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In vitro culture facilitates the selection of healthy spermatids for assisted reproduction

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Objective: To evaluate the potential usefulness of in vitro culture of germ cells for distinguishing between healthy and apoptotic spermatids.

Design: Prospective study.

Setting: Private assisted reproduction laboratories and a university department.

Patient(s): Men with secretory azoospermia who were candidates for assisted reproductive treatment.

Intervention(s): Testicular biopsy samples were cultured in the presence of FSH and testosterone for 48 hours. Germ cell apoptosis before and after culture was evaluated by terminal deoxyribonucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

Main Outcome Measure(s): The percentage of germ cells at different stages of spermatogenesis that showed apoptosis-related DNA damage.

Result(s): In fresh samples, high levels of apoptosis were detected at those stages of spermatogenesis at which major developmental blocks occurred, with a maximum at the most advanced stage detected. In contrast, apoptotic cells were considerably less well represented at the most advanced stage after culture.

Conclusion(s): In addition to the previously described facilitation of spermatid recognition and the progression of cytoplasmic maturation, in vitro culture of germ cells is useful to overcome the danger of inadvertent use of apoptotic spermatids for assisted reproduction. (Fertil Steril® 1999;72:809–13. ©1999 by American Society for Reproductive Medicine.)

Key Words: Spermatid conception, spermatid culture, apoptosis, healthy cell selection

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After the first reported birth of a child resulting from fertilization with a round spermatid (1), several other successful round spermatid injection (ROSI) treatment cycles have been reported (2–6). However, in contrast to the first published series, in which ROSI was improvised in situations of unexpected sperm absence when intracytoplasmic sperm injection was planned (1), ROSI outcomes were less encouraging in those cases in which no production of late elongated spermatids or spermatozoa was ever detected in the patients' histories (5, 7). The condition in which spermatogenesis is blocked at the round spermatid stage has been called complete spermiogenesis failure (7).

We investigated the possible causes of the unexpectedly low efficacy of ROSI in patients with complete spermiogenesis failure and demonstrated recently that apoptosis-related DNA fragmentation is markedly more frequent in germ cells recovered from men with complete spermiogenesis failure compared with those in whom late elongated spermatids or spermatozoa can occasionally be found (8). Because round spermatids carrying this kind of DNA damage often have most of the characteristics of living cells and cannot be distinguished from healthy round spermatids at the time of ROSI, the risk of inadvertent use of such apoptotic cells is high in patients with complete spermiogenesis failure. This can explain the low success rate of ROSI in this category of patients.

Preliminary experiments conducted in our laboratories showed that germ cells from some men with complete spermiogenesis failure

have a good survival rate during 1–2 days of in vitro culture in media supplemented with high concentrations of FSH and testosterone. Moreover, round spermatids from these patients can even resume morphologic changes that are characteristic of spermiogenesis and develop into functionally competent gametes that, after microinjection into oocytes, give rise to viable embryos and term pregnancies (9).

This contrasts with the presumed inability of supraphysiologic concentrations of FSH and testosterone to stimulate spermatogenesis in vivo. The mechanism that enables in vivo arrested germ cells to overcome the developmental block during in vitro culture is not known. Because apoptosis is expected to produce an irreversible arrest of cell differentiation, it can be hypothesized that, despite the high degree of apoptosis among germ cells from men with complete spermiogenesis failure, some healthy germ cells can still be recovered by testicular biopsy, and these cells can be selected by allowing them to resume differentiation during in vitro culture in media with supraphysiologic hormone concentrations.

This study was undertaken to confirm this hypothesis. Apoptosis-related DNA fragmentation was evaluated in different stages of germ cells freshly recovered from patients with different types of spermatogenesis arrest and compared with that of germ cells from the same patients after in vitro culture. Data show that in vitro culture of testicular cells can be used to select healthy spermatids for ROSI.

MATERIALS AND METHODS

Samples of seminiferous tubules were obtained by open testicular biopsy from 11 men with different types of spermatogenesis arrest as determined by previous histologic examination (see Results). The experiments described in this study were approved by the Ethical Committee of the European Hospital. Testicular biopsy samples were disintegrated mechanically by stretching tissue pieces between two sterile microscope slides followed by subsequent repeated aspiration into a 1-mL tuberculin syringe. After homogenization, each sample was divided into two parts; one was used for immediate analysis and the other was allocated to in vitro culture. The part of each sample that was destined for immediate analysis was centrifuged at $500 \times g$ for 10 minutes, resuspended in 1 mL of Gamete-100 medium (Scandinavian IVF, Gothenburg, Sweden) containing collagenase I (1,000 U/mL; Sigma, St. Louis, MO) and elastase (10 U/mL; Sigma), and incubated for 30 minutes at 37°C as described (10) to achieve disintegration of cell clusters into single cells.

