

Micro- and macro-consequences of ooplasmic injections of early haploid male gametes

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This review refers to the evolution of ooplasmic injections of round spermatid nuclei (ROSI) or intact round spermatids (ROSI). Conclusions from their preliminary application in the hamster, rabbit, mouse and human are discussed. Criteria for identification of round spermatids and guidelines/quality control for application of ROSNI/ROSI techniques are emphasized. Although all the animal offspring and the human newborns delivered after ROSNI/ROSI are

healthy, additional research efforts are necessary to confirm the safety of these procedures and improve their outcome.

Key words: ROSNI/spermatid/spermatogenesis

Spermatogenesis/spermiogenesis

Spermatogenesis is the sequence of cytological events that result in the formation of mature spermatozoa from precursor cells. In most of the mammals, this process takes place within the seminiferous tubule throughout the reproductive life span of the male. The process of spermatogenesis involves a continuous replication of precursor stem cells to produce cells that can undergo successfully the subsequent changes. A reduction of the number of chromosomes to the haploid state occurs in spermatogenesis. The diploid state is restored on syngamy.

There are three fascinating events that together constitute spermatogenesis: (i) stem cell renewal by the process of mitosis, (ii) reduction of chromosomal number by meiosis, and (iii) the transformation of a conventional cell into the spermatozoon (spermiogenesis). During spermiogenesis no cell division is involved. This process is a metamorphosis in which a round cell is converted into a highly motile structure. The changes can be grouped into (i) formation of the acrosome, (ii) nuclear changes, (iii) development of the flagellum, (iv) reorganization of the cytoplasm and cell organelles (De Kretser and Kerr, 1988). The acrosome structure arises from the Golgi complex. Pro-acrosomal granules are established in the Golgi complex. They coalesce to form a single, large granule that comes into contact with the nuclear membrane and spreads over ~25–60% of the nuclear surface. The caudal region of the acrosome is partly attenuated and is termed the equatorial segment. Although the reason for this specialization is un-

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known, this region of the acrosome persists after the acrosome reaction and it represents the region in which the spermatozoon binds to the plasma membrane of the oocyte during the normal fertilization process. Alterations in the final structure of the spermatid head occur during spermiogenesis. Coalescence of chromatin granules is accompanied by chemical changes in the DNA, which is stabilized and becomes resistant to digestion by the enzyme DNase. This stabilization occurs at a time when lysine-rich histones are being replaced by arginine-rich, testis-specific histones in the spermatid nuclei. During the chromatin condensation, there is a progressive reduction in nuclear volume and dramatic changes in cellular shape. At the stage of the elongated spermatid the histones of round spermatids are replaced by protamines. The transition proteins determined in elongated spermatids consist of two major types (Fawcett *et al.*, 1971). They occupy the nucleus between the removal of histones and their replacement by protamines. After fertilization the sperm DNA becomes devoid of protamines again and associated with histones of oocyte origin (Perreault and Zirkin, 1982; Perreault *et al.*, 1987).

The central core of the sperm tail, the axial filament, develops from the pair of centrioles lodged at the periphery of the spermatid cytoplasm and subsequently moves centrally to be lodged at the caudal pole of the nucleus. Generation of spermatid flagella *in vitro* has been observed (Gerton and Millette, 1984). The basic structure of the axial filament is common to flagella and cilia and consists of nine peripheral double microtubules. The last steps in spermiogenesis are characterized by additional changes in the relationship of the nucleus and cytoplasm and movement of organelles within spermatids (De Kretser and Kerr, 1988; Sofikitis *et al.*, 1997a).

