0	18 ± 5.0	<0.001[a]
Testosterone (ng/dl)	355 ± 105.2	359 ± 94.8	NS[a]
Estradiol (pg/ml)	29 ± 14.2	27 ± 9.3	NS[a]
T<300 ng/dl	5 (38.5%)	6 (40.0%)	NS[a]
Number of patients with successful sperm retrieval at the second micro-TESE	2 (15.4%)	4 (26.7%)	NS[b]

All the data except for sperm retrieval rate were measured before the first micro-TESE. rhFSH, recombinant human FSH.
[a]Unpaired Student's *t*-test.
[b]χ^2 test.

Patterns of overnight secretion of gonadotrophins in men with NOA

Representative LH and FSH secretion patterns in a normal man versus a man with NOA are drawn in Fig. 3. Despite the blunted curve that results from evaluation only of the 20-min interval (and not of the 10-min interval), on average a pulsatile secretion was observed every 90–120 min in normal men (Fig. 3A and B). On the other hand, pulsatile secretion was less frequent in men with NOA (Fig. 3C and D). Absolute amplitudes of LH and FSH pulses were similar in control and NOA groups, but because of the lower basal levels, pulse heights expressed as a percentage of the basal level were significantly greater in the control group (Fig. 3E and F).

Predictive factors for sperm retrieval at the second micro-TESE

Among the men given hormonal treatment, there was no significant difference in age, pretreatment testicular volume or pretreatment levels of LH, FSH, testosterone or E_2 before the first micro-TESE attempt in men testing positive and those testing negative for the presence of sperm at the second micro-TESE (Table III). Hypospermatogenesis was found in 8 of the 28 men (29%) at the first micro-TESE and four of them (50%) had sperm present at the second micro-TESE. The prevalence of hypospermatogenesis, which contained various degrees of germ cell maturation, at the first micro-TESE was significantly higher in men where sperm were found at the second micro-TESE than in men who failed (67 versus 18%; Table III). There were no significant differences in the pre-operative values between the men with hypospermatogenesis at the first micro-TESE and those without it (data not shown). Data regarding maturation of the germ

