

# DEBATE

## Are spermatid injections of any clinical value?

### ROSNI and ROSI revisited

Sherman J.Silber<sup>1,3</sup> and Larry Johnson<sup>2</sup>

<sup>1</sup>Infertility Center of St Louis, St Luke's Hospital, 224 South Woods Mill Road, St Louis, MO 63017 and

<sup>2</sup>Department of Veterinary Medicine, Texas A & M University, College Station, TX 77843-4458, USA

<sup>3</sup>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

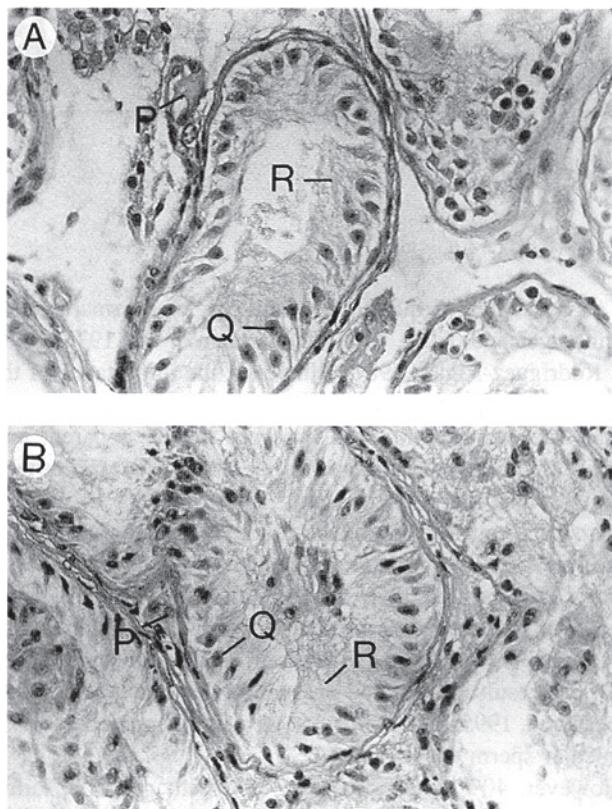
the early studies of quantitative analysis of spermatogenesis (Steinberger and Tjioe, 1968; Zuckerman *et al.*, 1978; Silber and Rodriguez-Rigau, 1981). However, the importance of this finding for helping azoospermic men with testicular failure have their own genetic child, was not readily apparent until 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.