After further centrifugation, the resulting cell pellet was used to prepare smears that were allowed to air-dry and then were fixed with 5% glutaraldehyde in 0.05 M of cacodylate buffer (pH 7.4). Fixed smears were stored at −40°C for up to 2 weeks before further processing using the previously described protocol (8) in which terminal deoxyribonucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) is combined with immunocytochemical visualization of the specific germline marker proacrosin. The commercially available In Situ Cell Death Detection Kit, Fluorescein (Boehringer, Mannheim, Germany), containing fluorescein isothiocyanate–labeled deoxyuridine triphosphate, was used for TUNEL, whereas the proacrosin immunocytochemical analysis was performed with 4D4 monoclonal antibody (11) as the primary antibody and tetramethylrhodamine B isothiocyanate–conjugated antimouse immunoglobulin G (Sigma) as the secondary antibody.

The use of glutaraldehyde instead of formaldehyde as the fixative in the TUNEL protocol augments the specificity of the test for apoptotic DNA fragmentation as opposed to DNA damage caused by necrosis, and obviates the requirement for an additional supravital stain (8, 12). The 4D4 monoclonal antibody previously had been shown to react with human germ cells from the pachytene stage onward (11). The green fluorescence of TUNEL-positive cell nuclei resulting from the addition of fluorescein isothiocyanate–labeled deoxyuridine triphosphate to exposed free ends of DNA fragments then was evaluated simultaneously with the red fluorescence resulting from proacrosin immunolabeling in double fluorescence preparations (8).

The second part of each homogenized sample was not exposed to any enzymatic treatment. Instead, it was resuspended in 2 mL of Gamete-100 medium supplemented with 50 IU/L of recombinant FSH (Puregon; Organon, Oss, the Netherlands) and with 1 μM of testosterone added in the form of a water-soluble complex (Sigma). The cells were cultured for 2 days at 30°C as described previously (13). No additional centrifugation or medium change was done during the culture period. At the end of the culture period, the cells were disintegrated with collagenase I and elastase and then processed for TUNEL and proacrosin immunocytochemical analysis as outlined earlier.

Fluorescence microscopy preparations were evaluated in a Leica DMRXA fluorescence microscope (Leica France, Rueil-Malmaison, France). Postzygotene germ cells were detected based on the presence of proacrosin immunoreactivity in developing proacrosomal granules that gave rise to an acrosomal vesicle or in a fluffy reticular area that corresponded with the Golgi apparatus in which the proenzyme is processed before passage to the acrosomal structures. Among proacrosin-positive cells, individual stages of spermatogenesis were distinguished according to the cell size and shape and the distribution of proacrosin immunoreactivity as described previously (11, 14, 15).

The percentage of TUNEL-positive (apoptotic) and TUNEL-negative (healthy) cells was determined for individual stages of germ cells, including postzygotene primary spermatocytes, secondary spermatocytes, round spermatids (Sa stage), and elongating spermatids (grouping together the Sb1, Sb2, and Sc stages of normal spermiogenesis as well as

TABLE 1

Incidence of apoptotic DNA fragmentation at different stages of germ cell development in fresh and cultured samples from patients with predominant meiotic maturation arrest.

Patient no.	Blocking stage		Fraction (%) of germ cells that showed TUNEL positivity					
			Fresh samples			Cultured samples		
	Primary*	Secondary†	Scyte I	ROS	ELS	Scyte I	ROS	ELS
1	Scyte I	ROS	136/200 (68)	20/24 (83)	—	157/200 (79)	3/30 (10)	0/13 (0)
2	Scyte I	ROS	122/200 (61)	56/70 (80)	—	115/200 (58)	14/56 (25)	1/24 (4)
3	Scyte I	ROS	151/200 (76)	33/35 (94)	—	153/200 (77)	28/49 (57)	—
4	Scyte I	Scyte II	45/100 (45)	20/33 (61)	—	31/100 (31)	2/50 (4)	1/10 (10)
5	Scyte I	ROS	81/200 (41)	15/22 (68)	—	92/200 (46)	3/63 (5)	0/27 (0)
6	Scyte I	ELS	32/200 (16)	31/100 (31)	8/9 (89)	41/200 (21)	14/50 (28)	17/100 (17)

Note: If possible, 100 or 200 cells were evaluated in each group. When $<$100 cells were available, all cells at the given stage that could be identified on two microscope slides were included. ELS = elongating spermatid; ROS = round spermatid; Scyte I = primary spermatocyte; TUNEL = terminal deoxyribonucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

* The most represented stage among germ cells from fresh samples.