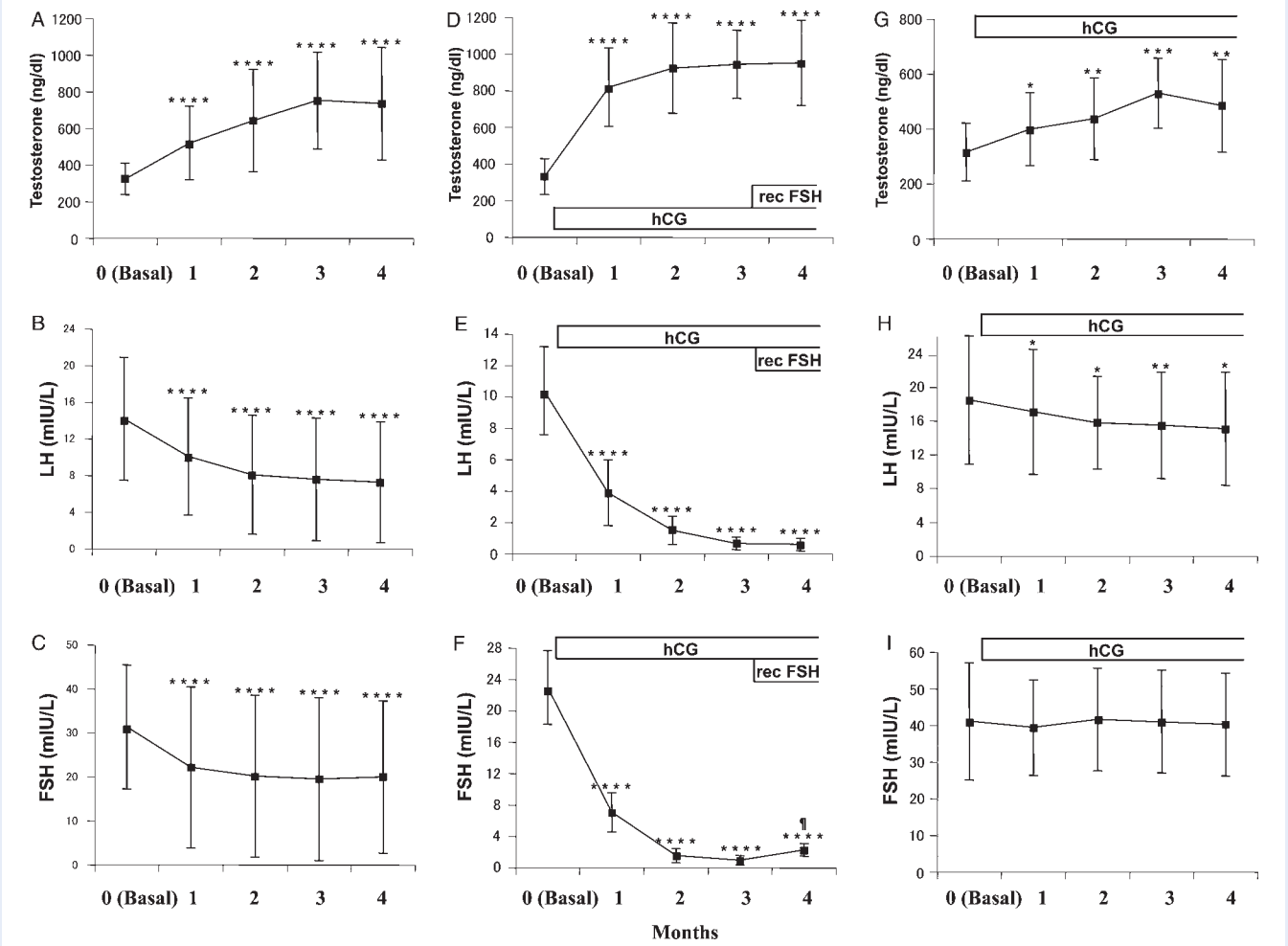

Figure 2 Effects of hCG-based hormonal therapy on the serum concentrations of testosterone (**A**, **D** and **G**), LH (**B**, **E** and **H**) and FSH (**C**, **F** and **I**) in all patients ($n = 28$, A–C), patients treated with hCG/rhFSH ($n = 15$, D–F) or with hCG alone ($n = 13$, G–I). Data are expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared with basal. ¶ $P < 0.05$ compared with the value at 3 months.

cells at the first micro-TESE are summarized in Table III. No mature sperm was obtained at the second micro-TESE if the initial histology was arrest at spermatogonia stage, Sertoli cell only or hyalinization of seminiferous tubules (Table III).

Effects of micro-TESE with or without hormonal treatment on testicular histology

Histological patterns at the first and second micro-TESE were summarized in Table IV. All men who could successfully obtain sperm at second micro-TESE after hormonal therapy had various degrees of normal tubules. Deterioration of spermatogenesis was observed in some men who failed the second micro-TESE; however, there were no significant differences in the testicular histology between the first and the second micro-TESE (Table IV). Representative histological alterations in the testes of men who failed the second micro-TESE are shown in Fig. 4. In addition to the tubular lesions, increased thickness of basement membrane (arrow), Leydig cell hyperplasia (arrowhead), and interstitial fibrosis/edema were often observed after the hormonal therapy (Fig. 4B and D). There were no specific alterations after hCG or hCG/rhFSH therapy.