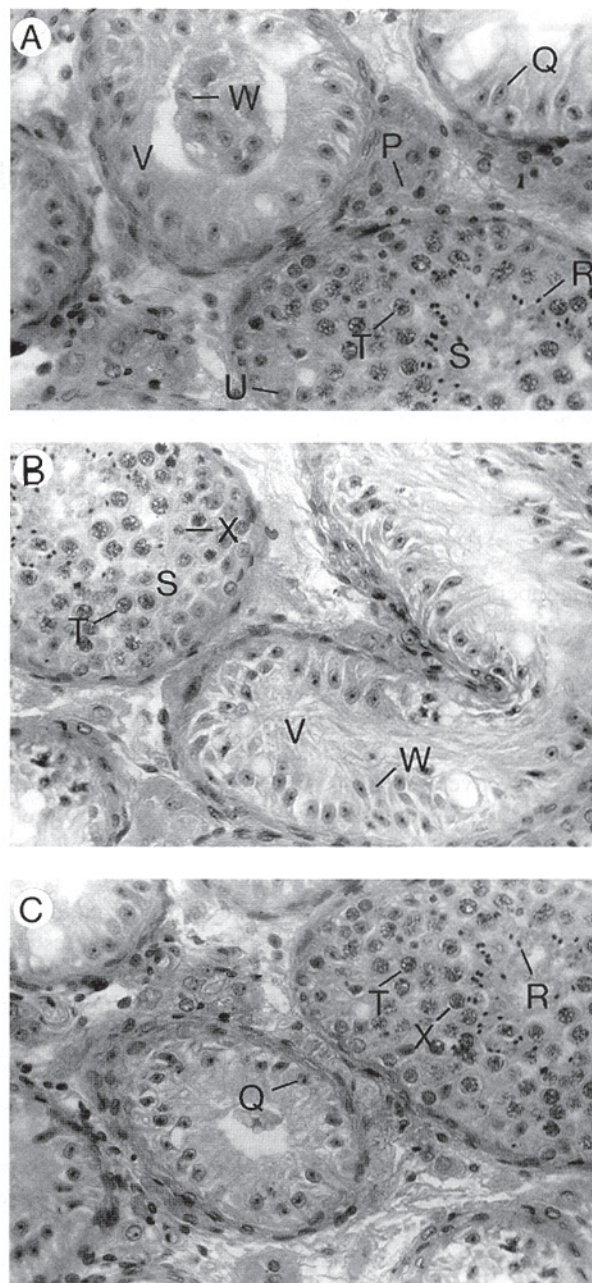


**Figure 1.** (A and B) Histological sections of testes with haematoxylin and eosin stain showing Sertoli cell only with no germ cell elements. P = Leydig cell cluster, Q = typical Sertoli cell nucleus with prominent nucleolus, R = amoeboid cytoplasm of Sertoli cell with no distinct boundary.

**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

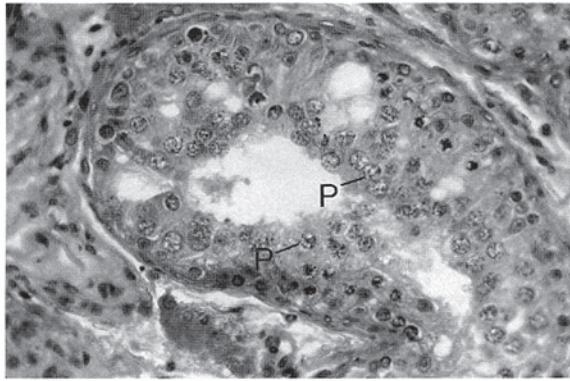


**Figure 2.** (A–C) Histological sections of testes with haematoxylin and eosin stain showing Sertoli cell only; in these cases, the empty tubules are next to a single tubule which exhibits normal spermatogenesis. The Sertoli cell-only tubules (the predominant pattern in each of these three patients) 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.

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 spermatogenesis.





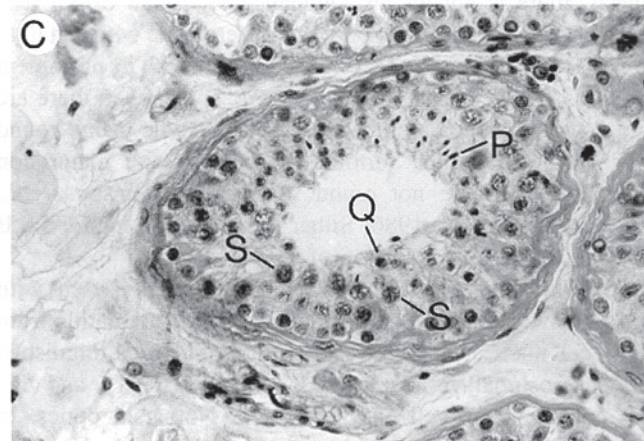
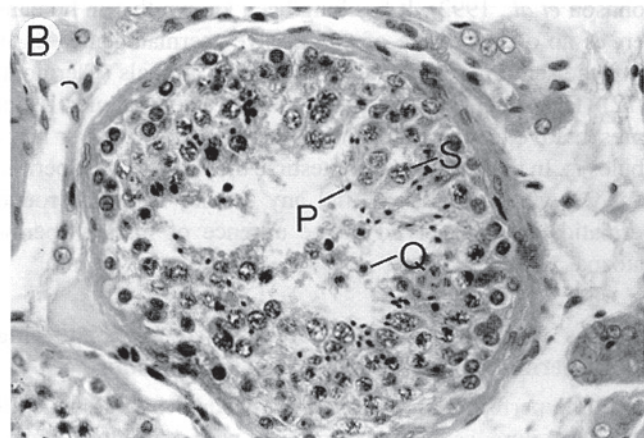
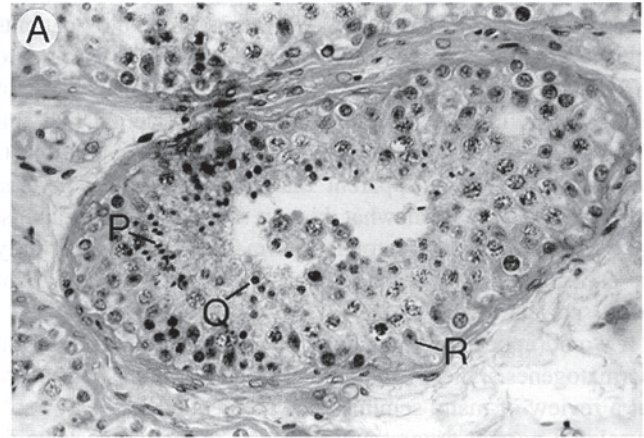
**Figure 3.** Histological section of testes with haematoxylin and eosin stain demonstrating maturation arrest. There is a full progression of spermatogonia, primary spermatocytes, but no secondary spermatocytes or round spermatids can be seen. P = pachytene spermatocyte.