† The most advanced stage among germ cells from fresh samples.

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the Saf, Sbp, and Scp abnormal forms). The previously described terminology (10) is used to denote different normal and abnormal spermatid forms.

The statistical significance of differences in the percentage of TUNEL-positive cells at individual stages of spermatogenesis between fresh and cultured samples was evaluated with the use of the χ^2 and Kruskal-Wallis tests.

RESULTS

By analyzing the prevalence of individual germ cell stages, identified with the use of the previously described criteria based on the size of the nucleus and the specific staining patterns with 4D4 antibody (11, 14, 15), the patients involved in this study were divided into two groups. In the first group (n = 6), testicular cell preparations showed an overwhelming majority of meiotic cells, mainly primary spermatocytes, with only a few, if any, postmeiotic round spermatids. This condition is referred to as predominant meiotic maturation arrest. Patients in the second group (n = 5) had overall low numbers of all types of germ cells, sometimes with a slight accumulation of round spermatids. This condition is referred to as predominant postmeiotic maturation arrest.

The distinction between the two types of maturation arrest was based on the results of the cytologic analysis performed in this study, and the allocation of individual patients into both groups was only partly in agreement with previous histologic diagnoses. In fact, meiotic maturation arrest had been diagnosed correctly in only four of the six patients in the first group, and postmeiotic maturation arrest had been detected histologically in only one of the five patients in the second group. The remaining patients in both groups had histologic findings indicative of germinal aplasia (Sertoli cell–only syndrome).

Patients With Predominant Meiotic Maturation Arrest

Among the six patients in the group with predominant meiotic maturation arrest, round spermatids were the most advanced germ cells detected in fresh samples from four patients and secondary spermatocytes were the most advanced cells from one patient. Despite their paucity, the secondary spermatocytes could be identified in this patient according to their nuclear size and the typical partitioning pattern of 4D4 immunoreactivity into two distinct acrosomal precursors (11, 14, 15). In one patient in this group, rare elongating spermatids (up to the Sc stage) were found (Table 1).

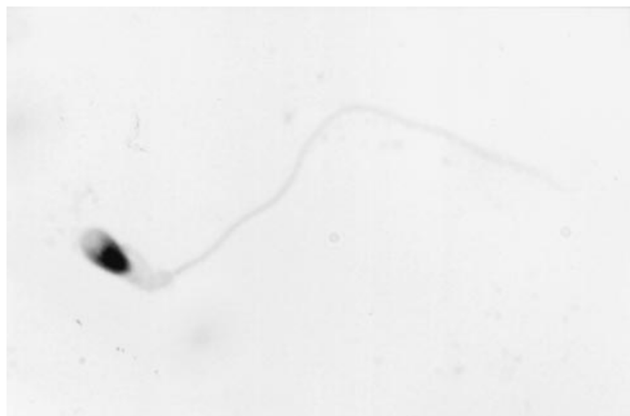
At the end of the culture period, variable numbers of germ cells from five patients in this group showed morphologic progression up to elongating spermatids, mostly the Sbp and Scp atypical forms. However, rare Sd elongated spermatids (Fig. 1) also were detected in the culture sample from one of these men (patient 5, Table 1). The mean percentage of TUNEL-positive cells was high (79%) at the most advanced stages detected in fresh samples. On the other hand, it was low (15%) at the most advanced stages detected after in vitro culture (Table 1). This difference was highly statistically significant ($P<.001$).

Patients With Predominant Postmeiotic Maturation Arrest

Three of the five patients in the group with predominant postmeiotic maturation arrest had complete spermiogenesis failure, with round spermatids (Sa stage) being the most advanced stage detected in fresh samples. The other two patients had rare elongating spermatids (up to the Sc stage)

FIGURE 1

Elongated (Sd) spermatid observed in a Papanicolaou-stained smear of in vitro cultured testicular cells from a man with meiotic maturation arrest with a complete absence of elongating and elongated spermatids in the fresh sample (patient 5).



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in their fresh samples (Table 2). All but one of these patients, however, had elongating spermatids by the end of the in vitro culture period (Table 2). Also in this group, the percentage of TUNEL-positive cells was significantly higher ($P < .001$) at the most advanced stage of germ cell development detected in fresh samples (65%), whereas it was low (10%) at the most advanced stage after in vitro culture (Table 2).