Discussion

Treatment with GnRHa or exogenous testosterone to trigger a 'gonadotrophin reset' is a reasonable strategy to decrease the hypersecretion of gonadotrophins in men with NOA (Foresta *et al.*, 2004). In this study, we observed that Leydig cells produced increased amounts of testosterone in response to high-dose hCG stimulation even under a hypergonadotropic condition and that in over half the men treated (15/28), endogenous gonadotrophins were suppressed below pre-adolescent levels through a negative feedback mechanism of elevated serum testosterone. In contrast with the hormonal environment created by GnRHa or exogenous testosterone, treatment by high doses of hCG may elevate intratesticular testosterone while resetting FSH action, a unique hormonal environment to which the testes have never been naturally exposed. It has been shown that hCG administered prior to the first micro-TESE results in a greater

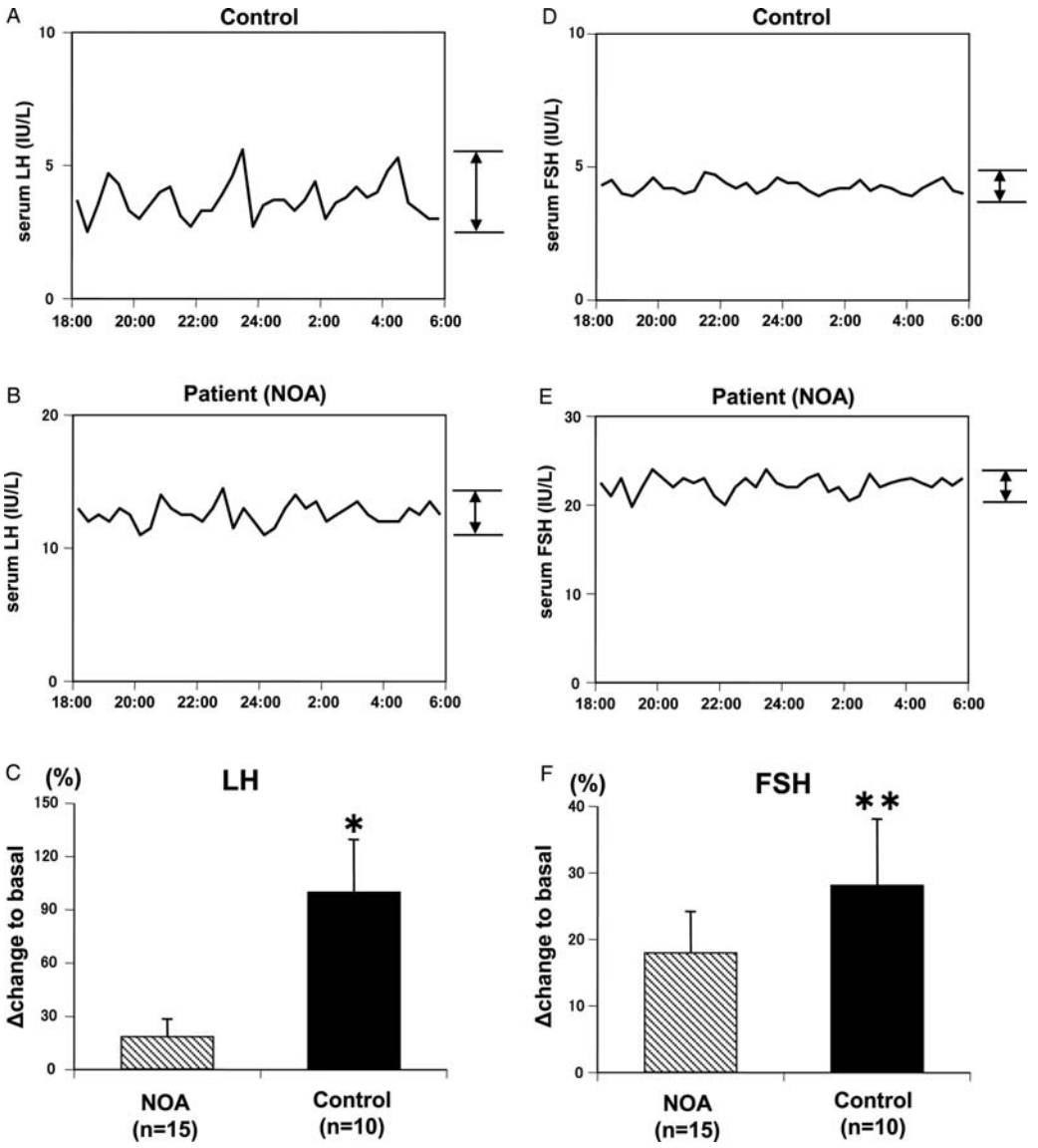

Figure 3 Overnight serum LH (**A** and **C**) and FSH (**B** and **D**) profiles of control ($n = 10$, A and B) and NOA patients ($n = 15$, C and D). Serum samples were obtained at 20 min intervals from 6:00 p.m. to 6:00 a.m. Bidirectional arrows indicate the pulse amplitude and Δchanges to basal secretion was calculated for each patient. Mean ± SEM of Δchanges to basal secretion of LH (**E**) and FSH (**F**). One hundred percent indicates that the highest value is twice from the basal (bottom) value. $^{*}P < 0.01$, $^{**}P < 0.05$ compared with NOA.

likelihood of sperm retrieval in some patients (Ramasamy *et al.*, 2009), but there is no established method to obtain sperm in the case of a failed micro-TESE. Unlike the 'gonadotrophin reset', this hCG-based hormonal therapy may not impair the testosterone-dependent steps during spermatogenesis (e.g. spermiogenesis). By creating this unique hormonal environment, 6 of 28 patients (21%) who failed to yield sperm at the first micro-TESE succeeded at the second attempt (Table I). The precise mechanism underlying promotion of spermatogenesis still remains unclear, but this hCG-based hormonal therapy provides a new opportunity for men with NOA for whom sperm retrieval via micro-TESE has failed.