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 prominent 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 postmeiotic. 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

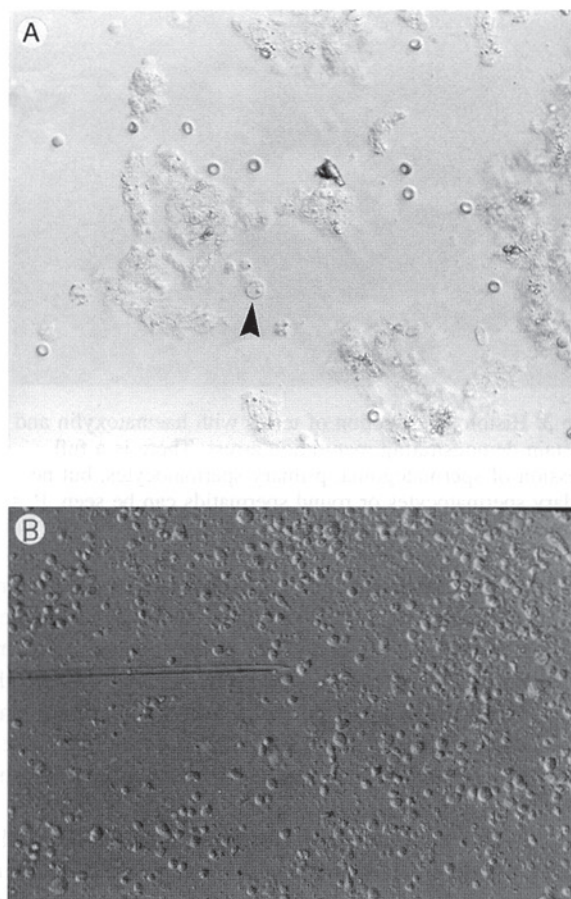
When one performs a TESE–ICSI procedure in patients with testicular failure, as well as in patients with normal spermatogenesis, there is always an abundance of ‘round cells’. It is very difficult with Hoffman optics to differentiate with certainty a round spermatid from a Sertoli cell nucleus with its 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 ham-



