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Developmental potential of elongating and elongated spermatids obtained after in-vitro maturation of isolated round spermatids

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BACKGROUND: Round spermatid injections are associated with disappointing clinical outcomes, and although these cells have been shown to mature into late spermatids *in vitro*, the developmental potential of such gametes remains to be demonstrated. METHODS: Round spermatids were isolated from 12 testicle samples of patients with obstructive azoospermia, hypoplasia, complete maturation arrest, and incomplete Sertoli cell-only syndrome. They were cultured for 7 days at 32°C, 5% CO_2 in air, in microdrops of Vero cell-conditioned medium containing 10% synthetic serum substitute. RESULTS: From the 238 round spermatids cultured, 25.2% attained the elongating and 5.5% the elongated spermatid stage (3–4 days per step). Relatively higher maturation rates were found in cases with obstructive azoospermia, but differences were significant only for elongated spermatids (9.3%). No differences were found in maturation rates between cases with non-obstructive azoospermia (4.3% of elongated spermatids). Experimental microinjections with elongating and elongated spermatids revealed a low fertilization rate (40.9%) but a normal blastocyst formation rate (60%). CONCLUSIONS: Late spermatids resulting from in-vitro culture of round spermatids in conditioned medium, either in controls in cases with a spermiogenetic block, appeared able to successfully fertilize the human oocyte and elicit normal embryo development.

Key words: in-vitro maturation/non-obstructive azoospermia/round spermatids

Introduction

In the treatment of non-obstructive azoospermic patients, several clinical trials were faced with the exclusive presence of round, elongating and elongated spermatids in treatment testicular biopsies. In those reports, whereas microinjection with elongating and elongated spermatids gave rise to relatively acceptable viable pregnancy rates, treatment cycles using round spermatids only enabled a very low pregnancy rate of ~3% per cycle (Fishel *et al*., 1995; Tesarik *et al*., 1995; Amer *et al*., 1997; Antinori *et al*., 1997a,b; Araki *et al*., 1997; Vanderzwalmen *et al*., 1997; Barak *et al*., 1998; Bernabeu *et al*., 1998; Kahraman *et al*., 1998; Sofikitis *et al*., 1998; Sousa *et al*., 1999).

Because of these rather disappointing results, in-vitro culture of round spermatids was then initiated in an effort to try to overcome the low clinical outcome obtained with the use of such immature haploid germ cells. The correct identification of round spermatids is technically difficult, and an inappropriate option could have a detrimental effect on the outcome of round spermatid injection. Although in recent years several guidelines on how to correctly recognize and test those cells have emerged (Sousa *et al*., 1998, 1999; Tesarik *et al*., 1998d), flagellar growth by in-vitro culture of round spermatids could help in the correct identification of live and viable round spermatids even if they ultimately do not show an improved developmental potential. It has been demonstrated (Aslam and Fishel, 1998) that a few cultured round spermatids can grow flagella without the presence of other germ cells or Sertoli cells, but their fertilizing and developmental capability remained extremely reduced (Fishel *et al*., 1997; Balaban *et al*., 2000). Similar experiments have also been conducted (Bernabeu *et al*., 1998; Sousa *et al*., 1999) confirming that no clinical beneficial effects could be obtained by previous in-vitro culturing of isolated round spermatids, besides showing an improvement in the fertilization rate.

On the contrary, by using more complex media, isolated round spermatids were then shown to be able to mature into elongated spermatids and spermatozoa in ~7–12 days (Cremades *et al*., 1999), which is near the expected physiological pace of in-vivo spermiogenesis (Heller and Clermont, 1963).

To further substantiate these findings, in the present study we present the first results showing that normal elongating and elongated spermatids in-vitro matured from isolated round spermatids can give rise to normal blastocyst formation rates.