A number of studies suggest that FSH rather than LH plays a crucial role in stimulating spermatogenesis (i.e. DNA synthesis in spermatogonia and preleptotene spermatocytes) indirectly through the FSH receptor in Sertoli cells. Fifteen of 28 patients (54%) enrolled in our hormonal treatment protocol showed decreased FSH levels (i.e. FSH reset) after hCG treatment (Fig. 2D–F), and subsequent rhFSH administration was a reasonable therapy to stimulate spermatogenesis. When histological examination detects hypospermatogenesis, the patient has a chance to respond to the hormonal therapy (Table III). Increased intratesticular testosterone following hCG treatment promotes spermiogenesis; the stages for which high intratesticular testosterone levels are essential (Matthiesson *et al.*, 2006). Considering that hCG alone is effective in 2 of 15 men who had sperm at the second micro-TESE, rhFSH is not always necessary if the germ cell maturation has progressed to near mature sperm. The

Table III Patients' characteristics and histological patterns at the first micro-TESE categorized by the second micro-TESE results for the hormone treatment group.

Histological patterns obtained at first micro-TESE	Sperm (+) at the second micro-TESE ($n = 6$)	Sperm (−) at the second micro-TESE ($n = 22$)	*P*-value
Patient age	36 ± 5.5	34 ± 5.3	NS[a]
Bilateral testicular volume (ml)	13 ± 6.5	18 ± 9.1	NS[a]
LH (IU/l)	9 ± 4.2	14 ± 6.7	NS[a]
FSH (IU/l)	26 ± 15.5	29 ± 15.0	NS[a]
Testosterone (ng/dl)	305 ± 59.2	395 ± 143.8	NS[a]
Estradiol (pg/ml)	29 ± 14.2	28 ± 11.9	NS[b]
Hypospermatogenesis	4 (67%)	4 (18%)	$P < 0.05$[c]
Uniform reduction in late spermatid number	0	0	
Moderate reduction in late spermatid number mixed with normal tubules	0	0	
Mixed with tubules showing no progression past spermatocyte stage	3	1	
Mixed with tubules showing no progression past spermatogonia	1	0	
Mixed with tubules containing no germ cells	0	0	
Mixed with hyalinized tubules	1	3	
Germ cell arrest at primary spermatocyte stage	2 (33%)	6 (27%)	
Germ cell arrest at spermatogonia stage	0	4 (18%)	
Sertoli cell only syndrome[b]	0	6 (27%)	
Complete hyalinization of seminiferous tubules[b]	0	2 (9%)	

All the data were the ones before the first micro-TESE.
[a]Unpaired Student's *t*-test.
[b]These cases were enrolled in the hormonal treatment protocol because the presence of germ cells was suspected in the first micro-TESE samples.
[c]After each group was categorized as hypospermatogenesis or the others, χ^2 test was performed.