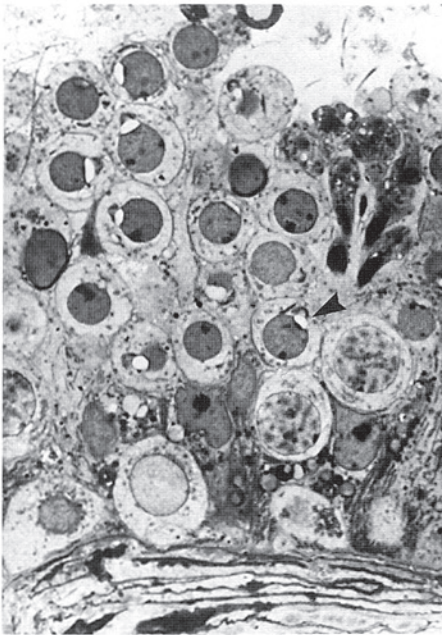
**Figure 5.** (A) Morselated testicular tissue in patient with Sertoli cell only seen under Hoffman optics in a microdroplet diluted for testicular sperm extraction–intracytoplasmic sperm injection. The arrow points to one of many prominent Sertoli cell nuclei with its nucleolus, which is often mistaken 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.

sters, 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.

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.

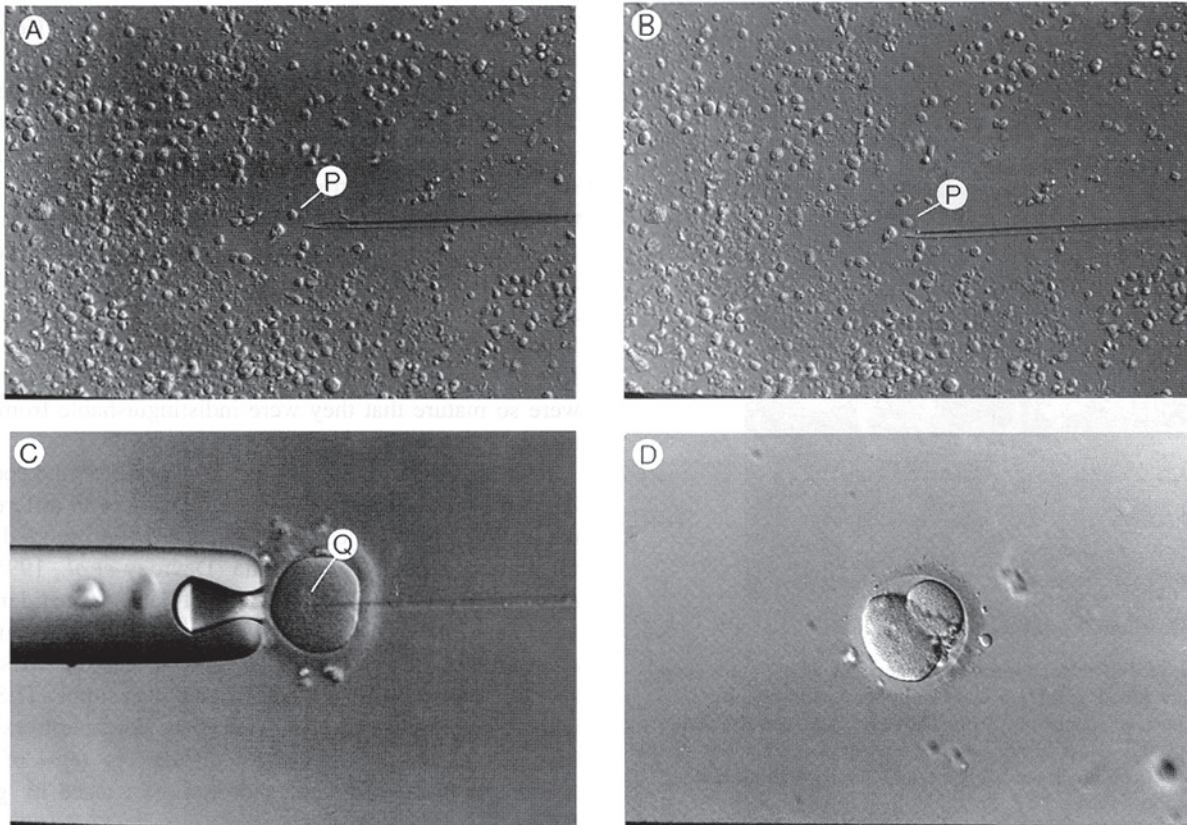
Because of the confusion, we elected, in patients undergoing TESE in whom absolutely no spermatozoa were recoverable after hours of exhaustive searching, to inject Sertoli cell nuclei into the oocytes (which would otherwise go to waste anyway). We were able to obtain non-specific 'fertilization' with 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.

