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In-vitro maturation of round spermatids using co-culture on Vero cells

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In an attempt to determine whether co-culture could promote sperm maturation, three patients with non-obstructive azoospermia, two with maturation arrest at the level of primary spermatocytes and one patient with <1% tubules showing complete spermatogenesis, and one patient with total globozoospermia, gave consent to experimentally co-culture round spermatids retrieved from the testicle on Vero cell monolayers. In all azoospermic patients elongating spermatids could be obtained from round spermatids. In one case of maturation arrest, of 37 round spermatids co-cultured for up to 5 days, 30% developed flagella, 46% matured to elongating and 19% to elongated spermatids, with one mature spermatozoon also obtained (3%). In the same patient, primary cultures of three round spermatids with flagella enabled development of one further mature spermatozoon. In the case with total globozoospermia, of six round spermatids co-cultured for up to 5 days, one mature spermatozoon was obtained, with a flagellum and normal head morphology. These preliminary findings suggest that it may be possible to overcome the round spermatid block, and even the triggering of morphological abnormalities arising at the spermiogenic level, by in-vitro maturation under special environmental conditions.

Key words: co-culture/in-vitro germ cell maturation/non-obstructive azoospermia/round spermatids/Vero cells

Introduction

After the suggestion made by Edwards *et al.* (1994) on the use of spermatids for the treatment of non-obstructive azoospermic patients, and the realization that round spermatids can effectively fertilize the human oocyte (Vanderzwalmen *et al.*, 1995; Sousa *et al.*, 1996), the intracytoplasmic injection of round (Tesarik *et al.*, 1995, 1996; Tesarik and Mendoza, 1996a) and elongated (Fishel *et al.*, 1995, 1996) spermatids on a clinical ground was successful. From the reported cases, 35 pregnancies have already been obtained with spermatid injection. Twenty seven were obtained with elongating and elongated spermatids (Fishel *et al.*, 1995, 1996; Mansour *et al.*, 1996; Amer *et al.*, 1997; Antinori *et al.*, 1997a; Araki *et al.*, 1997; Vanderzwalmen *et al.*, 1997; Barros *et al.*, 1998a,b; Bernabeu *et al.*, 1998; Kahraman *et al.*, 1998; Sofikitis *et al.*, 1998b; Sousa *et al.*, 1999). Of the successful eight cases using round spermatids, three were retrieved from the ejaculate (Tesarik *et al.*, 1995, 1996; Tesarik and Mendoza, 1996a; Barak *et al.*, 1998) and five from the testicle (Antinori *et al.*, 1997a; Vanderzwalmen *et al.*, 1997; Kahraman *et al.*, 1998), with one cycle using frozen–thawed cells (Antinori *et al.*, 1997b). However, some other reports showed no success at all (Hannay, 1995; Chen *et al.*, 1996; Yamanaka *et al.*, 1997).

In spite of these achievements, the complete absence of elongated spermatids or spermatozoa from the ejaculate or diagnostic testicle biopsy in the previous history of the patient seems to have an adverse effect on the clinical outcome, with no present pregnancy having been obtained with round spermatids in these extremely severe cases (Amer *et al.*, 1997; Vanderzwalmen *et al.*, 1997; Barros *et al.*, 1998a,b; Bernabeu *et al.*, 1998; Kahraman *et al.*, 1998; Sofikitis *et al.*, 1998b).

There are many concerns about the clinical consequences of using round spermatids in micro-injection treatments, as well as about the correct recognition of those cells (Tesarik, 1996, 1997a,b; Tesarik and Mendoza, 1996b; Fishel *et al.*, 1996, 1997; Silber *et al.*, 1997; Aslam *et al.*, 1998a,b; Balet *et al.*, 1998; Devroey, 1998; Silber, 1998; Silber and Johnson, 1998; Sofikitis *et al.*, 1998a; Sousa *et al.*, 1998; Tesarik *et al.*, 1998a,b; Vanderzwalmen *et al.*, 1998). Some studies tried to validate the recognition of the round spermatid stage based on the application of non-vital staining techniques (Mendoza and Tesarik, 1996; Mendoza *et al.*, 1996; Angelopoulos *et al.*, 1997). Although these methods cannot be applied in effective treatments, they showed that in the majority of the cases the correct cellular choice can be made. Simultaneously, others have described the live characteristics of round spermatids directly to guide clinical applications (Tesarik and Mendoza, 1996a; Vanderzwalmen *et al.*, 1997; Sousa *et al.*, 1998; Tesarik *et al.*, 1998a,b; Verheyen *et al.*, 1998).