Evolution of round spermatid nuclei injections (ROSNI) and intact round spermatid injections (ROSI)

Ogura and Yanagimachi (1993) have shown that round spermatid nuclei injected into hamster oocytes form pronuclei and participate in syngamy. DNA synthesis was found in these pronuclei. However, the developmental potential of the obtained zygotes was not evaluated in this study. In another study, Ogura *et al.* (1993) injected intact round spermatids into the perivitelline space of mature hamster or mouse oocytes and applied a fusion pulse attempting to fuse the intact spermatids with the oocytes. They also studied the behaviour of hamster and mouse round spermatid nuclei incorporated into mature oocytes by the above electrofusion process. It was found that the spermatid nuclei

commonly failed to develop into large pronuclei. In one additional study Ogura *et al.* (1994) confirmed that it is difficult to fuse successfully intact round spermatids with mature oocytes via the above electrical pulse-fusion method. However, they showed that when mouse intact round spermatids are successfully fused with oocytes, some of the resulting zygotes develop into normal offspring. The overall success rate of the electrofusion of intact spermatids with oocytes was low, attributable to the difficulty to fuse large cells like oocytes with small cells like spermatids without lysis of the larger cells (Bates, 1987; Ogura *et al.*, 1993).

To avoid oocyte damage due to the fusion process we chose a microsurgical approach to transfer round spermatid nuclei into rabbit ooplasm. By this method we achieved three pregnancies after ROSNI in rabbit oocytes (Sofikitis *et al.*, 1994a). In that study the proportion of implanted embryos to the number of injected oocytes and the ratio of offspring to the number of injected oocytes were low. The low values of these parameters may be attributable to the low developmental potential of the injected oocytes due to inadequate mechanical stimulation applied to activate oocytes prior to ROSNI. For this reason we designed another study the objective of which was to evaluate the effects of electrical stimulation of oocytes before ooplasmic ROSNI on oocyte activation and subsequent embryonic development (Sofikitis *et al.*, 1996a). That study provided information on the optimal stimulation necessary for oocyte activation, fertilization, and normal embryonic development when ooplasmic ROSNI-embryo transfer procedures are scheduled. We showed that electrical stimulation of oocytes prior to ooplasmic ROSNI and embryo transfer procedures has beneficial effects on oocyte activation, fertilization, and subsequent embryonic development and results in 13% live birth rate per activated oocyte (Sofikitis *et al.*, 1996a). We also assessed the possibility of achieving fertilization and embryonic development *in vitro* after ROSNI into the perivitelline space, speculating that ooplasmic injections may occasionally damage the oocytes and evaluating the possibility of increasing fertilization rates by avoiding injuries of the oocytes. However, injections of two round spermatid nuclei into the perivitelline space of non-stimulated oocytes did not result in fertilization. The proportion of 2–4-cell stage embryos to the successfully injected oocytes after ROSNI was much lower in electrically stimulated oocytes treated with perivitelline space injections than in electrically stimulated oocytes treated with ooplasmic injections. Therefore, it was concluded that the entrance of the round spermatid nucleus into the ooplasm is more effective than

nuclear injections into the perivitelline space for achieving fertilization.

After the above successful trials to produce offspring by microsurgical transferring round spermatid nuclei into rabbit oocytes, a question was raised: could round spermatid nuclei selected from subjects with various testicular disorders have similar fertilizing capacity? That question was of great concern because in our two previous studies the round spermatids had been harvested from healthy male rabbits. Furthermore, the probability that humans/animals with primary testicular failure may not have anatomically and physiologically normal spermatids cannot be excluded. To answer the above question we induced an experimental varicocele model in the rabbit, isolated round spermatid nuclei from the testicles of varicocelized rabbits, injected the nuclei into healthy mature oocytes, and proved that these nuclei had fertilizing potential. The overall fertilization rate was 23%. However, embryo transfer procedures did not result in pregnancies (Sofikitis *et al.*, 1996b). In contrast, Sasagawa and Yanagimachi (1997) achieved delivery of normal offspring after ooplasmic injections of round spermatid nuclei recovered from cryptorchid mice. Delivery of healthy offspring after ooplasmic injections of round spermatid nuclei in the mouse has also been reported by Kimura and Yanagimachi (1995a). However, healthy mice were used in that study.