## References

- Antinori, S., Versaci, C., Dani, G. *et al.* (1997) Fertilization with human testicular spermatids: four successful pregnancies. *Hum. Reprod.*, **12**, 286–291.
- Araki, S., Motoyama, M., Yoshida, A. *et al.* (1997) Intracytoplasmic injection with later spermatids: a successful procedure in achieving childbirth for couples in which the male partner suffers from azoospermia due to deficient spermatogenesis. *Fertil. Steril.*, **67**, 559–561.
- Clermont, Y. (1972) Kinetics of spermatogenesis in mammals; seminiferous epithelium cycles and spermatogonial renewal. *Physiol. Rev.*, **52**, 198–236.
- Devroey, P., Liu, J., Nagy, Z. *et al.* (1995) Pregnancies after testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) in non-obstructive azoospermia. *Hum. Reprod.*, **10**, 1457–1460.
- Edwards, R.G., Tarin, J.J., Dean, N. *et al.* (1994) Are spermatid injections into human oocytes now mandatory? *Hum. Reprod.*, **9**, 2217–2219.
- Fishel, S., Green, S., Bishop, M. *et al.* (1995) Pregnancy after intracytoplasmic injection of spermatid. *Lancet*, **345**, 641–642.
- Holstein, A.F. and Roosen-Runge, E.C. (eds) (1981) *Atlas of Human Spermatogenesis*. Grosse Verlag, Berlin.
- Johnson, L. (1986) Review article. Spermatogenesis and aging in the human. *J. Androl.*, **7**, 331–354.
- Johnson, L., Petty, C.S. and Neaves, W.B. (1981) A new approach to quantification of spermatogenesis and its application to germinal cell attrition during human spermiogenesis. *Biol. Reprod.*, **25**, 217–226.
- Johnson, L., Chaturedi, P.K. and Williams, J.D. (1992) Missing generations of spermatocytes and spermatids in seminiferous epithelium contribute to low efficiency of spermatogenesis in human. *Biol. Reprod.*, **47**, 1091–1098.
- Kimura, Y. and Yanagimachi, R. (1995) Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development*, **121**, 2397–2405.
- Mendoza, C. and Tesarik, J. (1996) The occurrence and identification of round spermatids in the ejaculate of men with nonobstructive azoospermia. *Fertil. Steril.*, **66**, 826–829.
- Mendoza, C., Benkhalifa, M., Cohen-Bacrie, P. *et al.* (1996) Combined use of proacrosin immunocytochemistry and autosomal DNA *in situ* hybridisation for evaluation of human ejaculated germ cells. *Zygote*, **4**, 279–283.
- Ogura, A. and Yanagimachi, R. (1993) Round spermatid nuclei injected into hamster oocytes form pronuclei and participate in syngomy. *Biol. Reprod.*, **48**, 219–225.
- Ogura, A., Yanagimachi, R. and Usui, N. (1993) Behavior of hamster and mouse round spermatid nuclei incorporated into mature oocytes by electrofusion. *Zygote*, **1**, 1–8.
- Ogura, A., Matsuda, J. and Yanagimachi, R. (1994) Birth of normal young after electrofusion of mouse oocytes with round spermatids. *Proc. Natl. Acad. Sci. USA*, **91**, 7460–7562.
- Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A. (1992) Pregnancies after intracytoplasmic injection of single spermatozoan into an oocyte. *Lancet*, **340**, 17–18.
- Silber, S.J. and Rodriguez-Rigau, L. (1981) Quantitative analysis of testicle biopsy: determination of partial obstruction and prediction of sperm count after surgery for obstruction. *Fertil. Steril.*, **36**, 480–485.