DISCUSSION

A previous study showed that patients with spermatogenesis arrest usually have high percentages of apoptotic germ cells at those stages at which spermatogenesis is blocked (8). In another study (9), the possibility of obtaining in vitro development of spermatids from some men with complete spermiogenesis failure was demonstrated. The present study confirms these observations. The possibility that the cells with a higher degree of maturity observed at the end of in vitro culture might already be present in the samples before culture is unlikely; if this were the case, such cells could hardly escape detection in the fresh samples in which the seminiferous tubules are disintegrated completely by the enzymatic treatment.

The results of the present study thus support the working hypothesis that only nonapoptotic germ cells can undergo in vitro postmeiotic morphologic differentiation. Thus, the use of in vitro culture, in addition to yielding spermatids with a higher degree of cytoplasmic maturation compared with fresh samples (10), provides a means for easy distinction of healthy spermatids from apoptotic ones. Such a distinction is hardly possible in fresh samples without the use of invasive methods of examination.

The selection of healthy spermatids in cultured samples is based on morphologic criteria that reflect individual changes that occur during spermiogenesis (nuclear condensation and protrusion, cell body elongation, flagellar growth) and whose application does not demand a special skill. The risk of a mistake in the distinction of living round spermatids in fresh testicular biopsy samples from non-germ cells thus also is circumvented by in vitro culture. The unusual rapidity of germ cell in vitro development observed in this study confirms the previous observations (9, 10, 13) and other investigators (16–18).

It is of interest that the frequency of apoptotic germ cells detected after culture was lower than before culture even in those patients in whom no morphologic progression of germ

TABLE 2

Incidence of apoptotic DNA fragmentation at different stages of germ cell development in fresh and cultured samples from patients with predominant postmeiotic maturation arrest.

Patient no.	Blocking stage		Fraction (%) of germ cells that showed TUNEL positivity					
			Fresh samples			Cultured samples		
	Primary*	Secondary†	Scyte I	ROS	ELS	Scyte I	ROS	ELS
1	ROS	—	15/50 (30)	96/100 (96)	—	78/100 (78)	55/100 (55)	2/23 (9)
2	ROS	—	85/100 (85)	21/50 (42)	—	26/52 (50)	11/48 (23)	—
3	ROS	—	47/100 (47)	56/100 (56)	—	55/100 (55)	21/100 (21)	3/50 (6)
4	ROS	ELS	24/100 (24)	23/100 (23)	9/14 (64)	28/100 (28)	39/100 (39)	2/54 (4)
5	ROS	ELS	35/100 (35)	39/100 (39)	25/36 (69)	41/100 (41)	21/100 (21)	5/50 (10)

Note: If possible, 100 or 200 cells were evaluated in each group. When <100 cells were available, all cells at the given stage that could be identified on two microscope slides were included. ELS = elongating spermatid; ROS = round spermatid; Scyte I = primary spermatocyte; TUNEL = terminal deoxyribonucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

* The most represented stage among germ cells from fresh samples.

† The most advanced stage among germ cells from fresh samples.

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cell differentiation occurred during the culture period (patients 3 and 6 in Table 1, and patients 2, 4, and 5 in Table 2). This observation can be explained by further progression of the apoptotic process in those germ cells that were only at its beginning before culture, leading to complete disintegration of these cells by the end of the culture period. At the same time, the ongoing differentiation of healthy cells at earlier stages of spermatogenesis, especially at the primary spermatocyte stage (13), may yield in vitro developed secondary spermatocytes and spermatids that can substitute for the disappearing apoptotic cells at the respective stages.

The eventual effects of the enzymatic treatment that was used to dissociate cells just before their final preparation for TUNEL and immunocytochemical analysis could not contribute to the differences between individual groups because the cells in all the groups were treated in the same way and at the same time before fixation. Moreover, a similar treatment is used routinely to increase the yield of testicular spermatozoa for assisted reproductive techniques without any negative effect on success rates (19). Hence, in vitro culture may improve the results of spermatid conception even in those cases in which no morphologic progression can be detected by simple repeated observations.

In conclusion, this pilot study suggests a possible approach to overcoming the problem of germ cell apoptosis, one of the main currently known factors that limit the success of spermatid conception. Four normal infants were born after the microinjection of in vitro developed elongated spermatids into oocytes, two of them (twins) in a case of complete maturation arrest at the primary spermatocyte stage and two in cases with complete maturation arrest at the round spermatid stage (9, 20). Karyotype analysis, performed in the first three infants born after the clinical application of this method, did not reveal any numeric or structural chromosomal abnormalities (20). Further study is needed to determine the relation between the reproductive capacity of in vitro cultured spermatids, the degree of morphologic progression of germ cell differentiation during in vitro culture, and the percentages of apoptotic cells at different stages of spermatogenesis before and after culture.

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