Goto *et al.* (1996) examined the possibility of utilizing in-vitro derived spermatids for intracytoplasmic injection. There were no significant differences in the development of bovine oocytes injected with various types of male gametes (testicular spermatozoa, spermatids, or spermatids obtained after in-vitro divisions of secondary spermatocytes). It was demonstrated that bovine oocytes injected with in-vitro derived spermatids were capable of developing to blastocyst stage. In another study, fertilization and embryo development *in vitro* has been achieved after ooplasmic injections of nuclei extracted from frozen-thawed rabbit round spermatids (Ono *et al.*, 1996).

Clinical application of ROSNI/ROSI techniques

After the encouraging message from the above animal investigations an attractive challenge was to apply ooplasmic injections of round spermatid nuclei selected from testicular biopsy material for the treatment of non-obstructed azoospermic men (Edwards *et al.*, 1994; Sofikitis *et al.*, 1994a). The first pregnancies in the international literature via ROSNI techniques were achieved in 1994 and reported in 1995 (Sofikitis *et al.*, 1995a; Hannay, 1995). However, these pregnancies resulted in abortions. A few months later, Tesarik *et al.* (1995) reported delivery of two healthy

children after round spermatid injections into oocytes. The mean fertilization rate was 45% in that study. Fishel *et al.* (1995) reported a pregnancy and birth after elongated spermatid injections into oocytes. Vanderzwalmen *et al.* (1995) and Chen *et al.* (1996) reported successful fertilization of human oocytes by intracytoplasmic injections of late-stage spermatids or round spermatids, respectively.

The first ROSNI procedures in the USA were performed in California, Louisiana and Florida (Sofikitis *et al.*, 1995b). Fertilization and development up to 10-cell stage embryos was achieved in non-obstructed American couples. The overall fertilization rate per injected oocyte was 31% in that study. The peak of the two-pronuclei (2PN) appearance curve in the group of oocytes injected with round spermatid nuclei was 9 h post-injections. At that time all normally fertilized oocytes revealed 2PN, whereas 2 h later both pronuclei disappeared in 20% of the oocytes. Considering that the peak of 2PN appearance after intracytoplasmic sperm injection (ICSI) is 16 h post-injection (Nagy *et al.*, 1994) it appears that the speed of human embryo development after ROSNI is faster compared with ICSI and that oocytes injected with round spermatid nuclei should be checked for pronuclei earlier than oocytes injected with spermatozoa. This difference in the speed of embryo development is compatible with previous studies in the hamster (Ogura and Yanagimachi, 1993) and the rabbit (Sofikitis *et al.*, 1996a) and may be attributable to differences in the protein status of the nucleus (Perreault and Zirkin, 1982; Ogura and Yanagimachi, 1993).

Yamanaka *et al.* (1997) reported an oocyte cleavage rate of 61% after ROSNI procedures and confirmed that the appropriate time for assessment of fertilization after human ROSNI techniques is 9 h post-injection. Additional pregnancies achieved by ROSNI or ROSI techniques were recently reported by Tanaka *et al.* (1996), Mansour *et al.* (1996a,b), Antinori *et al.* (1997a and 1997b), Vanderzwalmen *et al.* (1997), Amer *et al.* (1997) and Sofikitis *et al.* (1997b). Average fertilization rates were >25% in all the above studies.

