Human Reproduction vol.13 no.3 pp.509–523, 1998

DEBATE

Are spermatid injections of any clinical value?

ROSNI and ROSI revisited

Sherman J.Silber[1,3] and Larry Johnson[2]

[1]Infertility Center of St Louis, St Luke's Hospital, 224 South Woods Mill Road, St Louis, MO 63017 and [2]Department of Veterinary Medicine, Texas A & M University, College Station, TX 77843-4458, USA

[3]To whom correspondence should be addressed

Tiny numbers of spermatozoa can be extracted from an extensive testis biopsy and be used successfully for intracytoplasmic sperm injection (ICSI) in ~60% of cases of non-obstructive azoospermia caused by testicular failure (e.g. maturation arrest, Sertoli cell only, cryptorchid atrophy, post-chemotherapy, or even Klinefelter's syndrome). However, no sperm are recoverable in 40% of cases even after a very extensive testicular sperm extraction (TESE)–ICSI attempt. Round spermatid nucleus injection (ROSNI) and round spermatid injection (ROSI) would be an appropriate alternative if no elongated spermatozoa, or elongated spermatids were recoverable. Round cells are abundant in morselated testicular tissue of almost all azoospermic men, but difficulties arise in distinguishing under Hoffman or Nomarski optics whether they are haploid round spermatids, diploid spermatocytes or spermatogonia, or even somatic cells like Sertoli cell nuclei or Leydig cells. This paper attempts to clarify such confusion by reviewing data on 143 consecutive testis biopsies of men with non-obstructive azoospermia due to germinal failure, and 62 controls with obstructive azoospermia and normal spermatogenesis. In no cases were round spermatids found in the absence of elongated spermatozoa, and maturation arrest was found always to be a failure of progression beyond meiosis (not at maturation from round spermatid to mature elongated spermatid). Errors arising after injecting somatic or other round cells could result in an appearance resembling fertilization and cleavage, and explain reports of finding 'round spermatids' in azoospermic men where no 'spermatozoa' were retrievable. The use of TESE–ICSI to achieve pregnancies in azoospermic men with deficient spermatogenesis is more concerned with finding tiny foci of spermatozoa, rather than searching for 'round spermatids', which are recoverable only if elongated forms are also available.

Key words: ICSI/maturation arrest/spermatids/TESE

Introduction

The discovery that azoospermic men with germinal failure often have minute foci of spermatogenesis, was observed in the early studies of quantitative analysis of spermatogenesis (Steinberger and Tjioe, 1968; Zuckerman *et al*., 1978; Silber and Rodriguez-Rigau, 1981). However, the importance of this finding for helping azoospermic men with testicular failure have their own genetic child, was not readily apparent until the era of intracytoplasmic sperm injection (ICSI) (Palermo *et al*., 1992; Van Steirteghem *et al*., 1993). In 60% of cases of azoospermia caused by testicular failure (e.g. maturation arrest, Sertoli cell only, cryptorchid testicular atrophy, post-chemotherapy azoospermia, or even Klinefelter's syndrome), a tiny number of spermatozoa can often be extracted from an extensive testicle biopsy, and these few retrieved spermatozoa, using ICSI, can result in a normal pregnancy (Devroey *et al*., 1995; Silber *et al*., 1995a,b,c, 1996). We termed this procedure TESE (testicular sperm extraction).

However, 40% of azoospermic men with germinal failure have no sperm recoverable during an extensive TESE–ICSI procedure. Recently the possibility has been investigated of using 'round spermatids', or 'round cells', derived from testicular tissue (or even from the ejaculate), that are presumably early spermatids, to inject for ICSI for such cases when no elongated spermatozoa are recoverable. Many infertility clinics have attempted ICSI with ROSNI (round spermatid nucleus injection) or ROSI (round spermatid injection). The concept behind this is to provide an option for those patients in whom mature spermatozoa cannot be identified in the TESE–ICSI procedure.

This approach was first suggested by Yanagimachi and Ogura (1993) and led to similar efforts in humans (Ogura and Yanagimachi, 1993; Ogura *et al*., 1993, 1994; Edwards *et al*., 1994). Specifically, 'round cell injection' in human cases of azoospermia followed the comment in their discussion section, 'if spermatids can be obtained in an acceptable way from azoospermic patients, these cells can be used to construct zygotes with full developmental potential.'

The intention of this article is to present the outcome of an extensive series of testicle biopsies in all varieties of azoospermic men, to review our previously published histological findings in azoospermic men suffering from 'maturation arrest', and to give a view of our attempts to understand the ROSI procedure. It is much easier to discern the various stages and progression of spermatogenesis and of 'spermiogenesis' in well-prepared, stained histological slices than the unstained cytological specimens found at TESE–ICSI. Photographs are included to help in-vitro (IVF) laboratory personnel to identify the many various types of 'round cells' seen in a dissected testicular specimen, relying to some extent not only on present efforts, but also on well-established previously published reports.

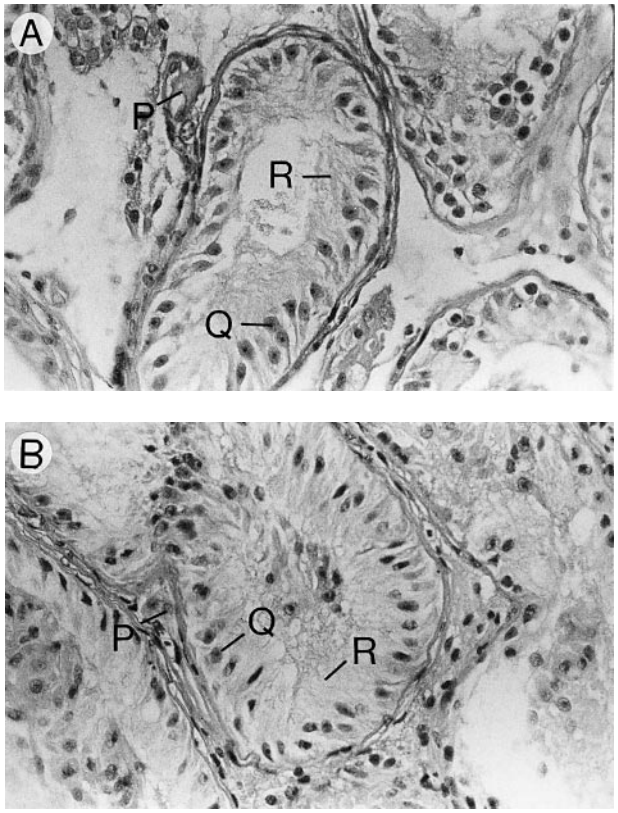

Figure 1. (**A** and **B**) Histological sections of testes with haematoxylin and eosin stain showing Sertoli cell only with no germ cell elements. P = Leydig cell cluster, Q = typical Sertoli cell nucleus with prominent nucleolus, R = amoeboid cytoplasm of Sertoli cell with no distinct boundary.

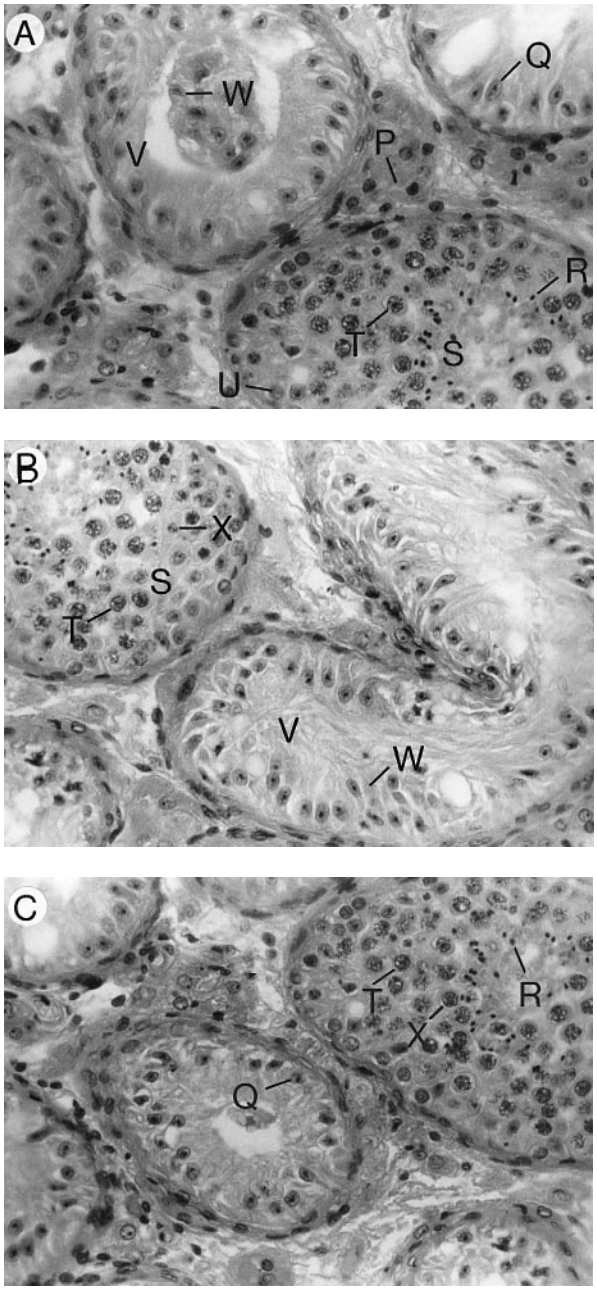

Figure 2. (**A**–**C**) Histological sections of testes with haematoxylin and eosin stain showing Sertoli cell only; in these cases, the empty tubules are next to a single tubule which exhibits normal spermatogenesis. The Sertoli cell-only tubules (the predominant pattern in each of these three patients) demonstrate distinct, round Sertoli cell nuclei with a prominent dark-staining nucleolus, approximately the size of round spermatids. In the juxtaposed tubule with normal spermatogenesis, note the normal progression of spermatogonia, spermatocytes and spermatids. P = Leydig cell cluster, Q = typical Sertoli cell nucleus with prominent nucleolus, R = amoeboid cytoplasm of Sertoli cell with no distinct boundary, S = adjacent seminiferous tubule with normal spermatogenesis, T = (pre-meiotic division) pachytene spermatocyte, U = spermatogonia, V = seminiferous tubule with 'Sertoli cell only'(W), X = early round spermatid.