Table IV Histological changes before and after the hormonal therapy.

	No treatment ($n = 20$)		Hormonal therapy sperm (+) ($n = 6$)		Hormonal therapy sperm (−) ($n = 22$)	
	1st	2nd[a]	1st	2nd[a]	1st	2nd[a]
Hypospermatogenesis	4 (20%)	2 (10%)	4 (67%)	6 (100%)	4 (18%)	3 (14%)
Uniform reduction in late spermatid number	0	0	0	0	0	0
Moderate reduction in late spermatid number mixed with normal tubules	0	0	0	6	0	0
Mixed with tubules showing no progression past spermatocyte stage	3	0	3	0	1	0
Mixed with tubules showing no progression past spermatogonia	1	0	0	0	0	0
Mixed with tubules containing no germ cells	0	0	0	0	0	1
Mixed with hyalinized tubules	0	2	1	0	3	2
Germ cell arrest at primary spermatocyte stage	7 (35%)	4 (20%)	2 (33%)	0	6 (27%)	2 (9%)
Germ cell arrest at spermatogonia stage	3 (15%)	4 (20%)	0	0	4 (18%)	8 (36%)
Sertoli cell only syndrome	4 (20%)	6 (30%)	0	0	6 (27%)	7 (32%)
Complete hyalinization of seminiferous tubules	2 (10%)	4 (20%)	0	0	2 (9%)	2 (9%)

1st, first micro-TESE, 2nd, second micro-TESE.
[a]After each group was categorized as hypospermatogenesis or the others, χ^2 test was performed between the first and the second micro-TESE.

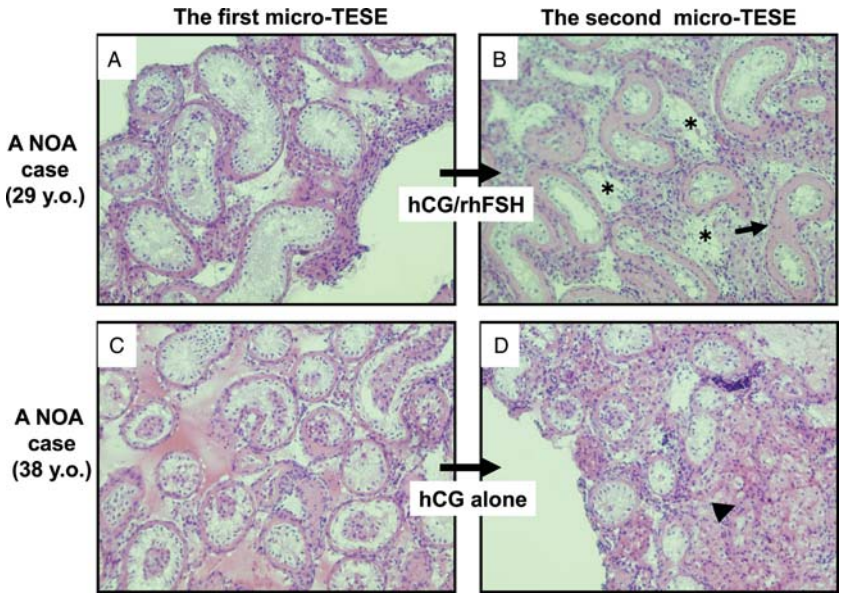

Figure 4 Effects of hCG-based hormonal therapy on the testicular histology. Photomicrographs (hematoxylin–eosin staining, ×200) of representative two cases are shown. Note that there were no specific changes between hCG/rhFSH and hCG alone. An arrow indicates the increased thickness of the basement membrane. An arrowhead indicates the Leydig cell hyperplasia. *Indicates interstitial edema.