In-vitro maturing of round spermatids could be one method for the study of the round spermatid block and an effective way to solve the clinical failures. In-vitro culturing with current media has already been evaluated. Barros *et al.* (1998a,b) and Bernabeu *et al.* (1998) have clinically applied this technique, but no morphological changes were observed and no beneficial effects were noticed on the clinical outcome, except an improvement of the fertilization rate. On the contrary, and although performed outside clinical application, Aslam and Fishel (1998) have demonstrated that about 22% of round spermatids can grow flagella under in-vitro culturing.

In the present short report we describe the degree of maturation of round spermatids under Vero cell co-culture,

showing that, at least in some cases, maturation blocks can be overcome up to the formation of spermatozoa.

Materials and methods

Round spermatids were taken for experimental purposes with informed consent from patients in whom testicular open biopsy or percutaneous testicular sperm extraction was needed for their treatment protocol. Testicular spermatid extraction and spermatid isolation were performed as previously described (Barros *et al.*, 1998a,b; Bernabeu *et al.*, 1998).

Three patients with non-obstructive azoospermia, two with maturation arrest at the level of primary spermatocytes and one patient with <1% tubules showing complete spermatogenesis, and one patient with total globozoospermia, gave consent to experimentally co-culture round spermatids on Vero cells monolayers, in an attempt to see if co-culture could promote normal maturation. In no cases were in-vitro matured cells used for clinical treatment.

Round spermatids were retrieved from the testicle by open biopsy (cases with maturation arrest at the primary spermatocyte level) or percutaneous testicular aspiration. In the case of the patient with total globozoospermia, the criterion for testicular extraction was the presence of bacteria in the ejaculate. In all cases, the karyotype was normal.

Testicle samples were diluted in a large microdrop containing sperm preparation medium (SPM; with HEPES buffer; Medicult, Copenhagen, Denmark) on a plastic tissue culture plate covered by light mineral oil (embryo tested, Medicult). Cells were selected with a micropipette with 7–8 μm inner diameter (Swemed, Frolunda, Sweden), washed in SPM and then transferred to microdrops containing Vero cell monolayers. Samples of each group of isolated round spermatids used for co-culture were also transferred to a glass slide, fixed and stained (Schorr) to ensure the proper recognition of the round cells.

Isolated round spermatids were in-vitro cultured under co-culture conditions using Vero cell (American Type Culture Collection, 81-CCL, Rockville, MD, USA) monolayers grown in 20 μl microdrops on a plastic tissue culture plate covered by light mineral oil (embryo tested, Medicult) at 37°C with 5% CO_2 in air.

