

Pharmacological concentrations of follicle-stimulating hormone and testosterone improve the efficacy of in vitro germ cell differentiation in men with maturation arrest

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Objective: To examine whether in vitro differentiation of germ cells from men with maturation arrest is improved by augmenting FSH and T concentrations above the values effective in samples from men with normal spermatogenesis.

Design: Prospective, controlled in vitro study.

Setting: Private assisted reproduction centers and a university department.

Patient(s): Men with meiotic or postmeiotic maturation arrest.

Intervention(s): Testicular spermatid extraction, in vitro culture of testicular biopsy samples, intraoocyte injection of elongated spermatids, embryo culture and transfer.

Main Outcome Measure(s): Progression of in vitro germ cell differentiation, fertilization, and pregnancy outcomes with in vitro cultured germ cells.

Result(s): In some cases of meiotic and postmeiotic maturation arrest, more advanced germ cell stages were achieved by in vitro culture in the presence of 500 IU/L FSH as compared with 50 IU/L FSH. The beneficial effect of 500 IU/L FSH was further potentiated by a simultaneous increase of T concentration from 1 to 10 μ M. Fertilizations with germ cells recovered after incubation with these pharmacological hormone concentrations gave rise to viable embryos and the births of five healthy babies.

Conclusion(s): Pharmacological concentrations of FSH and T are beneficial for in vitro maturation of germ cells from some men with in vivo maturation arrest. (Fertil Steril® 2002;77:245–51. ©2002 by American Society for Reproductive Medicine.)

Key Words: In vitro spermatogenesis, FSH, testosterone, maturation arrest, nonobstructive azoospermia

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Recent reports have shown that in vivo maturation arrest can be overcome, in some cases, by incubation of partially disintegrated testicular tissue in media containing FSH and T (1–3). Accordingly, a small subpopulation of germ cells arrested in the first meiotic prophase (primary spermatocytes) completed the two meiotic divisions and started postmeiotic differentiation in as little as 48 hours of culture. Similarly, postmeiotically arrested round spermatids entered the elongation phase of spermiogenesis in these conditions (4). Births of healthy infants were achieved by assisted re-

production techniques using in vitro matured spermatids from men with meiotic and postmeiotic maturation arrest (1, 2).

In spite of these encouraging data, in vitro maturation is known to fail in 50%–75% of cases, depending on the in vivo blocking stage (5). The reason why at least a few blocked cells from some men resume development in vitro while all cells from other men are completely refractory to this treatment is unknown. Nevertheless, the association of this germ cell refractoriness with higher serum FSH concentrations as compared with culture-responsive

cases (5) suggests that desensitization or a defective function of the FSH receptor may be involved. If this is the case, the receptor function may be restored by increasing the concentration of FSH in culture medium to pharmacological levels.

The dependence of *in vitro* maturation of human male germ cells on the concentration of FSH in culture medium was only studied in samples from men with normal spermatogenesis (6, 7), and the concentration-response relationship for T has never been addressed. To fill this gap, this study compares the effects of increasing FSH concentration in culture medium from 50 IU/L, a concentration at which the maximal effect of this hormone in testicular samples from men with normal spermatogenesis was reached (7), to 500 IU/L. Furthermore, the higher concentration of 500 IU/L FSH was tested either together with the previously used T concentration of 1 μM (7) or with a 10 times higher concentration (10 μM) of this hormone. Finally, first experience with assisted reproduction using germ cells matured in the presence of these elevated hormone concentrations is reported.

MATERIALS AND METHODS

Patients

This study involves 19 men with histological diagnosis of maturation arrest confirmed by immunocytochemical analysis of smeared testicular cells with 4D4 monoclonal antibody (8) as described elsewhere (6, 7). Eight of the participants suffered from premeiotic (spermatogonium stage) or early meiotic (primary spermatocyte stage) maturation arrest, whereas the maturation arrest was postmeiotic (round or early elongating spermatid stage) in the remaining 11 patients. Eighteen patients participated fully in the study protocol, including both *in vitro* culture of testicular cells with different concentrations of FSH and T and an assisted reproduction attempt with *in vitro* matured germ cells taken from the culture group in which the best progression of spermatogenic events had been achieved. The remaining one patient only underwent an assisted reproduction attempt after germ cell culture with a high concentration of FSH without T addition. Institutional Review Board approval was obtained for this study.