exact mechanism that restores the function of Sertoli cells after FSH reset is not fully understood. High plasma FSH levels, determining a down-regulation of FSH receptors, impair tubular function. Foresta *et al.* (2004) supported this hypothesis, demonstrating an improvement in Sertoli cell function in severe testicular damage after reduction of high FSH plasma concentration by administration of a GnRHa. The Sertoli cell is also regulated by testosterone, and its function is restored by elevated intratesticular testosterone (O'Shaughnessy *et al.*, 2010). The hCG response of testosterone production has been shown to predict the success of sperm retrieval (Madgar *et al.*, 2002; Ramasamy *et al.*, 2009), indicating that stimulating Leydig cell function even under hypergonadotropic conditions causes favorable effects on spermatogenesis.

A possible method to restore Leydig cell function is the generation of an hCG pulse through exogenous high-dose hCG administration. *In vitro* studies suggest that subsequent hCG stimulation of Leydig cells, even under hypergonadotropic conditions, restores their function (also seen in cells cultured with high concentrations of gonadotrophin) (Aggarwal *et al.*, 2009; Wistuba *et al.*, 2010). In men, LH secretion is determined by the frequency, amplitude and duration of its secretory pulses with large between individual variations (Spratt *et al.*, 1987). Continuous monitoring of gonadotrophin secretion showed a decreased relative amplitude in NOA because the basal gonadotrophin levels were much higher in the NOA group (Fig. 3E and F), indicating that the gonadotrophin stimulation of Leydig and Sertoli cells is paradoxically weak because of desensitization. In fact, decreased LH pulses in humans are associated with a lower Leydig cell sensitivity and reduced testosterone production (Keenan *et al.*, 2004). To obtain high amplitude of hCG pulses, we started subcutaneous injection of 5000 IU hCG, three times a week, which is the highest dose in the human study. Even in the hCG-alone group, the increase in serum testosterone level was significant during the treatment (Fig. 2G) but was not sufficient to cause the negative feedback regulation that would suppress gonadotrophin secretion (Fig. 2H and I). Pretreatment serum LH and FSH levels were significantly higher in the hCG-alone treatment group (16.2 and 38.4 IU/l, respectively) than in the hCG/rhFSH group (9.5 and 18.1 IU/l, respectively), indicating that severely impaired testes (Leydig cells) did not produce testosterone in response to high doses of hCG (Table II). Higher hCG doses (e.g. 10000 IU, three times per week) may stimulate the production of testosterone in men with high gonadotrophin levels.

Although deteriorations of testicular histology were observed in some men regardless of hormonal therapy (Table IV, Fig. 4), there was no significant difference of histological changes between the first and the second micro-TESE (Table IV). One of the prominent changes after hCG stimulation is the increased number of Leydig cells; several specimens showed Leydig cell hyperplasia (Fig. 4), which is in agreement with the *in vitro* studies indicating that hCG stimulation not only promotes proliferation (Shiraishi and Ascoli, 2006, 2007) but also inhibits apoptosis of Leydig cells (Tai *et al.*, 2009). Increased numbers of Leydig cells itself is not harmful, but it has been shown that high LH levels increase vascular permeability and induce edematous change in the testis (Bergh and Damber, 1993). Further study is needed to determine whether hCG stimulation may be optimized to effectively treat male infertility but avoid some of the commonly observed adverse effects of supraphysiological hCG doses, such as increased thickness of basement membrane and interstitial fibrosis.

Tournaye *et al.* (1997) reported that testicular histology is the only effective predictor of successful sperm recovery. Taken together with our observation that the presence of hypospermatogenesis categorized by McLachlan's criteria (2007) in the histology at the first micro-

TESE is a predictor of success in the second micro-TESE (Table III), we strongly recommend the hormonal therapy in these men prior to second micro-TESE. Future studies should investigate better protocols of hormonal therapy to improve the sperm retrieval rate.

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Authors' roles

K.S. designed the study, performed the micro-TESE and hormonal therapy, analyzed data and wrote the paper. C.O. and T.S. supported the micro-TESE and performed histological analysis. H.M. supervised the study and edited the manuscript.

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Conflict of interest

None declared.

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