Vero cell monolayers were prepared as described (Ouhibi *et al.*, 1989; Menezo *et al.*, 1990, 1992; Schillaci *et al.*, 1994). Briefly, after thawing at 37°C, newborn calf serum (NCS, Sigma N-4637, Barcelona, Spain) was added to the cell suspension, which was then centrifuged for 10 min at 800 *g*. The pellet was resuspended with 7% NCS in BM1 (Ellios Bio-Media F-75008, Paris, France), and the cells (2×10^6/25cm^2 flask) were cultured at 37°C with 5% CO_2 in air for 2–3 days, with a change of the medium being performed at 24 hours. At confluence, growth medium was removed and monolayers were dispersed with a 2.5% trypsin solution (BioMerieux 89651, Marcy L'Etoile, France) diluted in EDTA solution (1:5000; BioMerieux 89651) (1:48 v/v) or with a ×10 trypsin-EDTA solution (Sigma, T-4174) diluted in Hanks' balanced salt solution without calcium and magnesium (Sigma, H-6648) (1:9 v/v), and incubated twice for 2–3 min at 37°C until the cells rounded and detached from the bottom of the tissue culture flask (as controlled by inverted microscopy with Hoffman optics). Trypsin was then inactivated by the addition of NCS (1:1 v/v). Cells were then washed with NCS (800 *g*, 10 min), and cultured as above. Cells (10^5/ml) were cultured in microdrops with BM1 supplemented with 10% synthetic serum substitute (SSS-99193, Irvine Scientific, Santa Ana, CA, USA). Cell concentrations were adjusted after vital staining with the 0.2% Trypan blue (Sigma, T-8154) exclusion method, using the viability range of 85–98%. Micrographs were taken on a Nikon inverted research microscope equipped with Hoffman optics, temperature controlled stage, and Narishige micromanipulators. Freezing of Vero cells was as described in Ouhibi *et al.* (1989).

Results

Case 1

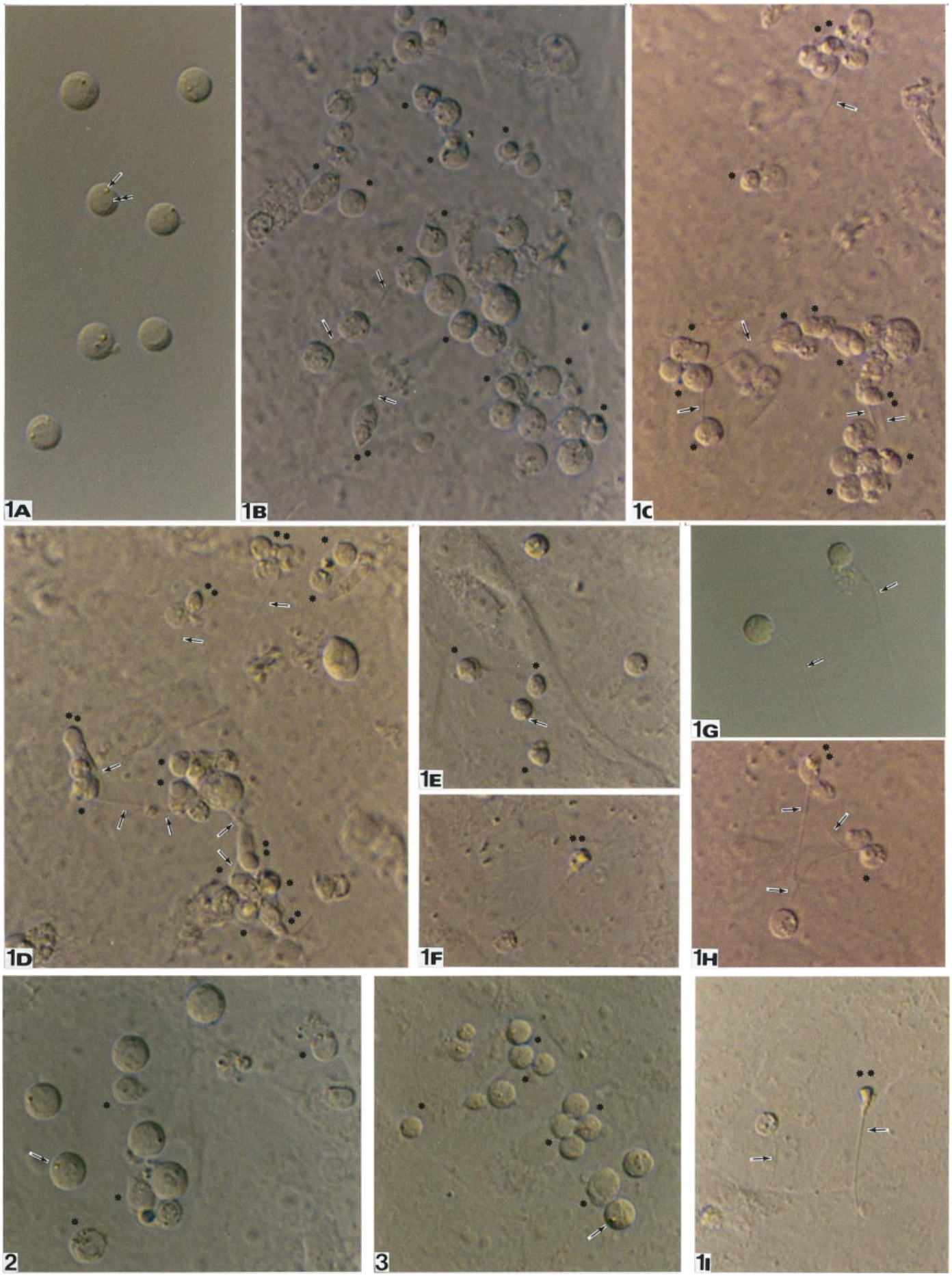
This patient had a diagnostic testicular biopsy showing maturation arrest at the primary spermatocyte level. The open testicular biopsy enabled us to recover elongated spermatids for injection and cryopreservation. We co-cultured 37 round spermatids without flagella (Figure 1A) for up to 4–5 days. Overall, 11 (30%) developed flagella, 17 (46%) became elongating spermatids as shown by the peripheral displacement of the nucleus, seven (19%) further developed to elongated spermatids with flagella, and in one case a spermatozoon was formed (Figure 1B–F). We also have co-cultured three round spermatids with flagella (Figure 1G), of which two became elongating (Figure 1H) and one, by day 5, reached the form of a mature spermatozoon (Figure 1I).