Testicular Tissue Recovery and Preparation

Testicular tissue was obtained by open testicular biopsy from multiple sites in each testis. Tissue pieces were disintegrated mechanically to a mixture of small segments of the seminiferous tubules, free cell clusters and individual cells as described (7). The tissue from different locations in both testes was then mixed together. This tissue preparation was carried out in gamete medium (Scandinavian IVF Science, Gothenborg, Sweden). Homogenized tissue in gamete medium was distributed into aliquots corresponding to individual treatment groups. One aliquot was used immediately for determination of the state of spermatogenesis before culture.

The other aliquots were cultured *in vitro* with different medium supplementations before performing the same analysis (see below).

Germ Cell In Vitro Culture

The basic features of the culture system used in this study were described elsewhere (6, 7). Briefly, a crude mixture of partially disintegrated testicular tissue was incubated at 30°C in gamete medium to which different concentrations of FSH and T had been added. The preparations of FSH and T used in this study were recombinant human FSH (Puregon, Organon, Oss, The Netherlands) and water-soluble, cell culture tested T (Sigma, St. Louis, MO), respectively. Both preparations were originally dissolved in gamete medium and then diluted to the final concentration by adding the appropriate volume of this solution to aliquots of mechanically disintegrated testicular tissue in the same medium (see above). Thus, no centrifugation step was necessary before testicular tissue samples were put to the final culture medium. The duration of *in vitro* culture was 48 hours unless otherwise indicated.

Evaluation of In Vitro Maturation Outcomes

In vitro maturation outcomes were evaluated by comparing the presence of individual stages of germ cell development in the aliquot of homogenized testicular tissue that was processed before the beginning of *in vitro* culture (as described above) and in individual aliquots of the same homogenized sample after culture with different concentrations of FSH and T. Each pre- and postculture sample was divided into two aliquots. One aliquot was digested with collagenase I (1,000 units/mL) and elastase (10 units/mL; both purchased from Sigma) as described elsewhere (7) and then subdivided into two subaliquots. One subaliquot was evaluated by standard cytologic analysis after smearing on microscope slides, fixation with 100% ethanol, and staining with the use of Papanicolaou method (9). The other subaliquot was processed by immunocytochemistry with 4D4 monoclonal antibody (6, 7). The 4D4 monoclonal antibody was used as an immunocytochemical germline marker, which had previously been shown to react with human male germ cells from the pachytene spermatocyte stage onward (6–8). A previously described terminology (6, 7), adapted after de Kretser and Kerr (10), is used to denote individual stages of postmeiotic differentiation. This classification distinguishes the Sa (round spermatid) Sb (early elongating spermatid), Sc (late elongating spermatid), and Sd (elongated spermatid) stages of postmeiotic maturation.

The other aliquot of the original enzyme-treated testicular cell suspensions was inspected by an inverted microscope with the use of Hoffman modulation contrast optics and a 40 \times objective lens. Spermatogonia and primary spermatocytes at individual stages of the first meiotic prophase were distinguished in these native preparations using criteria described elsewhere (11). The accuracy of this method was checked by using the classical silver-staining method for the

TABLE 1

Effects of different concentrations of FSH on in vitro development of germ cells from men with premeiotic or meiotic maturation arrest cultured in the presence of 1 μ M testosterone.