Materials and methods

About 5 µl of washed and concentrated testicle tissue sample was diluted in 100 µl of sperm preparation medium (SPM; with HEPES buffer; Medicult, Copenhagen, Denmark) on a plastic tissue culture plate (Falcon, Barcelona, Spain) covered by light mineral oil (embryo-tested; Medicult). Round spermatids were isolated from this drop with a micropipette of 7–8 µm inner diameter (Swemed, Frolunda, Sweden), washed in SPM and then transferred to 20 µl 'microdrops' containing conditioned medium from Vero cell monolayers supplemented with 10% synthetic serum substitute (SSS-99193; Irvine Scientific, Santa Ana, CA, USA). Round spermatids were cultured under light mineral oil at 32°C with 5% CO_2 in air, for 6–7 days. Vero cell monolayers (American Type Culture Collection, 81-CCL; MD, USA) were prepared as described (Cremades *et al*., 1999).

Twelve male patients with azoospermia and normal karyotypes participated in the present experiments. The clinical evaluation, which included a diagnostic testicular biopsy, showed two cases of incomplete Sertoli cell-only syndrome, one case of complete maturation arrest at the primary spermatocyte stage, four cases of hypoplasia, and five cases of obstructive azoospermia [one congenital bilateral absence of the vas deferens (CBAVD), four vasectomy]. Recovery of male gametes for clinical treatment was performed on the day of oocyte retrieval by percutaneous fine needle testicular sperm aspiration in cases of obstructive azoospermia and by open testicular biopsy in cases of non-obstructive azoospermia. Testicular tissue preparation was performed as previously described (Bernabeu *et al.*, 1998; Sousa *et al*., 1999). In all cases of obstructive azoospermia and hypoplasia, testicular spermatozoa could be recovered for treatment. In the case of maturation arrest and in one case of Sertoli cell-only syndrome, elongated spermatids were the most mature germ cells found for treatments. In the other case of Sertoli cell-only syndrome, only a focus of spermatocytogenesis was found. Notwithstanding, in the latter case some round spermatids escaping from the primary spermatocyte stage block could be retrieved for experiments. In all patients, round spermatids were taken for experimental purposes after informed consent.

Oocytes used for testing the developmental potential of in-vitro cultured spermatids were given by couples that had oocyte retrieval for other reasons at the day matured spermatids became available. For ovarian stimulation, female patients electing for intracytoplasmic sperm injection (ICSI) clinical treatment cycles due to male factor infertility were treated with a long gonadotrophin-releasing hormone (GnRH) analogue suppression protocol combining triptorelin (Decapeptyl depot, 3.75 mg; Lasa, Barcelona, Spain), pure FSH (Neo-Fertinon 75 UI; Serono S.A., Madrid, Spain) and human menopausal gonadotrophin (HMG-Lepori 75 UI; Farma-Lepori, Barcelona, Spain). Ovulation was induced with human chorionic gonadotrophin (HCG, Profasi 2500 UI; Serono). Oocytes were recovered from large ovarian follicles by ultrasonically guided follicular aspiration, 36 h after HCG, using flush medium (Medicult). In all cases, female patients had normal karyotypes.

After denuding the cumulus–oocyte complexes with fine glass pipettes (Swemed), spare mature and immature metaphase I oocytes were given for research purposes after informed couple consent. Mature and in-vitro matured oocytes (on Vero cell monolayers, for up to 6 h) donated for research purposes were microinjected (Tesarik and Sousa, 1995) with in-vitro matured spermatids using glass ICSI pipettes (Swemed) and then cultured for 2 days in IVF medium (Medicult). Normal fertilization was assessed 14–18 h after injection by the presence of two pronuclei and two polar bodies. Embryo cleavage and quality were evaluated 42 h after ICSI, according to the blastomere size equality and the relative proportion of anucleate fragments (Staessen *et al.*, 1995). Embryos were then in-vitro cultured to the blastocyst stage in Vero cell monolayers.

Micrographs were taken on a Nikon inverted research microscope equipped with Hoffman optics, temperature-controlled stage, and Narishige micromanipulators.

Hormone concentrations in media were determined as follows. FSH and LH (BioMérieux kit; Vidas Systems, Madrid, Spain), and progesterone and oestradiol (Abbot kit; Ax sym Systems, Madrid, Spain) were assayed by enzyme-linked immunosorbent assay. Testosterone was assayed by radioimmunoassay (Tecam). Assays were repeated for three separate experiments.