Histological examinations of testicle biopsy slides in patients with non-obstructive and obstructive azoospermia

Our library of testicular histology for non-obstructive azoospermia due to germinal failure totalled 143 patients. Sixty-six of these 143 men had a diagnosis of 'Sertoli cell only' with no other known cause for infertility. Fifty-nine of these patients had a diagnosis of either pure 'maturation arrest' or a combination of 'maturation arrest' and 'Sertoli cell only' in different areas of the same slide. Eighteen patients had other causes for non-obstructive azoospermia, such as mumps orchitis, sex chromosomal anomaly, cryptorchidism, or previous chemotherapy.

For controls with 'normal spermatogenesis' we used 62 men with obstructive azoospermia, caused either by congenital absence of the vas or vasoepididymostomy failure, who over the course of the last 3 years, have undergone TESE–ICSI procedures (because of either an absence of epididymis or a failure to find motile spermatozoa in the epididymis). Thus, we were able to review a library consisting of 205 testes biopsies, 143 having non-obstructive azoospermia due to testicular failure, and 62 with obstruction and normal spermatogenesis. These slides were all reviewed in a quantitative fashion as has already been described (Steinberger and Tjioe, 1968; Zuckerman *et al*., 1978; Silber and Rodriguez-Rigau, 1981; Johnson *et al*., 1992). We found no cases of classic 'hypospermatogenesis' (as we define it) in azoospermic patients. Classic hypospermatogenesis implies a diffuse reduction in quantitative spermatogenesis throughout the testis and it is generally associated with oligozoospermia, not azoospermia.

Figures 1A,B and 2A–C illustrate the findings of spermato-

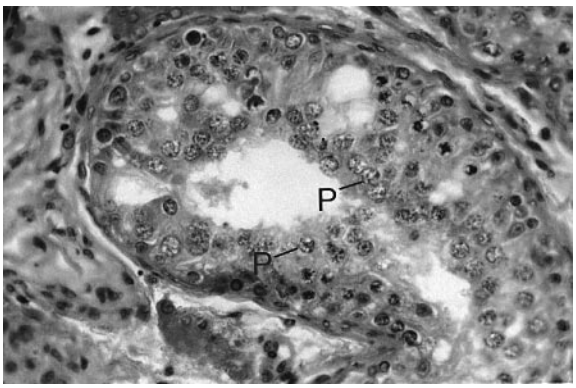

Figure 3. Histological section of testes with haematoxylin and eosin stain demonstrating maturation arrest. There is a full progression of spermatogonia, primary spermatocytes, but no secondary spermatocytes or round spermatids can be seen. P = pachytene spermatocyte.

genesis in all categories of non-obstructive azoospermia studied. In the case of Sertoli cell only, there is, of course, an absence of germ cells. If one looks at an entire slide of more than 20 tubules with 'Sertoli cell only,' in many cases there will be an occasional tubule with normal spermatogenesis (Silber, 1995b; Silber *et al*., 1996). The minimum number of tubules counted on both sides was 40 per patient, and usually >100 tubules were counted.

The Sertoli cell is a large, formless amoeba-like cell in which the germ cells would normally be nourished. The nuclei of the Sertoli cells are located along the basement membrane circumferentially at the base of the seminiferous tubule, and each contains a very prominant nucleolus. The Sertoli cell nucleus is a dominant presence in the histology of Sertoli cell only. It is important to keep this picture in mind when searching for 'round spermatids' in men with no spermatozoa found at TESE–ICSI.

Figure 3 shows representative histology from a patient with 'maturation arrest'. In all cases, the arrested development was found to occur in meiosis, either at zygotene or pachytene. No round spermatids were found except in those cases (partial) where elongated spermatids and mature spermatozoa also occurred. Thus, in none of the 125 cases of idiopathic non-obstructive azoospermia was there any evidence of 'spermiogenic' arrest, i.e. arrest in the development of mature elongated spermatids from round spermatids. The germinal defect in non-obstructive azoospermia, as already reported, was either an absence of germ cells (Sertoli cell only), or a failure of germ cells to progress beyond meiosis (maturation arrest) (Silber *et al*., 1996).

In the other miscellaneous causes of non-obstructive azoospermia, whether from chemotherapy, cryptorchidism, or mumps, we found varying degrees of fibrosis and tubular atrophy that were not seen in the idiopathic examples previously discussed. However, once again the defect in spermatogenesis in all these cases involved either an absence of germ cells, or a failure of the germ cells to progress beyond meiosis. Thus, in all 143 cases of non-obstructive azoospermia caused by testicular failure, spermiogenic arrest, i.e. failure of round spermatids to develop into mature spermatozoa, was never found. Such a condition must be fairly uncommon.

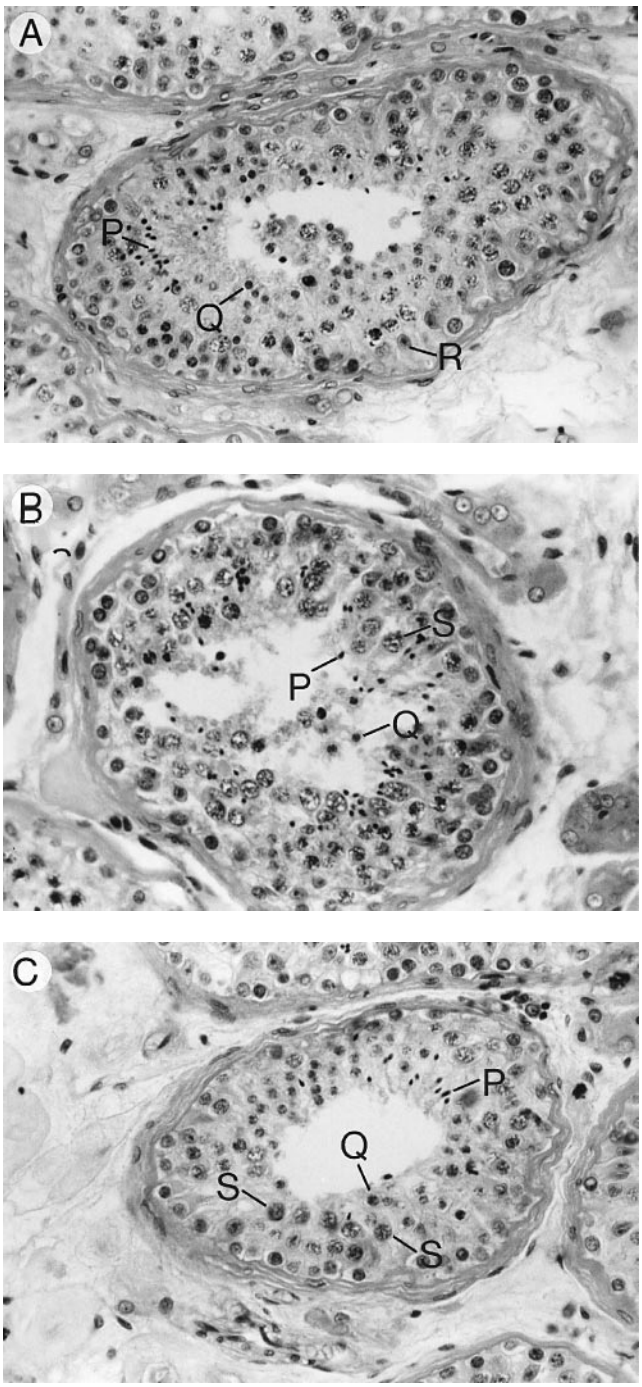

Figure 4. (**A**–**C**) Histological sections of testes with haematoxylin and eosin stain in patients with obstructive azoospermia and normal spermatogenesis. Note the Sertoli cell nuclei, along with a full progression of spermatogonia, primary spermatocytes, secondary spermatocytes, round spermatids and mature, darkly staining condensed oval spermatids. Because of the distribution of the six stages of spermatogenesis in humans, in sections of the tubule where the round spermatids are prominent, elongated spermatids are not seen. In the majority of the areas of each tubule where elongated spermatids are present, round spermatids are not seen. However, in any section of testes, whether normal or pathological, round spermatids are not seen in the absence of elongated spermatids. P = elongated, mature spermatid, Q = round spermatid, R = Sertoli cell nucleus, S = pachytene spermatocyte.