Patient	Serum FSH (IU/L)	In vivo blocking stage	Most advanced stage after culture with different FSH additions		
			No FSH	50 IU/L FSH	500 IU/L FSH
1	12.7	Leptotene	Leptotene	Leptotene	Leptotene
2	25.4	Pachytene	Pachytene	Pachytene	Pachytene
3	15.1	Pachytene	Pachytene	Pachytene	Sa spermatid
4	36.8	Spermatogonium	Spermatogonium	Spermatogonium	Spermatogonium
5	22.4	Leptotene	Leptotene	Leptotene	Leptotene
6	41.3	Leptotene	Leptotene	Leptotene	Pachytene
7	29.5	Pachytene	Pachytene	Pachytene	Sd spermatid
8	34.0	Pachytene	Pachytene	Pachytene	Sa spermatid

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visualization of meiotic chromosomes during the first meiotic prophase (12).

Assisted Reproduction Treatments

Controlled ovarian stimulation was performed with the use of recombinant human FSH (Puregon) after pituitary desensitization with a GnRH agonist beginning in the mid-luteal phase (13). Ovulation was induced by 10,000 IU hCG (Profasi, Serono, Rome, Italy), and oocytes were recovered by ultrasound-guided transvaginal follicle aspiration performed 35–36 hours later. Assisted reproduction treatment was performed only in those cases in which late elongating or elongated spermatids were found after in vitro culture of testicular biopsy specimens. Spermatids were injected into metaphase II oocytes using previously described techniques and instrumentations (14). Spermatid-injected oocytes were checked for the presence of pronuclei at three different time points—10, 13, and 16 hours after the injection. They were

considered normally fertilized when two pronuclei and two polar bodies were apparent at at least one of these checking times. Normally fertilized oocytes were cultured further to allow cleavage. Cleaved embryos were transferred to the patient's partner's uterus 2 days after spermatid injection when they had developed to the 2- to 4-cell stage.

RESULTS

Premeiotic and Meiotic Maturation Arrest

With an invariable T concentration of 1 μ M, increasing the concentration of FSH in culture medium from 50 to 500 IU/L led to an improvement of in vitro maturation outcomes in four out of eight patients with premeiotic or meiotic maturation arrest (Table 1). Moreover, with an invariable FSH concentration of 500 IU/L, increasing the concentration of T from 1 to 10 μ M led to further improvement in two

TABLE 2

Effects of different concentrations of T on in vitro development of germ cells from men with premeiotic or meiotic maturation arrest cultured in the presence of 500 IU/L FSH.

Patient ^a	In-vivo blocking stage	Most advanced stage after culture with different T additions		
		No T	1 μ M T	10 μ M T
1	Leptotene	Leptotene	Leptotene	Pachytene
2	Pachytene	Leptotene	Leptotene	Sd spermatid
3	Pachytene	Pachytene	Sa spermatid	Sd spermatid
4	Spermatogonium	Spermatogonium	Spermatogonium	Spermatogonium
5	Leptotene	Leptotene	Leptotene	Pachytene
6	Leptotene	Leptotene	Pachytene	Pachytene
7	Pachytene	Pachytene	Sd spermatid	Sd spermatid
8	Pachytene	Pachytene	Sa spermatid	Sd spermatid

^a Patients are identified by the same numbers as in Table 1.

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(patients 3 and 8) of these cases (Table 2). In addition, the combination of 500 IU/L FSH and 10 μ M T allowed in vitro maturation to occur in three cases (patients 1, 2, and 5) in which no in vitro differentiation was detected in the other culture groups (Tables 1 and 2).

With regard to the use of in vitro matured germ cells for assisted reproduction treatment, the improvement due to the increase in FSH concentration in culture medium (Table 1) was of immediate therapeutic benefit in three cases in which in vivo spermatogenesis was blocked at the pachytene stage of the first meiotic prophase. In these cases, the increase in FSH concentration made it possible to achieve meiosis and to form haploid cells (round and elongated spermatids) that could be used for micromanipulation-assisted fertilization (patients 3, 7, and 8). The concomitant increase in T concentration from 1 to 10 μ M enabled the development of postmeiotic cells in one additional case (patient 2) and improved postmeiotic development in patients 3 and 8 in whose testicular biopsy samples some germ cells reached the elongated spermatid (Sd) stage under these conditions, contrasting with the complete arrest at the round spermatid (Sa) stage when the culture was performed with the lower T concentration (Table 2).