Results

Vero cells were seeded in the presence of BM1 (synthetic medium for in-vitro cell culture; Ellios Bio-Medica, Paris, France) and newborn calf serum (NCS; Sigma, Barcelona, Spain). This medium was kept for up to 2 days, after which cell monolayer confluence occurred. After this period, the medium was changed to BM1 and SSS, with the cells remaining incubated for 2 further days. After this period, this medium (conditioned medium) was taken for round spermatid maturation. Hormone levels in conditioned medium were assayed for three different experiments and the mean values are shown in Table I. In all cases, values were very low except for oestradiol, which exhibited the highest concentrations, followed by FSH and testosterone. To ascertain if these hormones were secreted or were already present in basic media composition, all solutions used in the preparation of Vero cell monolayers were subsequently assayed. The comparative analysis shows (Table I) that oestradiol came essentially from NCS, but also, to a much lesser extent, from SSS and BM1. Oestradiol present in BM1 and NCS (63.1 pg/ml) was thus consumed by Vero cells during proliferation and monolayer formation (1 pg/ml when assayed at confluence, 2 days after seeding), ceasing after that period (15.3 pg/ml in BM1 + SSS versus 11.7 pg/ml in conditioned medium, 2 days after confluence and after changing NCS to SSS). No significant changes were noticed in relation to FSH and testosterone, whose levels appeared mainly due to their presence in SSS (Table I).

Round spermatids were isolated from patients with conserved spermatogenesis (obstructive azoospermia), from patients with partially disrupted spermiogenesis (hypoplasia), and from patients with non-obstructive azoospermia due to complete maturation arrest or incomplete Sertoli cell-only syndrome in whom a focus of spermiogenesis was found at treatment (one case of each) or just a focus of spermacytogenesis was found (one case of Sertoli cell-only syndrome).

In-vitro culturing of isolated round spermatids in Vero cell-conditioned medium containing 10% SSS (Figure 1) enabled progression of spermiogenesis up to elongating spermatids in all cases studied (Table II). In this period, that lasted for ~2–3 days, ~35% (83/238) of the round spermatids degenerated, which gave a maturation rate of ~65% (155/238). Of the elongating spermatids obtained *in vitro*, ~61% (95/155) appeared morphologically abnormal (without a flagellum), and only 39% (60/155) attained a correct morphological development (Figures 2 and 3). Abnormal elongating spermatids continued to evolve in culture, showing further elongation