Case 2

This patient also had a diagnostic testicular biopsy showing maturation arrest at the primary spermatocyte level. The open testicular biopsy enabled us to recover round spermatids for injection and cryopreservation, with no elongating or elongated spermatids found. We co-cultured ten round spermatids without flagella for up to 3 days. Overall, none formed flagella, but four showed a peripheral displacement (elongation) of the nucleus (Figure 2).

Figure 1. Case 1. (**A**) Isolated round spermatids without flagella (about 6–8 μm in diameter). Note the round nucleus (double arrow) and acrosomal vesicle (arrow) surrounded by a thin rim of cytoplasm. (**B–D**) Co-culture of the same population of round spermatids without flagella on a Vero cell monolayer at days 1, 2 and 3, respectively. Spatial changes and confluence of the cells occurred at all times, making individual follow-up and step characterization difficult. Note development of flagella (arrows), and maturation into elongating (peripheral displacement of the nucleus) (*) and elongated (**) spermatids. (**E, F**) Co-culture of another population of round spermatids without flagella (arrow), at days 2 and 4, respectively. Note maturation into elongating spermatids (*) and then to an almost mature spermatozoon (**). (**G**) Isolated round spermatids with flagella (arrows). (**H, I**) Co-culture of the same round spermatids with flagella at days 2 and 5, respectively. Note development into elongating (*) and elongated (**) spermatids at day 2, and of the elongated one into a mature spermatozoon (**) at day 5. Magnification ×800.

Figures 2 and 3. Cases 2 and 3, respectively. Co-culture of round spermatids without flagella (arrow) on Vero cell monolayers at day 3. Note maturation into elongating (*) spermatids (loss of the round acrosomal vesicle, decreased size and peripheral displacement of the nucleus). Magnification ×800.