Figure 4A–C shows examples of the histology of 62 patients with obstructive azoospermia undergoing TESE–ICSI, who presumably should have had normal spermatogenesis. All of these cases with a clinical diagnosis of obstruction had full progression of spermatogenesis, both premeiotic and post-meiotic. It is readily apparent that zygotene and pachytene spermatocytes are somewhat bigger than round spermatids, but Sertoli cell nuclei, leptotene spermatocytes and the briefly present secondary spermatocytes are all of similar size to round spermatids.

It would appear that in some tubules which exhibit normal spermatogenesis, there is a predominance of round spermatids, but a review of many seminiferous tubules in these cases still revealed a normal progression of round to mature spermatids (Johnson *et al*., 1992). It has long been known that in humans there is no orderly wave of progressive spermatogenic stages down the seminiferous tubules, as in most animals (Clermont, 1972). Therefore, the appearance of just a few tubules is not representative of the rest of the testicle, but of twenty or more tubules is. In these 205 cases of testicle biopsies in azoospermic men, we were not able to find any tubules in which round spermatids were observed in the absence of mature spermatozoa.

An atlas of male germ cells: to be used for identifying round spermatids during a TESE–ICSI procedure

When one performs a TESE–ICSI procedure in patients with testicular failure, as well as in patients with normal spermatogenesis, there is always an abundance of 'round cells'. It is very difficult with Hoffman optics to differentiate with certainty a round spermatid from a Sertoli cell nucleus with its prominent nucleolus, or even from a spermatocyte. Even when there are truly no spermatozoa at all, there will always be many 'round cells' seen with either Sertoli cell only or with maturation arrest, but these are not round spermatids (Johnson *et al*., 1981, 1992; Johnson, 1986; Silber *et al*., 1996). Figure 5A,B was taken from our collection of TESE cases.

With Hoffman and Nomarski optics normally used with ICSI, it is very difficult to distinguish round spermatids from Sertoli cell nuclei. The round spermatid should be distinguished by the acrosomal vesicle located on the periphery, and this does not show up well on Hoffman or Nomarski optics. In Figure 6 the round spermatid can be distinguished by the 'glow' of the early acrosomal cap (Holstein and Roosen-Runge, 1981). This can only be reliably and simply visualised with phase contrast.

There has been a great deal of effort extended towards improving this recognition of spermatids in a wet preparation (Mendoza and Tesarik, 1996; Mendoza *et al*., 1996; G.Verheyen, personal communication). This is not easy to accomplish, but the system of Verheyen (1998) appears to be very promising.

A review of the basic science of round spermatid injection in experimental animals

The landmark studies of Sofikitis *et al*. (1994, 1996), Ogura and Yanagimachi (1994) and others were performed in hamsters, mice and rabbits with normal spermatogenesis. In 1993, Ogura and Yanagimachi and their co-workers first published the results of their work with round spermatid nuclei injections in hamster and mouse oocytes. In their first study involving subzonal insertion of hamster round spermatids followed by electrofusion, only 5–10% of the fused oocytes had normal two-pronucleate fertilization. The rest of the spermatid-derived pronuclei were small and abnormal. For the mouse, electrofusion with spermatids was even less sufficient. Only 10% of the mouse oocytes fused with round spermatids and almost all the male pronuclei were small at the two-pronucleate stage.

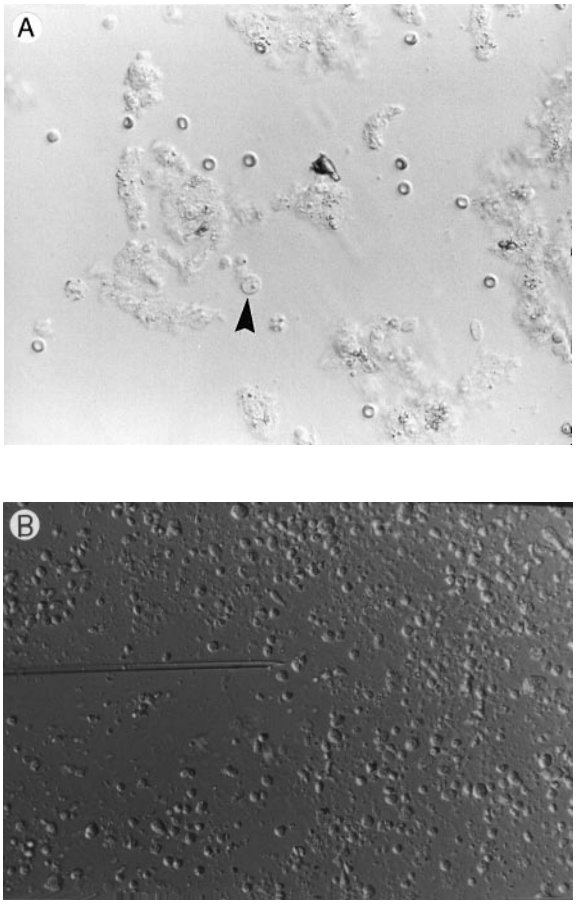

Figure 5. (**A**) Morselated testicular tissue in patient with Sertoli cell only seen under Hoffman optics in a microdroplet diluted for testicular sperm extraction–intracytoplasmic sperm injection. The arrow points to one of many prominent Sertoli cell nuclei with its nucleolus, which is often mistaken as a Golgi apparatus prior to forming the acrosome cap. Note also red blood cells and abundant Sertoli cell cytoplasm. (**B**) Microdroplet of testicular tissue dissolved in digestive enzyme in a patient with Sertoli cell only.

In these original papers, the identification of round spermatids was made by the presence of a prominent central nucleolus. 'The nuclei of round spermatids were small in size and each one had a centrally located nucleolus.' This description of the identification of the round spermatid could easily be confused by clinicians less familiar with the basic science of spermatogenesis, with a Sertoli cell nucleus and its prominent nucleolus.

A year later Yanagimachi's group were able to obtain live-born mice from round spermatid injection (Ogura *et al*., 1994).

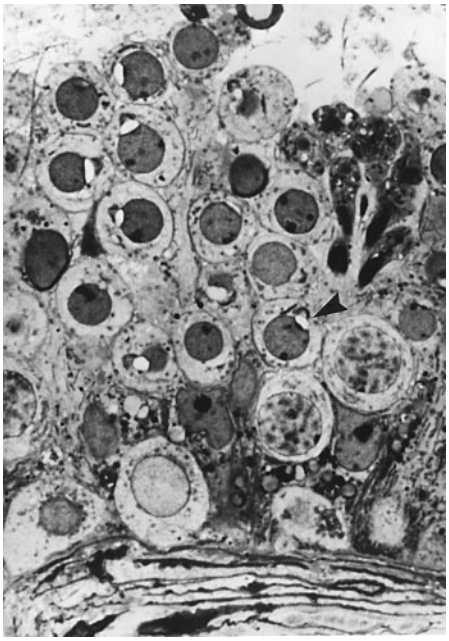

Figure 6. A field of normal spermatogenesis in which early round spermatids are noted to have a large peripheral, non-staining 'acrosomal vesicle'. Reprinted from Holstein, A.F. and Roosen-Runge, E.C. (eds), *Atlas of Human Spermatogenesis*, Grosse Verlag, Berlin, with permission from Medizinische Verlagsanstalt GmbH, Berlin, Germany (1981).

Only four live baby mice resulted from a total of 475 eggs so injected, representing a 1% live birth pregnancy rate. Clearly the pregnancy rate would have been dramatically higher with the selection of mature spermatids or spermatozoa. But this paper demonstrated that 'nuclei of round spermatids, like those of mature spermatozoa have reproductive potential.' Perhaps that inefficiency will eventually be improved (Kimura and Yanagimachi, 1995).

Clinical experience with ROSI and ROSNI

The first reports of success (Tesarik *et al*., 1995, 1996; Tesarik and Mendoza, 1996) described seven cases of azoospermic men, which, despite the absence of mature spermatozoa, had round spermatids in the ejaculate, and these round spermatids were injected (instead of mature spermatozoa), resulting in two out of seven successful pregnancies with viable births. This would be an incredible increase in efficiency compared to the 1% live birth rate that Ogura and Yanagimachi obtained in mice injected with round spermatids.

Perhaps more importantly, the finding of round spermatids in the absence of mature spermatozoa in an azoospermic man appears to contradict our observation that in humans, round spermatids are not found in the absence of mature spermatids. Nonetheless, a few other centres have been able to repeat this work (Sofikitis *et al*., 1996; Antinori *et al*., 1997a,b; Fishel *et al*., 1997).