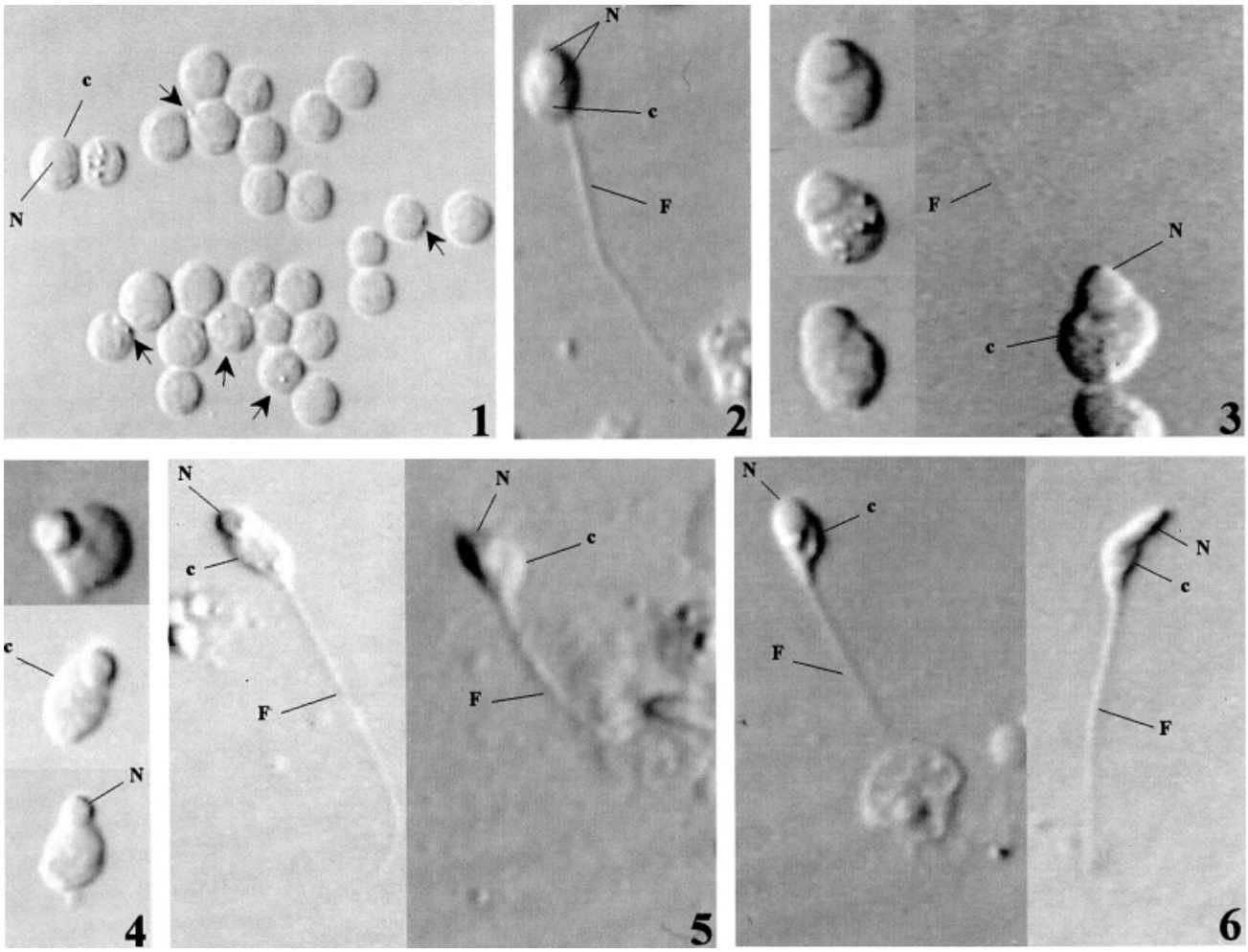

Figure 1. Isolated early (without a flagellum) round spermatids in culture (day 0). Note the round smooth nucleus (N), the narrow rim of cytoplasm (c), and the round acrosomal vesicle (arrows) in Golgi phase spermatids. Round spermatids in which no round acrosomal vesicle is visible are oriented out of the focal plane or are at the cap phase.

Figure 2. Normal early elongating spermatid (1–2 days of culture). Note the round nucleus at the cell periphery, and the presence of a flagellum (F).

Figure 3. Abnormal (cells at the left side: no flagellum) and normal (with a flagellum) late elongating spermatids (3–4 days of culture). Note that the cytoplasm has become elongated, and that the nucleus is smaller, denser and oval, and is protuding at one pole of the cell.

Figure 4. Abnormal (no flagellum) elongated spermatids that have evolved from abnormal elongating spermatids (6–7 days of culture). Note that the cytoplasm has further elongated, and that the nucleus is smaller, denser and clearly protudes out of the cytoplasm.

Figures 5 and 6. Normal early (Figure 5: 5–6 days of culture) and late (Figure 6: 6–7 days of culture) elongated spermatids. Note the decrease in cytoplasm volume and the mature nuclear appearance.

Table I. Hormone concentrations in culture media

Media	FSH (mIU/ml)	LH (mIU/ml)	Progesterone (ng/ml)	Oestradiol (pg/ml)	Testosterone (ng/ml)
BM1	1.7	<0.1	0	3.8	<0.1
Newborn calf serum (NCS)	0.1	<0.1	0	59.3	<0.2
Synthetic serum substitute (SSS)	3.7	<0.1	0.2	11.5	0.7
Vero cells + BM1	1.5	<0.1	0	0	<0.1
Vero Cells + BM1 + NCS[a]	1.3	<0.1	0	1	<0.2
Vero Cells + BM1 + SSS[b]	1.8	<0.1	0.2	11.7	0.9

[a]Media used for Vero cell growth up to confluence and taken for assays at the end of 2 days of culture.
[b]Media used for Vero cells after monolayer formation and taken for assays at the end of 2 days of culture.
The supernatant medium corresponds to the conditioned medium used for spermatid in-vitro culture.
BM1 = synthetic medium for in-vitro cell culture.