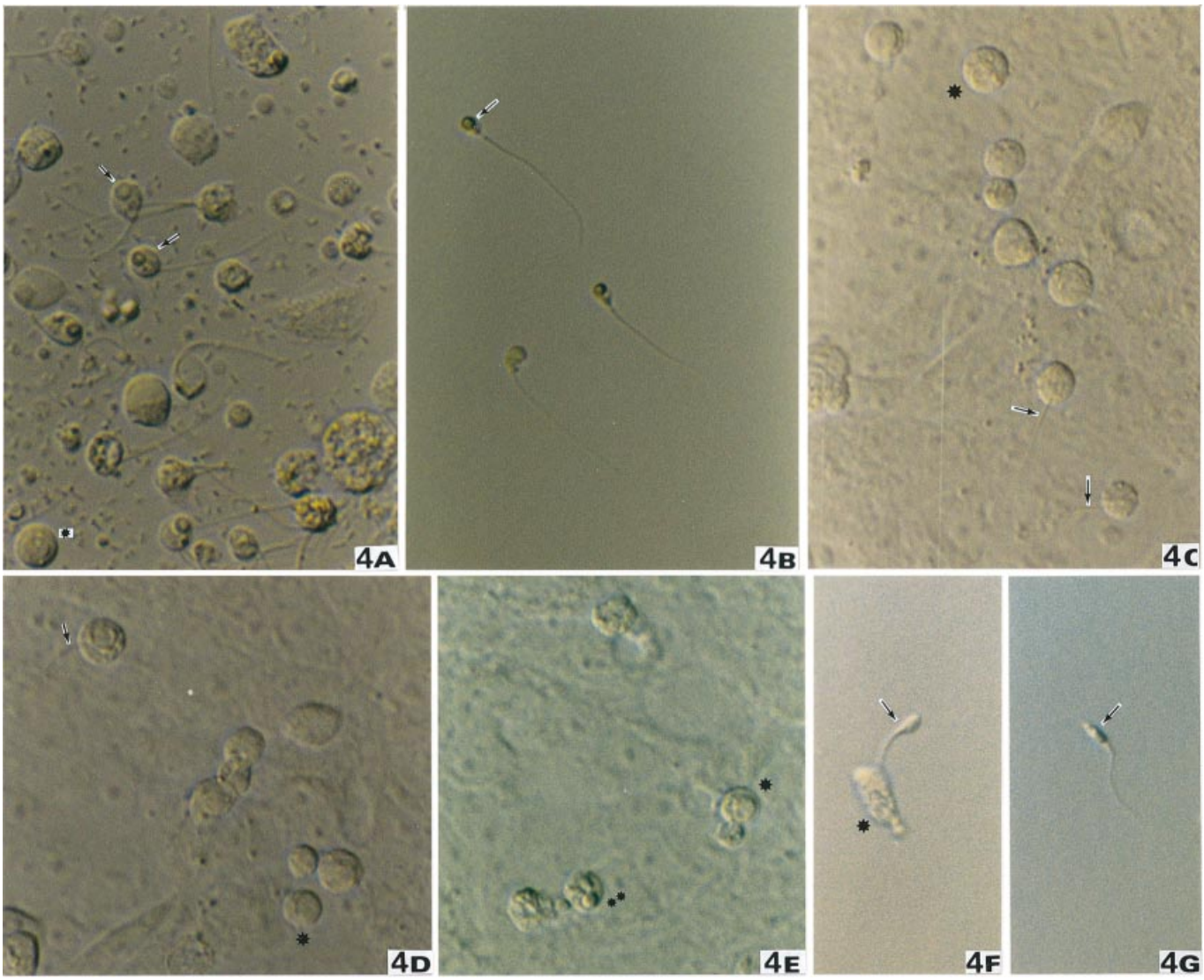


Figure 4. Case 4. (**A**) Ejaculate sample. Note round-head spermatozoa (arrows), round spermatids (*), leukocytes and bacteria. (**B**) Isolated round-head spermatozoa (arrow) from the testicle specimen. (**C**) Co-culture of six round spermatids without flagella and two with flagella (arrows) on a Vero cell monolayer at day 1. Note that round spermatids without flagella are maturing into elongating (peripheral displacement of the nucleus) spermatids (*). (**D**) Day 2. One elongating spermatid has developed a flagellum (arrow). Of the two round spermatids originally with flagella, one is now elongating and seems to have lost the flagellum (*), whereas the other has detached and was lost. (**E**) Day 4. The remaining round spermatid originally with flagellum has detached and was lost. Of the 5 round spermatids originally without flagella, one is still elongating (*), whereas the others developed dense granules in the cytoplasm (**). (**F**) Day 5. The elongating spermatid that developed a flagellum has matured into a spermatozoon (arrow). The head is slightly out of focus because the flagellum is attached to a Vero cell (*). (**G**) The same mature spermatozoon after removal of the Vero cell, showing elongated head morphology (arrow). Magnification ×800.