A further report of the use of spermatid injection came from Fishel *et al*. (1995). Their successful pregnancy resulted from injection of a spermatid retrieved from the testicle, rather than the ejaculate, but these were not round spermatids. The author suggests that spermatozoa were not available and, therefore, they had to resort to choosing earlier spermatids. However, in this case report, some of the ejaculates of the patient actually had a few spermatozoa (crypt-azoospermia), and other ejaculates were azoospermic. An ill-conceived attempt apparently was made to retrieve spermatozoa from the epididymis (rarely successful in non-obstructive azoospermia), but then finally a testicular biopsy was performed and an attempt at TESE was made. Apparently, nine spermatozoa were actually recovered, but the morphology was deemed by the authors to be abnormal and, therefore, instead they chose to inject what they called 'elongated spermatids,' which looked 'healthier than the few spermatozoa obtained.' It is possible that these 'spermatids' were so mature that they were indistinguishable from normal mature spermatozoa.

Although Fishel *et al*. (1995) discuss the 'round spermatid' injection, clearly what they were reporting is no different from the routine sperm injections that have been reported already for non-obstructive azoospermia (Devroey *et al*., 1995; Silber *et al*., 1995a; 1996). Similar reports of 'late spermatid injection' have been made by Vanderzwalmen *et al*. (1995) and Araki *et al*. (1997). However, most of these successful cases are just sporadic reports of what is no different than simply TESE–ICSI with 'elongated spermatids', i.e. testicular sperm extraction, finding occasional spermatozoa present in 60% of testicle specimens from men with azoospermia caused by germinal failure.

Nonetheless, there is still a great deal of interest in attempting round cell injection in cases of azoospermia where no spermatozoa or elongated spermatids are found during the TESE procedure. However, it is very difficult to decide what really constitutes a round spermatid, a secondary spermatocyte, a primary spermatocyte, and even spermatogonia, Leydig cells and Sertoli cell nuclei in the usual ICSI setting.

Because of the confusion, we elected, in patients undergoing TESE in whom absolutely no spermatozoa were recoverable after hours of exhaustive searching, to inject Sertoli cell nuclei into the oocytes (which would otherwise go to waste anyway). We were able to obtain non-specific 'fertilization' with the appearance of at least one pronucleus and often two pronuclei, although the second pronucleus was usually much smaller than the first. On day 2, many of these oocytes cleaved. Most of the 'embryos' resulting from this parthenogenic activation were quite abnormal (see Figure 7A–D). This non-specific activation could serve as a source of confusion to enthusiasts for ROSI and ROSNI.

There is no doubt that round spermatid injection works in patients in whom round spermatids are found, albeit with extremely low efficiency. But it would appear that, in the vast majority of cases where true round spermatids are found, mature spermatids or spermatozoa should also be retrievable and would certainly be preferable for injection.

Conclusion

One of the problems for IVF clinics using the TESE–ICSI procedure is that the embryologist and clinician may possibly have little input from either a urologist or an endocrinologist who is experienced with spermatogenesis and testicular histo-

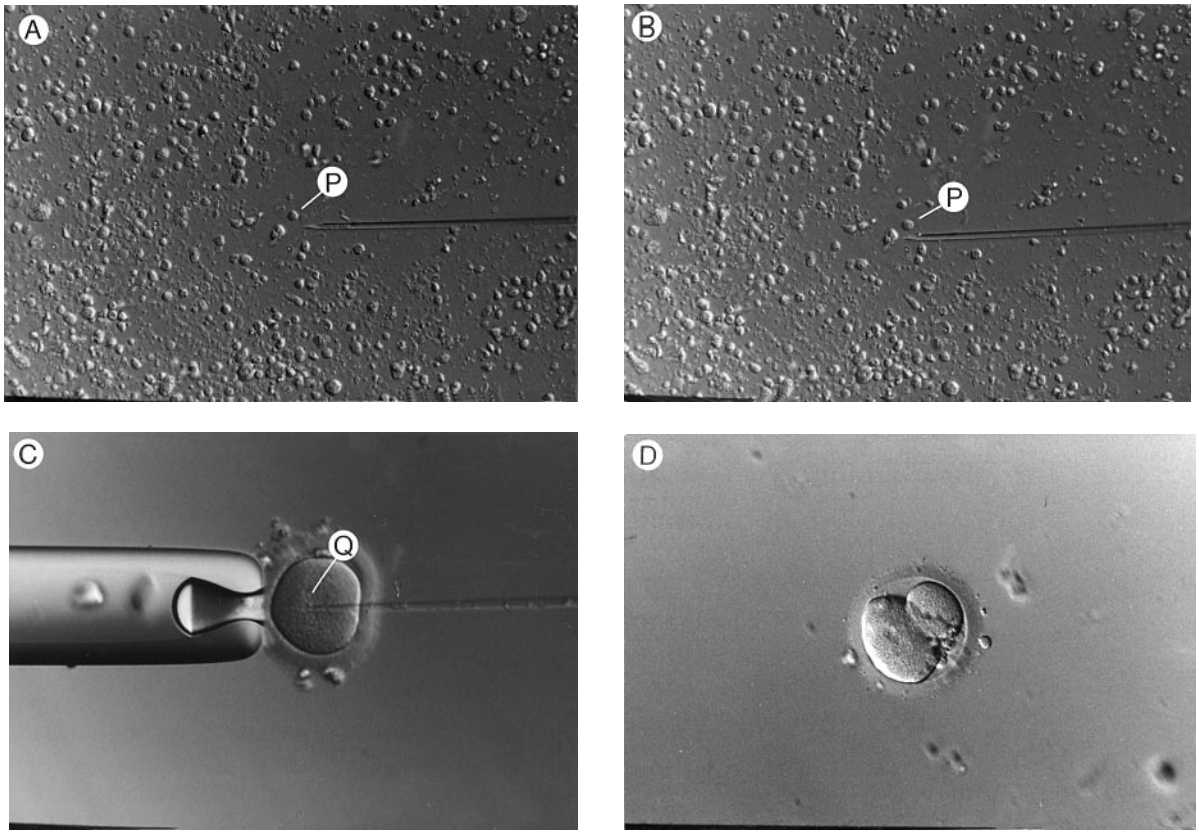

Figure 7. (**A**) Sertoli cell nucleus at testicular sperm extraction (Hoffman optics). (**B**) Sertoli cell nucleus (Hoffman optics) about to be picked up in intracytoplasmic sperm injection pipette. (**C**) Injected Sertoli cell nucleus. (**D**) Cleaving, poor quality 'embryo' resulting from injection of Sertoli cell nucleus in a patient in whom no spermatozoa were found. P = Sertoli cell nucleus (Q = injected).

logy. Our discovery that small numbers of spermatozoa sufficient for ICSI can be found in the testes of azoospermic men, does not mean that the testicle is a matzoh ball full of spermatozoa and round cells just waiting for injection.

We conclude that the ability to use TESE–ICSI to achieve pregnancies and babies in azoospermic men with deficient spermatogenesis is related to the ability to find tiny foci of spermatozoa in a testicle that otherwise is grossly deficient in spermatogenesis (such that not enough spermatozoa are being produced to reach the ejaculate), and not upon the ability to find less mature forms such as 'round spermatids' in these patients.

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The problems of spermatid microinjection in the human: the need for an accurate morphological approach and selective methods for viable and normal cells

P.Vanderzwalmen[1,4], M.Nijs[1], R.Schoysman[1], G.Bertin[1], B.Lejeune[1], B.Vandamme[1], S.Kahraman[3] and H.Zech[2]

[1]Schoysman Infertility Management Foundation, Vaartsraat 42, 1800 Vilvoorde, Belgium, [2]Institut fur In Vitro Fertilisierung und Embryo Transfer, Bregenz, Austria, and [3]IVF Unit, Sevgi Hospital, Ankara, Turkey

[4]To whom correspondence should be addressed

Before 1994, there was no hope of helping a man to father a child if his testicular dysfunction was such that no spermatozoa could be found either in his ejaculate or in several testicular samples. From that time, encouraging results were published with the use of spermatids in rodents. Syngamy with oocytes was obtained as well as embryo development and after transfer healthy fertile young were produced (Ogura *et al.*, 1994; Kimura and Yanagimachi, 1995; Sofikitis *et al.*, 1996; Sasagawa and Yanagimachi, 1997).

In cases of men with defective spermatogenesis including partial Sertoli cell-only syndrome, partial maturation arrest or miscellaneous non-obstructive azoospermia (cryptorchidism, chemotherapy, combined maturation arrest and Sertoli cell-only syndrome), the chance of finding spermatozoa in a single biopsy is very limited. According to Tournaye *et al.* (1996) areas of some persisting spermatogenesis occasionally can be detected in the above situations after multiple testicular sampling. In our testicular sperm aspiration (TESA) programme, we experienced cases where too few or no spermatozoa at all could be found in different samples of testicular tissue, despite the presence of tubules with complete spermatogenesis in a previous biopsy. The persistence of active and complete spermatogenesis in even a few tubules allows the production of mature spermatozoa that are probably lost by phagocytosis before reaching the vas deferens (Schoysman and van de Casseye, 1995).