Serum FSH concentration was elevated to various degrees in all these patients (Table 1), but the number of cases involved in this study is too low to evaluate the possible correlation between the need for very high FSH concentrations in culture medium and the serum FSH level.

Postmeiotic Maturation Arrest

Out of the 10 cases involved in this group, the increase in FSH concentration in culture medium from 50 to 500 IU/L, with an invariable T concentration of 1 μ M, led to an improvement of in vitro postmeiotic differentiation in four cases (Table 3). With an invariable FSH concentration of 500 IU/L, the increase in T concentration from 1 to 10 μ M reactivated postmeiotic differentiation in an additional two cases (patients 2 and 5) in which in vitro culture with 1 μ M T failed to produce any effect (Table 4). Furthermore, this elevation of T concentration enabled the achievement of morphologically more advanced stages of postmeiotic development in two cases (patients 3 and 8) in which a limited response was already observed after culture with the lower T concentration (Table 4). Similar to the cases of premeiotic and meiotic maturation arrest (see above), no apparent relationship between serum FSH concentration and the requirement for the higher FSH concentration in culture medium was noted (Table 3).

Reproductive Capacity of Germ Cells Matured In Vitro with High Concentrations of FSH and T

Out of eight patients with premeiotic or meiotic maturation arrest (Tables 1 and 2), assisted reproduction was attempted in four cases in which germ cells had achieved

TABLE 3

Effects of different concentrations of FSH on in vitro development of germ cells from men with postmeiotic maturation arrest cultured in the presence of 1 μ M T.

Patient	Serum FSH (IU/L)	In vivo blocking stage	Most advanced stage after culture with different FSH additions		
			No FSH	50 IU/L FSH	500 IU/L FSH
1	21.8	Sa spermatid	Sa spermatid	Sa spermatid	Sd spermatid
2	14.0	Sa spermatid	Sa spermatid	Sa spermatid	Sa spermatid
3	9.6	Sa spermatid	Sa spermatid	Sa spermatid	Sc spermatid
4	19.3	Sa spermatid	Sa spermatid	Sa spermatid	Sd spermatid
5	21.2	Sa spermatid	Sa spermatid	Sa spermatid	Sa spermatid
6	15.3	Sc spermatid	Sd spermatid	Sd spermatid	Sd spermatid
7	24.8	Sa spermatid	Sa spermatid	Sa spermatid	Sa spermatid
8	33.3	Sa spermatid	Sa spermatid	Sa spermatid	Sc spermatid
9	27.1	Sb spermatid	Sd spermatid	Sd spermatid	Sd spermatid
10	14.9	Sa spermatid	Sa spermatid	Sa spermatid	Sd spermatid

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postmeiotic stages of development after in vitro culture (patients 2, 3, 7, and 8). Altogether, 25 metaphase II oocytes were injected with elongated (Sd) spermatids. These injections resulted in the formation of 16 normal appearing zygotes (two pronuclei and two polar bodies). Fourteen zygotes cleaved and were transferred to the patients' partners 2 days after spermatid injection when they had reached the 2- to 4-cell stage of preimplantation development. ET was performed in all four cases. The numbers of embryos that were actually transferred to patients 2, 3, 7, and 8 were 3, 3, 4, and 4, respectively. One pregnancy was established (patient 8) and resulted in the birth of a healthy girl.

TABLE 4

Effects of different concentrations of T on in vitro development of germ cells from men with postmeiotic maturation arrest cultured in the presence of 500 IU/L FSH.