Case 3

This patient had a diagnostic testicular biopsy showing foci (<1% tubules) of complete spermatogenesis. Percutaneous testicular aspiration enabled us to recover spermatozoa for injection and cryopreservation. We isolated 15 round spermatids without flagella from the sample where spermatozoa were retrieved. After 3 days in co-culture, none developed a flagellum, but most of them changed their internal morphology, showing loss of the round acrosomal vesicle, decreased diameter, and peripheral displacement (elongation) of the nucleus (Figure 3).

Case 4

This patient had total globozoospermia with numerous bacteria in the ejaculate on the day of treatment (Figure 4A). Percutaneous testicular aspiration enabled us to recover round-head spermatozoa for injection and cryopreservation (Figure 4B). Six round spermatids without flagella and two round spermatids with flagella could be isolated for co-culture. Round spermatids with flagella detached by days 2 and 3 and were lost. Of the round spermatids without flagella, five showed a progressive peripheral displacement of the nucleus (elongation) during the first 2 days of culture (Figure 4C, D), followed by

formation of dense granules in the cytoplasm by day 4 (Figure 4E). One of the round spermatids without a flagellum became an elongating spermatid with a flagellum on the second day of culture (Figure 4D), and finally matured into a normal-headed spermatozoon (Figure 4F, G).

Discussion

In cases of non-obstructive azoospermia showing in their previous history a complete absence of spermatozoa either in the ejaculate or testicular biopsy, the recovery of only round spermatids for in-vitro fertilization is a frequent finding. The use of such round cells has a very poor outcome, with no pregnancy having been obtained up to the present time in such extreme cases (Amer *et al*., 1997; Vanderzwalmen *et al.*, 1997; Barros *et al.*, 1998a,b; Bernabeu *et al.*, 1998; Kahraman *et al*., 1998; Sofikitis *et al.*, 1998b).

In-vitro maturing of round spermatids could be a powerful way for studying this round spermatid block and to try to see if it can be overcome so as to solve the present clinical failures. In-vitro culturing with conventional media of isolated round spermatids (Aslam and Fishel, 1998; Barros *et al.*, 1998a,b; Bernabeu *et al.*, 1998) or of mixed spermatogenic populations containing Sertoli cells, primary spermatocytes, and round and elongating spermatids (Aslam and Fishel, 1998) was recently evaluated in humans. Barros *et al.* (1998a,b) and Bernabeu *et al.* (1998) have clinically applied this technique to those cases where there was no history of the presence of elongated spermatids or spermatozoa in previous ejaculates and in diagnostic testicular biopsies, and for whom only round spermatids could be retrieved at treatment. After 1–3 days of culture no morphological changes were observed, and although the micro-injection of those cells elicited a much higher fertilization rate, no pregnancies could be obtained. On the contrary, and although being performed outside clinical application, Aslam and Fishel (1998) have demonstrated, in non-obstructive and obstructive azoospermic patients, that about 22% of round spermatids begin to grow flagella very early under in-vitro culturing, although primary spermatocytes and elongating spermatids did not show any maturation progression for up to 4 days.