Silber *et al.* (1997) showed recently that there is indeed a minimum quantitative threshold of spermatogenesis which must be exceeded for any spermatozoa to reach the ejaculate; they suggested that 4–6 mature spermatids per tubules must be present in the testis biopsy for any spermatozoa to reach the ejaculate. They showed that the cause of secretory azoospermia (partial Sertoli cell-only syndrome, partial maturation arrest or miscellaneous non-obstructive azoospermia) had no effect on this threshold concept. Complete absence of spermatozoa in the testes reflects a deterioration of spermatogenesis with absolute inability to produce mature spermatozoa or at best production of some spermatozoa in few seminiferous tubules. Since 1994, Edwards *et al*. (1994) already proposed using spermatid for the management of non-obstructive azoospermia. This new perspective encouraged some in-vitro fertilization (IVF) centres to obtain viable embryos after the injection of spermatids into mature oocytes.

What is the situation now, after 3 years of applying this technique in the human? In cases with very severe spermatogenetic defects, fertilization and multiple pregancies can be achieved with spermatids in the elongation phase, yielding an acceptable implantation rate (Fishel *et al.*, 1995; Vanderzwalmen *et al.*, 1995; Chen *et al.*, 1996; Tesarik *et al.*, 1996; Antiniori *et al.*, 1997a; Araki *et al.*, 1997; Fishel *et al.*, 1997; Vanderzwalmen *et al.*, 1997). On the other hand, the efficiency of round spermatids in achieving fertilization and ongoing pregnancy is disappointing (Hannay *et al.*, 1995; Tesarik *et al.*, 1996; Amer *et al.*, 1997; Antiniori at al., 1997a,b; Vanderzwalmen *et al.*, 1997; Yamanaka *et al.*, 1997).

With the growing experience of the different centres offering such microassisted technology, can we definitely consider the use of spermatids as a useful therapy? To evaluate the actual therapeutic potential of spermatid conception different aspects have to be analysed. Two important parameters are the type and quality of isolated male germ cells to be injected into oocytes.

Preparation of a testicular cell suspension is of critical

importance in the optimal classification and identification of sperm cell precursors. A first step in the procedure consists of tissue dissociation to obtain a homogeneous cell suspension. The isolation of spermatozoa or spermatids from the testicular tissue can be obtained in two different ways. Mechanical dissociation of testicular tissue using two glass slides or fine needles is one option but it provokes some damage to the cells and the germ cells are contaminated with free nuclei, damaged cells and residual tissue pieces (Blanchard, 1991). A greater number of intact cells can be obtained with more gentle procedures involving enzymatic digestion of the tissue. In a recent study, Crabbe *et al.* (1997) reported that cell suspensions obtained from a testis biopsy by means of enzymatic digestion with collagenase type IV provided the best dissolution of the cells from their tissue with a higher proportion of vitality. In order to enhance the efficiency of spermatid collection such enzymatic treatment of the testicular tissue, followed by an erythrocyte lysing buffer treatment, can improve the recovery of spermatids (Nagy *et al.*, 1997).

The second step consists in the separation of the different cells composing the suspension. Very few attempts have been realized to separate human spermatogenic cells. Several methods of cell separation are based either on cell size or on cell buoyant density. The development of more efficient techniques of testis cell separation would permit the obtention of homogeneous spermatogenic cells population. Blanchard *et al.* (1991) separated with success the different spermatogenic and spermiogenic human cell population, by centrifugal elutriation technique. Nearly pure populations of spermatids were available despite some contamination with blood cells. As mentioned by Blanchard (1991), one difficulty when working with the human species is to obtain material in sufficient quantities. The efficiency of such a technique needs to be tested in our clinical application where the percentage of seminiferous tubules containing spermatids (round, elongating and elongated) and the number of cells present in the tubules are dramatically low.

Spermatid classification

Fertilization is affected by the type of male germ cells found in the biopsies and injected into oocytes (Vanderzwalmen, 1997). Therefore, in order to avoid confusion, it is mandatory to define as precisely as possible the different types of spermatids that were handled (Tesarik, 1997). In fact, without a clear terminology of each stage of spermiogenesis, it is difficult to draw some conclusions and to compare results.

Spermiogenesis, the final phase of spermatogenesis, is the cytodifferentiation of spermatids into spermatozoa. During this process, no cell division is involved and the haploid round spermatids differentiate into highly specialized cells for motility and fertilization. The studies of Clermont (1993), Holstein (1976) and de Krester and Kerr (1969), using staining techniques under light or electronic microscopy clarified the cytological changes characterizing spermiogenesis. They established a classification making an accurate distinction between the different developmental stages involving the beginning of spermiogenesis (Sa, Sb1) (Golgi phase, cap phase and acrosome swelling) followed by the nuclear changes and development of the tail (Figure 1A). Unfortunately, *in situ*, without cytological techniques and under conventional light microscopic technique it is difficult to make a strict distinction between the different developmental stages.

Therefore, those using spermatids mainly for clinical use, need simple and appropriate criteria, based on size and morphological aspects. In fact, iconography so far shows only germinative cells after staining. In a wet preparation using an inverted microscope, without any specific staining methods, we can divide the haploid cells into four categories according to the shape, the amount of cytoplasm and the size of the tail: (i) the round (Sa, Sb1); (ii) the elongating (Sb2); (iii) the elongated (Sc, Sd1); and (iv) the late elongated (Sd2) spermatids (Figure 1B).

In the pathological situation we are dealing with, it is even more difficult to make a strict classification since the cells that we handle are retrieved from patients with abnormal spermiogenesis. Round spermatids include the Golgi phase, the cap phase and the acrosome phase (movement of the nucleus towards a peripheral position). There then follows the elongating spermatids, the elongated spermatids composing the beginning of the maturation phase and finally, a more controversal stage, the late elongated spermatids just before their delivery. Elongating and elongated are discriminated according to the length of the tail which is surrounded by cytoplasm. An elongating spermatid is slightly oval and consists in the transition between the round and the elongated form. The last category that we observed during its delivery from the germinal epithelium is more difficult to include in the classification. Since their appearance is similar to the spermatozoon, the morphological changes occurring during the release of the late elongated spermatids from the Sertoli cells are hardly discernable. It is therefore difficult to give a strict terminology for this late spermatid stage that we can label ‘mature spermatid or ‘immature spermatozoon’. However, we cannot exclude that the degree of maturity of the cytoplasm and the nucleus is not wholly identical to the true spermatozoon. Therefore, in order to avoid some confusion in the analysis of the results, we think, that on morphological basis, such spermatids should not be included in the results together with the terminology of ‘elongated spermatids’ but, as suggested by Silber *et al.* (1996), as ‘mature spermatids’.

After enzymatic treatment of the testicular biopsy or after longer dilaceration of the tubules, most male gamete are released from the Sertoli cells and can be used with success in conventional intracytoplasmic sperm insemination (ICSI)–testicular sperm extraction (TESE)–TESA programme, without distinction between the spermatozoon and the mature spermatid. The definitions stated above are then applied into life situation and this to identify spermatids in wet preparations.

Identification of spermatids

Spermatids in the process of elongation (elongating or elongated) are easy to recognize according to morphological criteria (Figure 1B). However, in a wet preparation, the identification of round spermatids, from the other various types

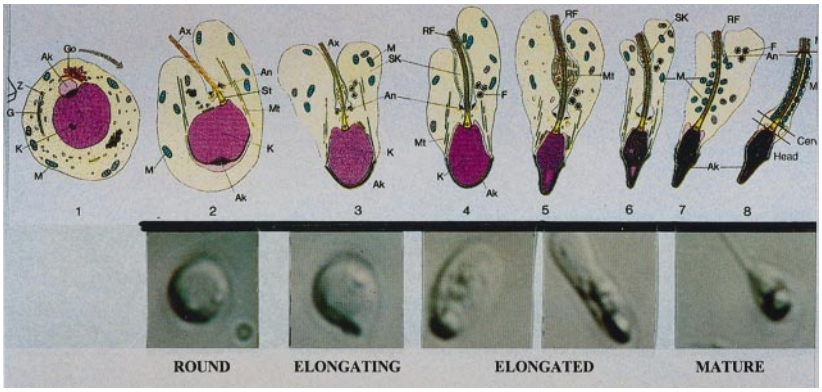

Figure 1. Spermiogenesis: differentiation of a spermatid into a spermatozoon. (**A**) Classification after staining techniques under light and electron microscopy. (**B**) Classification of spermatids in a wet preparation under Hoffman modulation contrast.

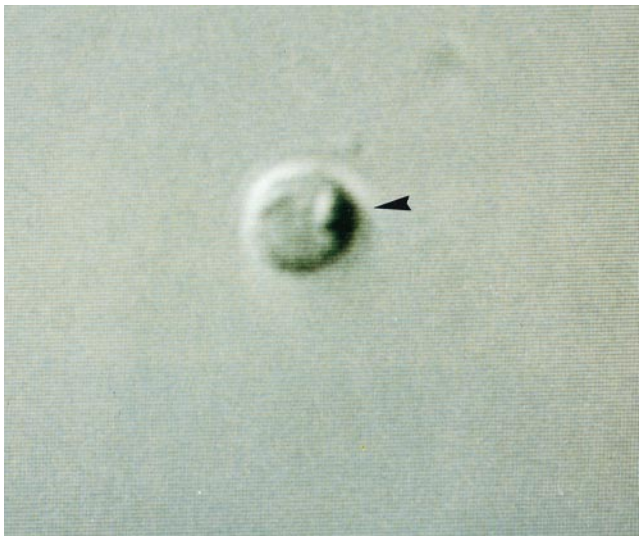

Figure 2. Round spermatid from testicular tissue exibiting a developing acrosomal structure as a bright spot on one side of the cell.