Table II. In-vitro maturation of isolated round spermatids

Type of spermatids	Obstructive	Non-obstructive azoospermia			Total
		HP	MA + SO	Total	
	(n = 5)	(n = 4)	(n = 3)	(n = 7)	(n = 12)
Round spermatids (n)	54	94	90	184	238
Elongating spermatids (%)	31.5	26.6	20	23.4	25.2
Early elongated spermatids (%)	13	9.6	10	9.8	10.5
Late elongated spermatids (%)	9.3	4.3	4.4	4.3	5.5

n = no. of cases; HP = hypoplasia; MA = maturation arrest; SO = Sertoli cell-only syndrome.

Table III. Fertilization and embryo development after injection of in-vitro matured spermatids

Total no. of injected oocytes	24
Degenerated oocytes after injection	2 (8)
No. of intact oocytes after injection	22
Non-fertilized (0PN/1PB)	5 (22.7)
Activated oocytes (0PN/2PB)	3 (13.6)
Abnormal fertilization rate (1PN/2PB zygotes)	5 (22.7)
Normal fertilization rate (2PN/2PB zygotes)	9 (40.9)
Embryo cleavage rate (from 2PN/2PB zygotes)	7 (77.8)
Embryo A + B grade (from 2PN/2PB zygotes)	5 (71.4)
Blastocyst rate (from 2PN/2PB zygotes)	3 (42.9)
Blastocyst rate (from 2PN/2PB zygotes and A + B grade embryos)	3 (60)

Values in parentheses are percentages.
PN = pronuclei; PB = polar bodies.

of the cytoplasm and elongation, condensation and bulging of the nucleus (Figure 4). Only normal elongating spermatids transformed into normal elongated spermatids, which occurred in the following 3–4 days of culture (Figures 5 and 6).

In total, of the 60 normal elongating spermatids obtained, 35 (58%) became arrested, and 25 (42%) progressed into elongated spermatids. In-vitro differentiation into elongated spermatids was not observed in four (33%) out of the 12 cases studied, which included two cases of obstructive azoospermia, one case of hypoplasia, and one case of Sertoli cell-only syndrome in which elongated spermatids were obtained at treatment. Elongated spermatids formed in the other eight cases (67%), which included three cases of obstructive azoospermia, three cases of hypoplasia, one case of maturation arrest, and the case of Sertoli cell-only syndrome in which no spermatids or spermatozoa were found at treatment. Considering only the eight cases where elongated spermatids formed, 54% of the normal elongating spermatids progressed to early elongated spermatids (25/46), whereas 46% remained arrested (21/46). Of the early elongated spermatids formed, 52% further matured into late elongated spermatids during the next day of culture (13/25), whereas 48% remained arrested in their development (12/25). Maturation into late elongated spermatids occurred in seven out of eight cases, with the single failure having occurred in one out of the three cases of hypoplasia.

In-vitro matured spermatids were injected into donated oocytes to assess their developmental capability (Table III). These experiments were performed whenever donated oocytes were available and after in-vitro matured spermatids showed no further signs of evolution after 24 h of culture. In total, of the 12 cases cultured, in only seven cases were there available oocytes for injection, which included three cases of obstructive azoospermia, two cases of hypoplasia, one case of maturation arrest, and the case of Sertoli cell-only syndrome where elongated spermatids were found at treatment. Of these cases, four had elongated spermatids for injection (one obstructive azoospermia, two hypoplasia, one maturation arrest), whereas in three cases injections were performed with arrested normal elongating spermatids (two obstructive azoospermia, one Sertoli cell-only syndrome).

Of the 24 oocytes that could be donated for research experiments, 12 oocytes were mature [at metaphase II (MII) of meiosis], while the other 12 oocytes were immature at the metaphase I stage of meiosis. These were first in-vitro matured in Vero cell co-culture conditions for ~6 h, and were micro-injected 4 h later. In the group of in-vitro matured oocytes, two were injected with arrested elongating spermatids (17%) and 10 with elongated spermatids (83%), whereas in the group of MII oocytes, 10 were injected with elongating spermatids, and two with elongated spermatids.