- Silber, S.J., Van Steirteghem, A.C. and Devroey, P. (1995a) Sertoli cell only revisited. *Hum. Reprod.*, **10**, 1031–1032.
- Silber, S.J., Van Steirteghem, A.C., Liu, J. *et al.* (1995b) High fertilization and pregnancy rate after intracytoplasmic sperm injection with sperm obtained from testicle biopsy. *Hum. Reprod.*, **10**, 148–152.
- Silber, S.J., Nagy, Z., Liu, J. *et al.* (1995c) The use of epididymal and testicular spermatozoa for intracytoplasmic sperm injection: the genetic implications for male infertility. *Hum. Reprod.*, **10**, 2031–2043.
- Silber, S.J., Van Steirteghem, A.C., Nagy, Z. *et al.* (1996) Normal pregnancies resulting from testicular sperm extraction and intracytoplasmic sperm injection for azoospermia due to maturation arrest. *Fertil. Steril.*, **66**, 110–117.
- Sofikitis, N.V., Miyagawa, I., Agapitos, E. *et al.* (1994) Reproductive capacity of the nucleus of the male gamete after completion of meiosis. *J. Assist. Reprod. Genet.*, **11**, 335–341.
- Sofikitis, N., Toda, T., Miyagawa, I. *et al.* (1996) Beneficial effects of electrical stimulation before round spermatid nuclei injections into rabbit oocytes on fertilization and subsequent embryonic development. *Fertil. Steril.*, **65**, 176–185.
- Steinberger, E. and Tjioe, D.Y. (1968) A method for quantitative analysis of human seminiferous epithelium. *Fertil. Steril.*, **19**, 960–970.
- Tesarik, J. and Mendoza, C. (1996) Spermatid injection into human oocytes. I. Laboratory techniques and special features of zygote development. *Hum. Reprod.*, **11**, 772–779.
- Tesarik, J., Mendoza, C. and Testart, J. (1995) Viable embryos from injection of round spermatids into oocytes (Letter). *N. Engl. J. Med.*, **333**, 525.
- Tesarik, J., Rolet, F., Brami, C. *et al.* (1996) Spermatid injection into human oocytes. II. Clinical application in the treatment of infertility due to non-obstructive azoospermia. *Hum. Reprod.*, **11**, 780–783.
- Vanderzwalmen, P., Lejeune, B., Nijs, M. *et al.* (1995) Fertilization of an oocyte microinseminated with a spermatid in an *in vitro* fertilization programme. *Hum. Reprod.*, **10**, 502–503.
- Van Steirteghem, A.C., Nagy, Z., Joris, H. *et al.* (1993) High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum. Reprod.*, **8**, 1061–1066.
- Verheyen, G., Crabbe, E., Joris, H. and Van Steirteghem, A. (1998) Simple and reliable identification of the human round spermatid by inverted phase-contrast microscopy: Does the real target group exist? *Hum. Reprod.*, **13**, in press.
- Zuckerman, Z., Rodriguez-Rigau, L.J., Weiss, D.B. *et al.* (1978) Quantitative analysis of the seminiferous epithelium in human testicle biopsies and the relation of spermatogenesis to sperm density. *Fertil. Steril.*, **30**, 448–455.

## 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<sup>1,4</sup>, M.Nijs<sup>1</sup>, R.Schoysman<sup>1</sup>, G.Bertin<sup>1</sup>, B.Lejeune<sup>1</sup>, B.Vandamme<sup>1</sup>, S.Kahraman<sup>3</sup> and H.Zech<sup>2</sup>

<sup>1</sup>Schoysman Infertility Management Foundation, Vaartsraat 42, 1800 Vilvoorde, Belgium, <sup>2</sup>Institut für In Vitro Fertilisierung und Embryo Transfer, Bregenz, Austria, and <sup>3</sup>IVF Unit, Sevgi Hospital, Ankara, Turkey

<sup>4</sup>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 pregnancies 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; Antinori *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; Antinori *et 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 controversial 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