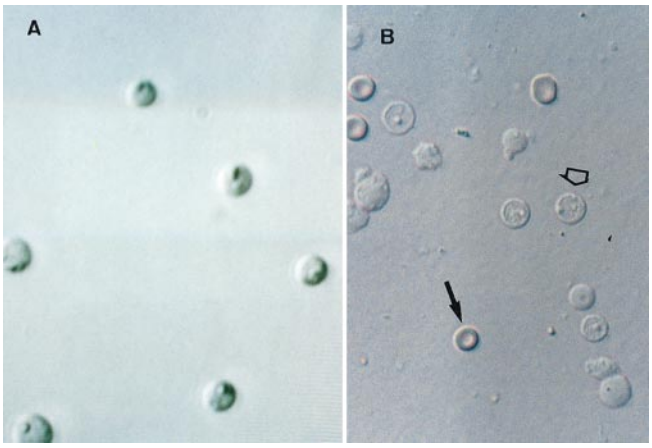

Figure 3. Identification of cells in a wet preparation from testicular tissue under Hoffman modulation contrast at ×400 magnification. (**A**) Round spermatid with visible acrosome; (**B**) erythrocytes (Φ), flat and transparent shaped Sertoli cells () with central nuclei containing the nucleolus.

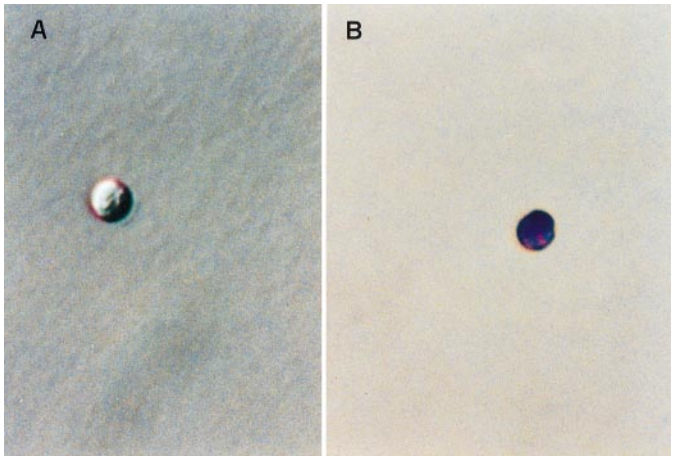

Figure 4. Round spermatid isolated from testicular tissue. (**A**) Observation of a wet preparation under Hoffman Modulation Contrast at ×600 magnification. (**B**) Testsimplet staining of the same round spermatids exhibiting a magenta-stained acrosome with adjacent nucleus and light coloured halo.

of cells present in the ejaculate or in the testicular tissue, is more difficult. In general, round spermatids can be distinguished from other round cells, such as spermatogonia, spermatocytes and polymorphonuclear lymphocytes, by their smaller size. The diameter of round spermatids (6.5–8 μm) is similar to that of erythrocytes (7.2 μm) and also to small lymphocytes.

Rotating and observing the round spermatids, we tried to ascertain the presence of a developing acrosomal structure as a bright spot or a small protusion on one side of the cell adjacent to the spermatid nucleus (Figure 2). This helpful criteria for selection of round spermatids is not observed in such cells immediately after the second meiotic division. Therefore round spermatids in the Golgi phase are extremely difficult to identify and can be confused easily with small lymphocytes as far as they have a similar size and shape.

Concerning the Sertoli cell nucleus, it is very unusual to confuse this cell with a round spermatid. In fact, unlike round spermatids which are three dimensionally round cells, the Sertoli cell nucleus is very flat, transparent and with a prominent centrally or adjacent localized nucleolus. Confusion may result from the fact that the prominent nucleolus of the Sertoli cell nucleus can be confused with the crescent-shaped dense

material or the developing acrosome bordering the plasma membrane of the round spermatid (Figure 3).

As mentioned by Yamanaka *et al.* (1997), it is clear that the methodology for round spermatid isolation is a major factor affecting the outcome of spermatid injection and that such morphological criteria are highly susceptible to intra- and extra-observer variation.

An embryologist should feel more confident with the research of haploid cells by standard microscopic technique and know this discriminative criteria before working with spermatid cells. Therefore, in order to ascertain that the identification of round spermatids based on morphological characteristics in the wet preparation are valid, we need tools to confirm our ability to select round spermatids.

After isolation and identification of one round cell based on morphometric parameters under an inverted microscope (Angelopoulos *et al*., 1997) or after using computer-assisted image analysis (Yamanaka *et al.*, 1997) isolated cells can be verified as round spermatids. Of the round cells chosen by this way, 80% were confirmed as being haploid cells after processing them with fluorescence in-situ hybridization technique (Angelopoulos *et al*., 1997) or by transmission electronic microscopy or confocal scanning laser microscopy (Yamanaka *et al.*, 1997).

Other techniques are also possible; Mendoza and Tesarik (1996) evaluated the occurrence of round spermatids in the ejaculate of men suffering from non-obstructive azoospermia, by three different staining methods: Papanicolaou, fluorescein-labelled *Pisum sativum* agglutinin binding and antiacrosin antiserum immunolabelling.

However, these staining techniques are expensive and are not always available in the majority of IVF laboratories. Therefore a more simple method for confirming round spermatid selection by qualitative criteria was recently proposed by Angelopoulos *et al.* (1997). A rapid correlation of the morphological features of a single stained cell by aspirating a cell with a micropipette was possible after staining it on a prestained slide (Testsimplets; Boehringer Mannheim, Mannheim, Germany) (Figure 4). For this method, cells <7.5 μm should be selected, since larger cells are likely to be secondary spermatocytes or white blood cells. These simple techniques offer the embryologist a direct control method of his selection procedure.

Quality of spermatids

The morphological identification and isolation does not however reveal anything about the viability or the genomical normality of the round spermatid. However, without staining or destroying the cell, it is currently impossible to distinguish the live from the dead cells and to differentiate genomically normal from abnormal spermatids. Actually, the only valuable parameter to evaluate the viability of the round spermatid, is its survival after aspiration in the micropipette; when no lysis is observed, the cells are considered to be alive.

Severe testicular damage may have consequences on the quality of the generated round spermatids. The lack of selection methods can explain the low reproductive capacity when spermatids were recovered from patients showing very severe defects of spermatogenesis. It is possible that such spermatids may not be wholly identical to those obtained from testes with partial failure of spermiogenesis or with focal complete spermatogenesis. Histopathological examination of testis exhibiting a complete failure of spermiogenesis showed an extremely low percentage of tubules ($<10\%$) containing a few round spermatids with picnotic nuclei, in degenerated or atrophic phase and also some desquamated spermatids.

After ICSI with mature spermatozoa, oocyte activation is an essential step and spermatozoa activated the majority of the oocytes by releasing a cytosolic oocyte-activating factor, oscillin (Swann, 1990; Parrington *et al.*, 1996). Souza *et al.* (1996) and Yamanaka *et al.* (1997) showed that in human round spermatids, the activity of the activating oocyte factor, developing between the secondary spermatocyte and the round spermatid stage, was sufficient to induce the Ca^{2+} oscillation mechanism into the oocyte. They observed an oscillation phenomena after the injection of round spermatid nuclei with no electrical or mechanical ooplasmic prestimulation. We (Vanderzwalmen *et al.*, 1997), have suggested that the cytoplasm of the round spermatid from severe pathological cases is not mature enough in comparison with its nucleus and it is possible that there is deficiency in oocyte activating factor (OAF) with as a consequence a lack of Ca^{2+} oscillation response. Our previous suggestion is recently confirmed by Tesarik who observed no Ca^{2+} oscillation mechanism after injecting spermatids of poor quality resulting from patients with extremely severe spermatogenesis dysfunction (personal communication).

In a recent report, (Vanderzwalmen, 1997) we showed that fertilization and embryonic development are affected by the type of male germ cells (round, elongating, elongated spermatids) found in biopsies and injected into oocytes. Spermatids in the elongation step should be the more suitable for injection, since our results show significantly higher fertilization, embryonic development and pregnancy rates after injection of elongating and elongated spermatids. The same parallelism can be made according to the maturity of the round spermatid. In fact, in a recent paper, Sofikitis *et al.* (1997) showed that the fertilizing and embryonic development capacities of rabbit testicular round spermatids is lower when round spermatids with proacrosomal granules (earlier stages of spermiogenesis) were used as compared with round cells exhibiting coalescence of the proacrosomal granules. To our knowledge, pregnancies and births of healthy babies were observed in patients where some spermatozoa had been observed before, either in the ejaculate or the testis (Tesarik *et al.*, 1995; Vanderzwalmen *et al.*, 1997). In the case of round spermatids retrieved from patients with a history of spermatogenetic block at the round spermatid stage, the pregnancies obtained resulted in abortion, increasing the suspicions of a genetic factor (Hannay *et al.*, 1995; Amer *et al.*, 1997).