Comparing the group of in-vitro matured oocytes with the group of MII oocytes, the degeneration rate was similar (8%) whereas the non-fertilization rate was higher (36 versus 9%); the activation rate (9 versus 18%), the abnormal fertilization rate (18 versus 27%), the normal fertilization rate (36 versus 45%) and the embryo cleavage rate (50 versus 100%) were all lower. In the group of in-vitro matured oocytes, normal fertilization and embryo cleavage occurred only with elongated spermatids, with all embryos having been of grade C and with no blastocyst having been formed. On the contrary, in the group of MII oocytes, all embryos were of grade B and the blastocyst formation rate was 60%. In this case, arrested elongating spermatids elicited a 44% fertilization rate (4/9) from which two blastocysts formed (2/4, 50%), whereas elongated spermatids showed a 50% fertilization rate (1/2) and gave rise to one blastocyst (1/1, 100%).

Discussion

In non-obstructive azoospermia, many patients show an absence of spermatozoa at testicle biopsies, which has forced the experimental use of spermatids in clinical treatments. Analysis of published reports led to the conclusion that elongating and elongated spermatids gave rise to relatively

acceptable pregnancy rates, whereas round spermatids appear unable to achieve viable pregnancies at an acceptable rate (Fishel *et al*., 1995, 1996, 1997; Tesarik *et al*., 1995; Vanderzwalmen *et al*., 1995, 1997; Amer *et al.*, 1997; Antinori *et al*., 1997a,b; Araki *et al.*, 1997; Barak *et al*., 1998; Bernabeu *et al*., 1998; Kahraman *et al*., 1998; Sofikitis *et al*., 1998; Sousa *et al*., 1998, 1999; Balaban *et al*., 2000).

In some patients with no spermiogenesis but with at least one focus of spermacytogenesis (maturation arrest at the primary spermatocyte stage), a few round spermatids can be isolated (Amer *et al.*, 1997; Antinori *et al*., 1997a,b; Vanderzwalmen *et al*., 1997; Barak *et al*., 1998; Bernabeu *et al*., 1998; Kahraman *et al*., 1998; Sousa *et al*., 1998, 1999). These round spermatids are supposed to have escaped the meiotic block and their culture *in vitro* was then thought as an alternative method to solve the clinical failures. In-vitro culturing of these cells demonstrated that ~22% of round spermatids can grow flagella in current IVF media after 1–2 days of culture (Aslam and Fishel, 1998), but their fertilizing and developmental capability remained extremely low (Fishel *et al*., 1997; Sousa *et al*., 1999; Balaban *et al*., 2000).

More recently, Tesarik *et al*. have shown that in-vitro culturing of the whole testicular tissue with rFSH (stimulates spermatogenesis) and testosterone (inhibits Sertoli cell apoptosis) can elicit differentiation of elongated spermatids from primary spermatocytes in 1–2 days (Tesarik *et al*., 1998b,c, 1999, 2000a,b), although this pace contrasted with the normal testicular cycle that needs more than 1 month to proceed through meiosis and spermiogenesis (Heller and Clermont, 1963; Steele *et al*., 1999a).

We have developed an alternative protocol where, to avoid any possibility of contamination by a hidden focus of elongated spermatids or spermatozoa, isolated round spermatids were cultured over Vero cell monolayers and individually followed (Cremades *et al*., 1999). With this system, it has been shown that a few round spermatids can differentiate *in vitro* up to the elongated spermatid and even spermatozoa stages in ~7–12 days, a more compatible time in comparison with the normal physiological duration of spermiogenesis of 16–22 days (Heller and Clermont, 1963).

Here, we have expanded upon those results. In the previous study, round spermatids were isolated from cases with conserved and disrupted spermatogenesis, cultured, isolated using conditioned medium from Vero cells, and in-vitro matured spermatids were microinjected into oocytes to study their developmental potential.