Patients ^a	In vivo blocking stage	Most advanced stage after culture with different T additions		
		No T	1 μ M T	10 μ M T
1	Sa spermatid	Sa spermatid	Sd spermatid	Sd spermatid
2	Sa spermatid	Sa spermatid	Sa spermatid	Sd spermatid
3	Sa spermatid	Sa spermatid	Sc spermatid	Sd spermatid
4	Sa spermatid	Sa spermatid	Sd spermatid	Sd spermatid
5	Sa spermatid	Sa spermatid	Sa spermatid	Sc spermatid
6	Sc spermatid	Sd spermatid	Sd spermatid	Sd spermatid
7	Sa spermatid	Sa spermatid	Sa spermatid	Sa spermatid
8	Sa spermatid	Sa spermatid	Sc spermatid	Sd spermatid
9	Sb spermatid	Sd spermatid	Sd spermatid	Sd spermatid
10	Sa spermatid	Sa spermatid	Sd spermatid	Sd spermatid

^a Patients are identified by the same numbers as in Table 3.

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Elongated spermatids (Sd) were used for assisted reproduction in eight of the nine cases with postmeiotic maturation arrest in which additional *in vitro* differentiation had been achieved (Tables 3 and 4). Late elongating spermatids (Sc) were used in the remaining case. Altogether, 76 metaphase II oocytes were injected, resulting in the formation of 51 normal appearing zygotes (two pronuclei and two polar bodies), of which 48 subsequently cleaved and were transferred to the patients' partners 2 days after spermatid injection when they had reached the 2- to 4-cell stage of preimplantation development. ET was performed in all nine cases.

The numbers of embryos that were actually transferred to patients 1, 2, 3, 4, 5, 6, 8, 9, and 10 were 5, 5, 6, 6, 5, 5, 6, and 5, respectively. Two pregnancies were achieved, one singleton (patient 3) and the other twin (patient 8). The singleton pregnancy resulted in the birth of a healthy girl. Two healthy children (boy and girl) were born from the twin pregnancy. As seen in Tables 3 and 4, the two cases were characterized by a complete postmeiotic maturation arrest *in vivo*, the most advanced germ cell stage found in freshly obtained testicular biopsy samples being the round spermatid at the Sa stage.

Another singleton pregnancy, resulting in the birth of a healthy boy, was achieved in a similar case of complete *in vivo* postmeiotic maturation arrest, which, however, is not included in Tables 3 and 4 because this patient was originally not involved in the study group. Unlike the other cases described in this study, the *in vitro* culture period was only 24 hours in this case, and the culture medium was supplemented with 500 IU/L FSH without any T added. After culture, a few germ cells developed up to elongated spermatids (Sd stage). Seven metaphase II oocytes from the patient's partner were injected with these *in vitro* matured elongated spermatids, resulting in the formation of four normal appearing zygotes (two pronuclei and two polar bodies), all of which subsequently cleaved and were transferred to the partner's uterus 2 days after spermatid injection when they were at the 4-cell stage.

DISCUSSION

This study confirms previously published findings showing the usefulness of culture medium supplementation with FSH and T for *in vitro* maturation of human testicular germ cells (4, 6, 7). Moreover, these data show that the dose-response relationship established for testicular samples from men with normal spermatogenesis (7) does not always apply to patients with severe spermatogenesis disorders. In fact, the concentrations of FSH and T (50 IU/L and 1 μ M, respectively) previously shown to be sufficient to produce a maximal effect on *in vitro* maturation of germ cells from men with normal spermatogenesis (7) are shown here to produce a maximal *in vitro* maturation response in some but not all patients with *in vivo* maturation arrest, regardless of

whether the cell differentiation is arrested during or after meiosis.

In some cases, the need for higher *in vitro* FSH concentrations was observed in patients with highly elevated (>20 IU/L) serum concentrations of this hormone, thus supporting the hypothesis of FSH receptor desensitization in men with testicular failure and a high compensatory FSH oversecretion (4, 5). However, the increase in the *in vitro* concentration of FSH was also beneficial in some cases in which serum FSH levels were elevated only moderately (10–20 IU/L). Hence, if a desensitization effect is involved in the differential response of germ cells from different patients to *in vitro* action of FSH, this effect may develop at different serum concentrations of FSH, possibly depending on the blood supply of seminiferous tubules in the diseased testis, on the functional state of the blood-testis barrier, and, consequently, on the local intratubular FSH concentration.