At the present state of this approach, the spermatid indication seems more favourable for azoospermic men who have proved their capacity to produce albeit only a few spermatozoa previously. However, in cases of severe patho-

logical cases, the usefulness of using round spermatids remains to be proven on a large scale. Therefore, these data suggest spermatid injection should be considered as an option in case of unexpected absence of spermatozoa. In the case of patients with complete block of spermiogenesis, actually, round spermatids should not be proposed to the couple as a therapy.

Couples entering this treatment should be advised about the safety and the extremely low efficacy of the procedure if round spermatids are used. Moreover, the risk of genetic transmission of Y chromosome deletions and of genomic imprinting anomalities should not be overlooked and care should be taken to avoid dramatic consequences of such pathologies.

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ROSI, instructions for use: 1997 update

Jan Tesarik[1,4], Ermanno Greco[2], and Carmen Mendoza[3]

[1]Laboratoire d'Eylau, 55 Rue Saint-Didier, 75116 Paris, France, [2]Center of Reproductive Medicine, European Hospital, Via Portuense 700, 00149 Rome, Italy, and [3]Institute of Biotechnology, University of Granada Faculty of Sciences, Campus Universitario 'Fuentenueva', 18071 Granada, Spain

[4]To whom correspondence should be addressed

Since the first report on the birth of a normal child resulting from a human oocyte fertilized with a round spermatid (Tesarik *et al.*, 1995), and the first full description of the human round

spermatid injection (ROSI) and elongated spermatid injection (ELSI) techniques (Tesarik and Mendoza, 1996), there have been many myths relating to the clinical use of these methods. One of these myths was characterized by the non-critical acceptance of ROSI as an established clinical treatment method to manage infertility due to spermiogenesis arrest without taking into account the underlying male pathology and, frequently, without the acquisition of adequate knowledge and technical skill. Logically, this approach led to frequent failures which, in turn, tended to discredit ROSI in the eyes of its further potential users (Silber and Johnson, 1998).

Here we delineate the main findings concerning ROSI that were achieved in 1997, discuss their relevance for the clinical application of ROSI and try to put ROSI in its correct place in the scale of assisted reproduction techniques that are available at the moment.

Recognition of spermatids – *condicio sine qua non*

All patients considered as candidates for ROSI treatment should have undergone a previous examination involving the search for spermatids in appropriately stained preparations. This can avoid situations in which the biologist is confronted with the problem of spermatid recognition for the first time only on the day of assisted reproduction treatment when he must entirely rely on his capacity to identify these cells in a fresh native sample. Relying on the probability that some spermatozoa can anyway be recovered in case of emergency by augmenting the volume of testicular tissue sampled by biopsy would be rather too optimistic (see below).

If a diagnostic testicular biopsy is being performed, it is advisable not to use the totality of the tissue obtained for histology but to reserve part of it for smear preparations that can be evaluated by more specific methods for spermatid identification. Enzymatic treatment of testicular biopsy samples (e.g. Crabbé *et al.*, 1997) can help dissociate testicular cells and thus facilitate the preparation of smears. Smears can then be evaluated by cytochemistry and immunocytochemistry using markers that selectively or specifically recognize the germ cell line (Mendoza and Tesarik, 1996; Mendoza *et al*., 1996), by fluorescent in-situ hybridization (FISH) to recognize haploid spermatids from higher-ploidy earlier germ cells and somatic cells (Mendoza *et al*., 1996; Angelopoulos *et al*., 1997) or by a combination of both (Mendoza *et al.*, 1996). These techniques also provide useful information about the quantitative ratio between round spermatids and small lymphocytes, the only cell type which can cause problems of spermatid recognition in fresh samples. This information can be used to predict the risk of erroneous injection of a non-spermatid cell into the oocyte.

If the patient has already undergone previous diagnostic testicular biopsy and there is no other indication for its repetition, the search for spermatids can be performed in the ejaculate. When spermatids are present in the ejaculate, there are certain to be spermatids, and perhaps of better quality, also in the testis. On the other hand, in a group of 66 patients with non-obstructive azoospermia, we found testicular spermatids only in nine patients (14%) in whom spermatids were not detected in semen (Tesarik *et al.*, 1998). In certain cases, in which relatively active persisting spermatogenesis is confined to only a small testicular region, spermatids can be found in the ejaculate although they fail to be recovered by testicular biopsy (Tesarik *et al*., 1998). The examination of the ejaculate thus appears to be the optimal way of assessing spermatid production in the testis while sparing the testicular tissue for eventual future therapeutic biopsies.

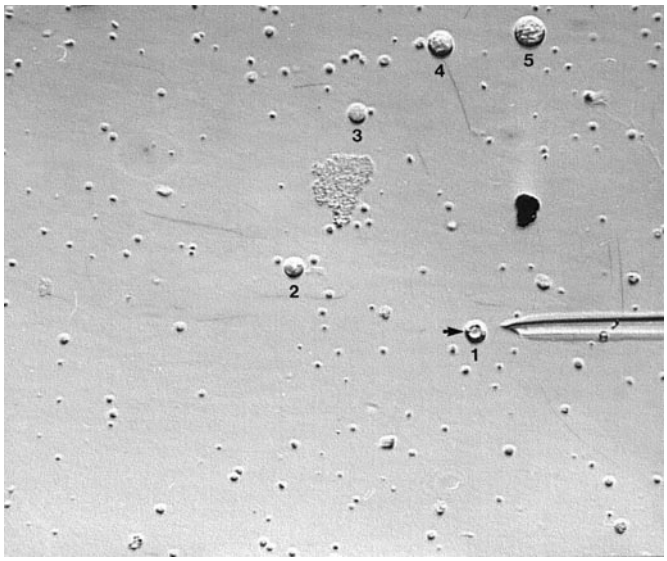

Figure 1. Native preparation of a spermatid-enriched fraction (Tesarik and Mendoza, 1996) of a fresh human ejaculate observed in an inverted microscope equipped with Hoffman optics. The size of the microinjection needle to the right of the image (7 μm, external diameter) can serve as a size standard. The cell next to the needle tip (1) is a round spermatid with a clearly visible nucleus, surrounded by a continuous zone of cytoplasm. An acrosomal granule appears as a bright spot adjacent to the nucleus (arrow). Another similar-sized cell (2) is also likely to be a round spermatid, but the fuzzy nuclear outline makes the identification uncertain and suggests cell degeneration accompanied by nuclear lysis. The cell of the similar size in which no cytoplasmic zone can be observed around the nucleus (3) is a small lymphocyte. Other nucleated cells (4, 5) are clearly distinguishable from round spermatids because of their larger size. Most leukocytes, together with the rare secondary spermatocytes, fall into this category. All the remaining round bodies seen in this picture are anuclear cell fragments and cellular debris. Part of this picture has been published previously (Tesarik, 1997b) and is reproduced with the permission of Baillière Tindall.

Identification of round spermatids in fresh samples is not as easy as in stained preparations. Notwithstanding, it should not represent a serious problem to a qualified and appropriately trained worker. The main distinctive signs for round spermatid recognition in fresh samples were described in detail previously (Tesarik and Mendoza, 1996; Sousa *et al.*, 1998). Briefly, the main characteristics of living round spermatids are the size (7–8 μm), the presence of a clearly visible nucleus, surrounded by a continuous zone of cytoplasm, and the acrosomal granule appearing in unstained living round spermatids as a spot of different optical density (bright or dark according to the actual setting of the optical system) adjacent to the nucleus (Figure 1). The latter, however, is only of relative value because very

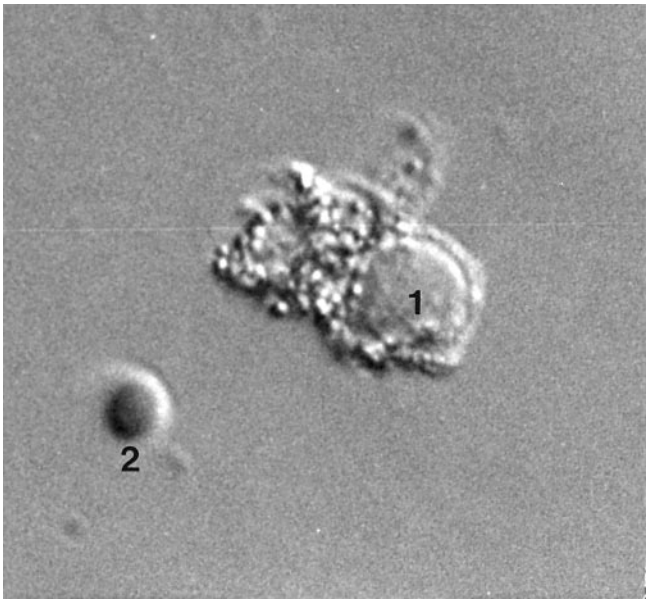

Figure 2. Partly disintegrated Sertoli cell showing a round nucleus (1) and characteristic granulated cytoplasm. The Sertoli cell nucleus is much larger than the round spermatids which would be similar to that of the adjacent red blood cell (2).

early round spermatids (Golgi phase of acrosomal development) have not yet developed an acrosomal granule, whereas the acrosome can assume a cap-like form (poorly visible in unstained cells) in more advanced stages of spermatid development (Tesarik, 1997a). The period during which the acrosomal vesicle is clearly visible in round spermatids is thus limited. On the other hand, the presence of the nucleus distinguishes spermatids from most round bodies in the preparation, most of which are cell fragments or dead cells in which the nucleus is difficult to visualize, probably as a sequela of cell organelle lysis (Figure 1).