The media used here for culturing showed a low (5.5%) in-vitro maturation rate of isolated round spermatids into normal elongated spermatids, which confirms our previous findings (Cremades *et al*., 1999). However, this system allowed diferentiation to occur at a physiological pace (~5–7 days per step), with a mean of 3–4 days from the round spermatid to the elongating spermatid stage and from the elongating spermatid to the elongated spermatid stage (Heller and Clermont, 1963).

Results also showed that, although a better differentiation rate was obtained in cases with conserved spermatogenesis (9.3 versus 4.3%), no significant differences between the different causes of non-obstructive azoospermia seemed to exist regarding the capability of round spermatids to differentiate into elongated spermatids. This finding is of particular clinical importance, especially as it included a case of incomplete sertoli cell-only syndrome where the treatment testicle biopsy did not show any focus of spermiogenesis but from which round spermatids were able to differentiate *in vitro* into elongated spermatids, which suggests that cases of non-obstructive azoospermia without advanced spermatids can be successfully used for in-vitro maturation.

Hormone level assays were also performed in the culture medium to assess if FSH and testosterone were present and thus could assist in the differentiation of round spermatids, but data only showed marginal values. This, therefore, suggests that those hormones might not be essential for spermiogenesis, although required for spermatocytogenesis (Tesarik *et al*., 1998b,c, 1999, 2000a,b), and that secreted factors from Vero cells, including small weight metabolites, interleukins and growth factors (Huang *et al*., 1997; Desai and Goldfarb, 1998) are probably needed to assist the normal differentiation of round spermatids. In the male seminiferous tubules, rFSH was shown to have a stimulatory role on the proliferating stages (spermatogonia population) and on meiosis (spermatocytes) (Hikim and Swerdloff, 1995; Foresta *et al*., 1998), whereas testosterone seems to act as an anti-apoptotic agent (Erkkila *et al*., 1997). Addition of both hormones to whole seminiferous cultures *in vitro* has been shown to accelerate highly the transmeiotic and postmeiotic differentiation of germ cells (Tesarik *et al*., 1998b,c, 1999, 2000a,b). However, if a 10–23% increase of elongated spermatids was observed in samples from obstructive azoospermic patients, in non-obstructive azoospermic cases the maturation rate was clearly much lower. Despite these considerations, it might be useful to try to culture round spermatids *in vitro* in the presence of rFSH and testosterone, as they may also improve the present maturation rates.

In the present experiments, although normal elongating, and elongated, spermatids showed a low fertilization potential (40.9%), normal blastocyst formation rates (60%) could be achieved with either type of spermatids. The low fertilization rate here obtained seems not to be due to the use of in-vitro matured metaphase I oocytes. In fact, in the present experiments we have not used oocytes arrested at prophase I nor metaphase I oocytes which have not achieved a full spontaneous maturation in the first 6 h of culture. As previously shown (Smitz and Cortvrindt, 1999), metaphase I oocytes that rapidly mature *in vitro* spontaneously show excellent rates of fertilization and embryo development after ICSI. Thus the low fertilization rate may instead be due to a lower developmental potential of the in-vitro matured spermatids used.

In the present experiments, although the morphology and rate of blastocyst development were normal, thus suggesting that in-vitro matured spermatids had a full developmental competence, there was no evidence that the blastocysts obtained were healthy, since due to the experimental nature of the procedures they were not transferred. In fact, studies have demonstrated that in-vitro cultured mouse testicular spermatids show abnormal DNA methylation and chromatin remodelling, and that testicular spermatids from patients with non-obstruct-

ive azoospermia also show increased DNA fragmentation (Jurisicova *et al*., 1999). Similarly, an increase in DNA degradation and apoptosis of germ cells during in-vitro culture has been observed (Tesarik *et al*., 1998a), and a lower developmental potential of embryos after round and elongated spermatid injection has been reported (Balaban *et al*., 2000). These results thus suggest that spermatids from non-obstructive azoospermic patients may carry a substantial risk for transmitting severe defects, especially if these are contained within otherwise morphologically normal gametes.