Even though the increase in FSH concentration from 50 to 500 IU/L led to an acceleration of *in vitro* maturation in four out of eight cases of premeiotic or meiotic maturation arrest, postmeiotic stages of spermatogenesis were achieved only in three of them. In the remaining one case, in which *in vivo* spermatogenesis was arrested in leptotene of the first meiotic prophase, some germ cells achieved pachytene after incubation with 500 IU/L FSH. Because, with the present state of the art, pachytene primary spermatocytes cannot be used for *in vitro* fertilization in humans, this limited improvement is not of any immediate clinical interest. Thus, the present data confirm previous findings showing that postmeiotic stages of human germ cell development that can be used for assisted reproduction can result from *in vitro* maturation of germ cells from men with meiotic maturation arrest only if *in vivo* meiosis continues up to pachytene in at least some germ cells (2, 4).

Unlike FSH, T concentration in serum is usually not altered in men with primary testicular failure. The beneficial effect of the high pharmacological concentrations of T (10 μ M) observed in some cases described in this study is thus difficult to explain. Previous studies, performed with testicular tissues from men with normal spermatogenesis, demonstrated a protective effect of T in the concentration range of 100 nM to 1 μ M (7, 15). The saturation level of nuclear high-affinity androgen receptors, mediating most of the classical, "transcriptional" responses to T, is known to be 1 nM or lower (16). However, because the testis is the main source of T in the organism, even the physiological intratesticular T concentrations are above this value (17). Hence, the intratesticular effect of T may be mediated by a nontranscriptional mechanism of action, implying low-affinity receptors on the cell surface.

This mechanism of action has also been shown to mediate the actions of T in rat Sertoli cells (18), of E₂ (19) and androstenedione (20) in human oocytes and of androstenedione in human granulosa luteinizing cells (21), and it appears

to be common in both the male and the female gonad where the local steroid concentrations greatly exceed the concentrations of the respective steroids in blood and in peripheral tissues (22). Pathologically decreased reactivity of the non-transcriptional response of human spermatozoa to progesterone has been found in some cases of unexplained male infertility (23). The mechanisms of nontranscriptional responses of cells to steroids are understood only partly (24), and the possibility that pharmacological steroid concentrations may reactivate a pathologically silenced receptor cannot be excluded.

Alternatively, a T metabolite, rather than T itself, may be required for in vitro survival and maturation of human germ cells. Indeed, T may be partly converted to E₂ and dihydrotestosterone during in vitro culture of testicular tissue samples. Both of these E₂ metabolites have been shown recently to protect human male germ cells against apoptosis during in vitro culture (25). The importance of the local action of estrogen in the human testes is further corroborated by case reports of two men suffering from infertility apparently due to homozygous inactivating mutation in the estrogen receptor α gene (26) or in the P-450 aromatase gene (27) and by experimental studies showing impairment of spermatogenesis and reduced fertility in male mice lacking a functional aromatase (cyp 19) gene (28).

Recently, point mutations in the androgen receptor gene were identified in three men with idiopathic testicular failure, and one of these mutations (Gln798Glu) led to a selective impairment of T-induced transcriptional activity with nearly normal response to dihydrotestosterone (29). It may thus be hypothesized that pharmacological concentrations of T in culture medium can partly compensate for pathologically decreased activities of enzymes responsible for the conversion of T to its two end metabolites E₂ and dihydrotestosterone. Further research is needed to elucidate the exact mechanism of the beneficial effect of pharmacological T concentrations on in vitro maturation of germ cells from men with in vivo maturation arrest.

The preliminary experience with assisted reproduction using human male germ cells matured in vitro under the influence of pharmacological concentrations of FSH and T shows that such cells can give rise to viable embryos that implant and develop normally after uterine transfer. Even though the fertilization rate does not appear to be improved as compared with that with germ cells cultured with lower hormone concentrations (1, 2), it has to be recalled that, with the lower hormone concentrations, germ cells from some men included in this study would not attain the developmental stages compatible with their use in assisted reproduction. With this in mind, these results are encouraging, although still too preliminary to make a definitive judgment about their clinical relevance.

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