The size of round spermatids can be evaluated by comparing them with the known size of the microinjection needle (Figure 1) or with that of red blood cells that are usually abundant in testicular biopsy samples. By simply excluding all cells whose size does not correspond to spermatids, confusion with most leukocytes (except small lymphocytes that do not show, however, a continuous rim of cytoplasm around the nucleus) is minimized. By respecting the size criterion, confusion is also impossible between spermatids and Sertoli cell nuclei whose injection into oocytes was reported by Silber and Johnson (1998). In fact, Sertoli cell nuclei are considerably larger than round spermatids and, of course, remnants of Sertoli cell cytoplasm that may still be present are morphologically quite different from the cytoplasm of round spermatids (Figure 2). On the other hand, we agree with Silber and Johnson (1998) in that two pronuclei can appear in some oocytes after injection of many non-spermatid cell types or their nuclei. Some of the injected oocytes can subsequently cleave although the normal timing of cleavage divisions and the occurrence of equal-sized, mononuclear blastomeres is very rare in these cases. Anyway, the development of two pronuclei and the observation of early cleavage divisions cannot be interpreted as a sign confirming that the cell previously injected into the oocyte was actually a spermatid.

Potential clinical usefulness of ROSI

The proportion of patients in whom only round spermatids but no later forms of spermiogenesis can be obtained for assisted reproduction, and who may thus represent a target group for ROSI, is still a matter of debate. Some workers fail to find round spermatids when later forms are absent (e.g. Silber and Johnson, 1998). However, most of those studies were published by the same group of workers and used standard histological preparations of testicular tissue in which tiny numbers of round spermatids can be missed. The identification of spermatids in such cases would be better done by analysing smear preparations with the use of specific immunocytochemical techniques to detect spermatid acrosomal materials or by FISH to detect the haploid status of spermatid nuclei (Mendoza *et al*., 1996).

In our experience, round spermatids can be detected in many patients in whom late spermatids or spermatozoa fail to be found (Amer *et al.*, 1997; Tesarik *et al.*, 1998). Similar findings were also reported by other, independently working groups (e.g. Antinori *et al*., 1997; Kahraman *et al*., 1997; Lee *et al*., 1997; Vanderzwalmen *et al.*, 1997; Yamanaka *et al*., 1997). Spermatogenic arrest at the round spermatid stage can also be induced experimentally in animal models (reviewed in Tesarik *et al.*, 1998). These experimental data suggest a genetic or non-genetic abnormalities of Sertoli cells, leading to premature spermatid detachment, as a possible pathogenetic mechanism underlying the complete spermiogenesis failure in humans. The prematurely released spermatids cannot continue their differentiation due to the lack of Sertoli cell support and are finally destroyed by apoptosis.

However, it is currently impossible to determine the relative incidence of the situation in which only round spermatids but no elongated spermatids or spermatozoa are produced in the testis among patients with non-obstructive azoospermia because results from different centres are dependent on the treatment policy of each centre. Centres that are ready to apply ROSI are likely to stop testicular tissue sampling as soon as a sufficient number of spermatids is recovered. It cannot be excluded that, in some cases, spermatozoa would be found by sampling more testicular tissue. Obviously, we have to choose here between two evils, that of massive destruction of testicular tissues by continuing tissue sampling without any guarantee that spermatozoa will eventually be found, on the one hand, and that of the still relatively low clinical efficacy and the potential additional risk factors (Tesarik, 1996; Tesarik *et al*., 1998) associated with ROSI.

It is clear that the expected fertilization rate with spermatids is significantly lower as compared with spermatozoa (Fishel *et al.*, 1997). On the other hand, it is important to warn against the illusion of testicular biopsy harmlessness which is tending to overwhelm in the world of assisted reproduction. In fact, transient adverse physiological effects, but also more serious complications leading to permanent devascularization of the testis, can be produced by testicular sperm extraction (Schlegel and Su, 1997), and the risk is increased when the intervention involves multiple incisions of the tunica albuginea (Jarow, 1991). Extensive sampling of testicular tissue in search of spermatozoa, while an adequate number of round spermatids

is already available, can thus be, in certain situations, in contradiction with the basic medical principle of *primum non nocere*. With the current state of the art, choosing ROSI as alternative to intracytoplasmic sperm injection (ICSI) with testicular spermatozoa would mean the reduction of the expected implantation rate by 50% (Vanderzwalmen *et al*., 1997), but would reduce the risk of irreparable testicular damage and spare the testicular tissue for eventual future attempts that can be performed when the ongoing research efforts will hopefully result in the improvement of ROSI success rates. The final choice must depend on a thorough evaluation of the couple's situation (age, social background, psychological condition, previous testicular biopsies, associated female pathology, etc.) in each case.

Spermatid quality – a major problem of ROSI

Even though the recognition of round spermatids is not an insurmountable problem, it is far from certain that any round spermatid can succesfully fertilize the oocyte to give rise to a viable embryo. Our recent data (Amer *et al*., 1997) show that this is particularly true for patients whose spermiogenesis is blocked at the round spermatid stage, the condition referred to as complete spermiogenesis failure. These data were corroborated by an independent group of workers (Vanderzwalmen *et al.*, 1997). Insufficiency of factors responsible for oocyte activation in spermatids originating from patients with complete spermiogenesis failure appears to be at least partly responsible for the poor results of ROSI in this category of patients (Tesarik *et al*., 1998). This is in contrast with spermatids from men with continuing spermiogenesis that can induce calcium signalling events similar to those induced by mature spermatozoa when injected into oocytes (Sousa *et al.*, 1996). Many patients with complete spermiogenesis failure also have an unusually high incidence of spermatids carrying DNA damage attributable to apoptosis (J.Tesarik, unpublished).

The oocyte-activating ability of spermatids can be evaluated by injecting them into mouse oocytes, using the technique previously described for assessing mature human spermatozoa (Rybouchkin *et al.*, 1995). Kits for the assessment of apoptosis are commercially available and can also be used for evaluation of spermatids before ROSI attempts. We believe that the application of these additional diagnostic methods will make it possible to distinguish cases in which the ROSI chance of success is acceptable from those in which the likelihood of failure is high. In the immediate future, this can enable the selection of patients for treatment according to these criteria. The possibility of addressing the low-chance group in a longer perspective should stimulate appropriate research efforts.

The DNA damage and the destruction of oocyte-activating factor(s) may be related, both resulting from germ cell apoptosis. Because neither of these abnormalities usually applies to the total population of spermatids recoverable from the patient (Tesarik *et al.*, 1998; J.Tesarik, unpublished), the problem of developmental failure after ROSI may be overcome by the application of methods for the selection of those few round spermatids which retain intact the relevant biological systems. Spermatid in-vitro culture is one possible approach to the solution of this problem. Our preliminary observations show that spermatids from some patients with complete spermiogenesis failure can resume spermiogenesis in culture. Such cells can thus be easily distinguished from those spermatids that remain blocked and do not show any morphogenetic progression at the end of the culture period. Moreover, ROSI can thus be replaced with ELSI which is technically much easier to perform (Tesarik and Mendoza, 1996). We are currently testing the possibility that only cells without apoptotic DNA damage are selected by this means. The oocyte-activating ability of cultured spermatids is also under investigation.

Practical recommendations

The principal message from the ROSI clinical trials reported so far, can be restated as follows: ROSI can give acceptable success rates in men in whom the production of at least a tiny number of spermatozoa has been previously detected. Regardless of whether or not spermatozoa could be retrieved by increasing the volume of testicular tissue sampled, this means that such cases are presently the most suitable ones for ROSI treatment (Tesarik, 1997b). It is important to remember this when extensive sampling of testicular tissue is not acceptable for various reasons. To be prepared for such situations, a search for spermatids, using ejaculated semen samples and appropriate cytological staining methods, should be done in all cases of non-obstructive azoospermia before the inclusion of the patient in an ICSI/ROSI programme.

For those patients in whom spermatozoa have never been detected, it should be made clear to them that their situation is different from most of the patients for whom successful ROSI treatment cycles have been reported in the literature and that their expected chance of success is considerably lower. In addition to spermatid identification, the examination of spermatids for apoptotic DNA damage and, eventually, for their oocyte-activating ability should be recommended in those cases. Finally, these patients should be informed about the ongoing research efforts in the field, which are likely to result in an improvement of ROSI efficacy in the near future.

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