However, other results point to a clearly different situation. Germ cell apoptosis has been shown to be a normal event during spermatogenesis, being increased in those conditions where genetic and exogenous factors cause disruption of the germinal epithelium (Lin *et al*., 1997; Steele *et al*., 1999b; Print and Loveland, 2000). In the present experiments, germ cells from obstructive and non-obstructive patients also exhibited high levels of cell degeneration during in-vitro culture, and round spermatids, which were unable to extrude a flagellum but were able to evolve into elongating spermatids without a flagellum, also began to degenerate at this stage. Abnormal spermatid maturation not associated with signs of cell degeneration could also be diagnosed by inspection of the morphology of the head and of the flagellum of elongating and elongated spermatids. These findings appear similar to those previously described (Tesarik *et al*., 1998b,c; Cremades *et al*., 1999), and may indicate that a complete morphologically normal maturation of spermatids may offer some guarantee concerning the viability and normal genetic constitution of gametes selected from the testis of azoospermic patients. This also applies to genomic imprinting, which has been shown recently to have been fully established by the time round spermatids are formed (Shamanski *et al*., 1999; Davis *et al*., 2000). That maturation of germ cells into elongated spermatids and spermatozoa with normal morphology may reflect a correct genetic constitution of the gametes is also supported by numerous clinical studies where normal viable pregnancies, without fetal abnormalities, were obtained after injection of testicular spermatozoa (Tarlatzis and Grimbizis, 1999) and of testicular elongating and elongated spermatids (Fishel *et al*., 1995, 1996, 1997; Vanderzwalmen *et al*., 1995, 1997; Amer *et al*., 1997; Antinori *et al*., 1997a,b; Bernabeu *et al*., 1998; Kahraman *et al*., 1998; Sofikitis *et al*., 1998; Sousa *et al*., 1999).

Thus, the current experimental and clinical data suggest that a low risk may be associated with injection of spermatids from azoospermic patients if strict criteria of selection are applied, e.g. after a correct discrimination between viable and non-viable spermatids, and with injection of spermatids being restricted to gametes showing a morphologically normal nucleus and flagellum (Sousa *et al*., 1998, 1999; Tesarik *et al*., 1998d). More recent data have indicated the occurrence of morphological and chromosomal fetal abnormalities (two out of four pregnancies) after elongated spermatid injection, thus suggesting that these immature haploid germ cells may still carry a substantial risk of inducing developmental errors (Zech *et al*., 2000). However, Sousa *et al*. (2000) presented a much larger series of term pregnancies after elongated spermatid injection, obtained in cases of Sertoli cell-only syndrome and maturation arrest, and have found no minor or major fetal abnormalities and no karyotype aberrations.

In the present study, we could demonstrate that round spermatids can mature *in vitro* into elongated spermatids and that these cells can successfully fertilize the human oocyte, giving rise to regular blastocyst formation rates. However, these results are preliminary, as many aspects of the process still need to be further evaluated and improved. First, although the present study was purely experimental, and the conditioned medium used for round spermatid in-vitro maturation did not contain any calf- or bovine-derived products, the growth phase of Vero cells has been achieved in the presence of newborn calf serum, which still poses the risk of prion transmission. Additionally, and although Vero cells were not used for round spermatid culturing, the conditioned medium used came from the supernatant medium covering Vero cell monolayer cultures, which may also carry the risk of transmission of virus diseases. Thus, in future experiments this medium should be replaced by complex synthetic elements. Second, in-vitro differentiation of round spermatids into elongated spermatids did not occur in all cases tested (4/12, 33.3%), which suggests that the process is still not entirely replicable and seems to vary individually from patient to patient. Failures occurred in two cases of normal spermatogenesis (obstructive azoospermia), in one case of limited disruption of spermatogenesis (hypoplasia), and in one case of incomplete Sertoli cell-only syndrome in which a focus of normal spermiogenesis up to the elongated spermatid stage was found for patient treatment during the open testicular biopsy. Thus, in these four cases, the isolated round spermatids had all the potential to differentiate into elongated spermatids and spermatozoa, but no differentiation was achieved *in vitro*. In conclusion, although, in all the 12 cases here analysed, elongating spermatids were obtained and successfully fertilized the human oocyte and then elicited normal embryo development, the absence of late differentiation in some cases without a genetic block may point to important deficiencies of the in-vitro culturing medium whose consequences still remain to be ascertained.

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