Our present preliminary results using round spermatid co-culture on Vero cell monolayers show that it is possible to mature these cells up to the elongating/elongated step and even to mature spermatozoa. In all three azoospermic patients studied, round spermatids without flagella could give rise to elongating spermatids. However, the growth of a flagellum and maturation through the elongation step of spermiogenesis up to mature spermatozoa could only be achieved in one patient with maturation arrest (primary spermatocyte level) from whom elongated spermatids were recovered. Because a complete maturation progression was not observed in the case of a patient in whom only round spermatids could be retrieved, we cannot presently conclude that co-culture is effective for cases with a very severe spermiogenic block. However, as the same disappointing results were obtained in a patient in whom spermatozoa were retrieved, no final conclusions can be taken before further cases are studied. One fourth patient, with total globozoospermia, and included as an internal control (presence of complete spermatogenesis), also gave surprising results, as a normal headed spermatozoon could be obtained from round spermatids without flagella after 5 days of co-culture. Although preliminary, this finding suggests that in-vitro maturing might also be used to overcome abnormal spermiogenesis.

Many concerns have been raised about the correct recognition of round spermatids and also about the possibility of finding such cells in cases where elongating or elongated spermatids are not simultaneously found (Silber *et al.*, 1997; Silber and Johnson, 1998; Verheyen *et al.*, 1998). As round spermatids can now be matured fully *in vitro*, our present results demonstrate that it is possible to recognise such round cells correctly, even in those severe cases. In support of our findings, recent studies on rat in-vitro spermatogenesis have also shown that maturation block at the round spermatid level can occur (Hue *et al.*, 1998).

In mammals other than the human, studies using long-term cultures of mixed spermatogenic populations in hormone supplemented media have also recently shown that meiosis can be completed *in vitro*. However, maturation then became arrested at the round spermatid step (Hue *et al.*, 1998). Our present preliminary results now show for the first time that co-culture on Vero cell monolayers can support full maturation of human round spermatids.

Although encouraging, these results should be interpreted as preliminary and are not intended to be directly applied to the treatment of infertile couples. In fact, studies are needed to further evaluate and improve the present system. First, the low success rate of sperm production in the present report may be related to the fact that we have cultured spermatids at 37°C, the ideal temperature for Vero cells. The ideal temperature for normal spermatogenesis is 32–34°C, and higher temperatures were shown to inhibit amino acid incorporation and tubulin polymerization (Nakamura *et al.*, 1978). It is generally assumed that the monolayer system in co-culture improves cell quality by removing toxic compounds from the culture medium, by supplying small molecular weight metabolites or by providing growth factors. Probably due to these factors, morphological changes were observed in round spermatids during co-culture with Vero cells even at 37°C. The importance of the action of the monolayer system in the present report is further supported by the previous work of Aslam and Fishel (1998) who could only obtain growth of flagella despite having added serum to the culture medium and working at 32°C. Future studies should ascertain whether Vero cells can grow normally at lower temperatures. If this will not inhibit monolayer formation and maintenance, then maybe the success rate of the system can be improved. Second, a high number of non-obstructive azoospermic patients should be studied to determine the real percentage of cases in whom the early spermatid block can be effectively overcome. Third, the intriguing observation that morphologically normal spermatozoa can be obtained from round spermatids in about half of the time that they are normally produced in the testicle (Heller and Clermont, 1963), suggests either that spermiogenesis can be accelerated if cells are cultured isolated in special media, or that an abnormal maturation is taking place. In support of

a normal accelerated process is the fact that Aslam and Fishel (1998) have also found a better maturation progression with isolated round spermatid culture than with culturing the whole seminiferous cell population. Furthermore, in the present results, maturation followed the morphological features described for the testis, which included: (i) for the cytoplasm: decrease in the diameter of the round spermatid, tail extrusion, elongation, and formation of a morphologically normal middle piece by loss of the residual cytoplasm at the nuclear base; and (ii) for the nucleus: decrease in diameter, migration towards the pole opposite tail extrusion, elongation and condensation. However, matured cells will have to be tested for viability, developmental potential, and genetic integrity. Fourth, although the monolayer system enabled us to demonstrate for the first time that it is possible to support full maturation of human round spermatids *in vitro*, co-culture may be considered a drawback for clinical application because of the inherent risks of transmitting infectious agents. For this reason, future efforts should lead to the establishment of efficient specially-supplemented free cell culture media.

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