Non-obstructive azoospermia and indications for ROSNI/ROSI procedures

Non-obstructive azoospermia may be due to secondary testicular damage or primary testicular damage. Secondary endocrine and exocrine testicular dysfunction may be due to (i) defects in the hypothalamic–pituitary–testicular axis, or (ii) systemic organic disease (i.e. chronic renal failure, liver insufficiency, sickle cell anaemia, diabetes mellitus). Primary testicular damage may be due to chromosomal

abnormalities, orchitis, trauma, varicocele, cryptorchidism, gonadotoxins, radiation or it may be congenital (i.e. Sertoli cell-only syndrome, myotonic dystrophy). Furthermore, genetic abnormalities affecting the function of germ cells or Sertoli cells may be among the causes of animal or human non-obstructive azoospermia. Thus, mutations in the white spotting locus of the mouse (Chabot *et al.*, 1988), the *Sl* locus encoding the c-kit ligand (Anderson *et al.*, 1990), and genes encoding retinoic acid receptor α (Akmal *et al.*, 1997) may impair spermatogenesis and result in azoospermia. Sex or autosomal chromosomal deletions are also involved in the aetiology of non-obstructive azoospermia. Involvement of at least three Y-linked genes in spermatogenesis has been suggested (Chai *et al.*, 1997). Several studies suggest that two gene families, *RBM* (RNA binding motif) and *DAZ* are present in Y-chromosomal regions that are deleted in some non-obstructed azoospermic men (Chai *et al.*, 1997). Both gene families show specific testicular expression and encode proteins with RNA binding motifs. There is also increasing evidence for a putative human male infertility DAZ-like autosomal gene (Chai *et al.*, 1997).

Recent studies have shown that a significant percentage of men with non-obstructive azoospermia have testicular foci of active spermatogenesis up to the stage of round spermatid, elongating spermatid, or spermatozoon (Sofikitis *et al.*, 1995a, 1997b, 1998a; Silber *et al.*, 1995a,b; Tesarik *et al.*, 1995; Silber, 1996; Mansour *et al.*, 1996a; Antinori *et al.*, 1997a; Vanderzwalmen *et al.*, 1997; Yamanaka *et al.*, 1997). Ooplasmic injections of spermatozoa offer a solution for men positive for spermatozoa in the therapeutic testicular biopsy material (Palermo *et al.*, 1992; Silber *et al.*, 1995b; Silber, 1996). When spermatozoa are not present, ROSNI/ROSI techniques re-present the only hope for treatment (Edwards *et al.*, 1994; Sofikitis *et al.*, 1994a).

Yamanaka *et al.* (1997) emphasized the need to collect an adequate amount of testicular tissue (>200 mg) for accurate demonstration of round spermatids by therapeutic testicular biopsy procedures in men who are negative for round spermatids in the routine diagnostic testicular biopsy specimen. It appears that some of the patients with a diagnosis of spermatogenic arrest at the primary spermatocyte stage or Sertoli cell-only syndrome may have rare foci of round spermatids somewhere in the testicles. Amer *et al.* (1997) used the term 'complete spermiogenesis failure' for men in whom the most advanced germ cell present in the testicular biopsy material is the round spermatid and the term 'incomplete spermiogenesis failure' for non-obstructed azoospermic men with a very limited number of elongated spermatids in testicular biopsy material. Several

studies have shown clearly that in men with spermatogenic arrest at the primary spermatocyte stage or Sertoli cell-only syndrome a number of germ cells in a limited number of seminiferous tubules can break the barrier of the premeiotic spermatogenic block and differentiate up to the stage of the round or elongating spermatid (Mansour *et al.*, 1996a; Tesarik *et al.*, 1996; Amer *et al.*, 1997; Antinori *et al.*, 1997a; Vanderzwalmen *et al.*, 1997; Yamanaka *et al.*, 1997; Sofikitis *et al.*, 1998a). Defects in the secretory function of the Leydig and Sertoli cells or other factors may not allow the round or elongating spermatids to complete the spermiogenesis. Silber *et al.* (1997) have demonstrated that non-obstructed azoospermic men have a mean of 0–6 mature spermatids per seminiferous tubule seen on a diagnostic testicular biopsy, whereas, 4–6 mature spermatids per tubule must be present for any spermatozoa to reach the ejaculate. In that study the authors claimed that there were no round spermatids in the therapeutic testicular biopsy material of men with maturation arrest if there was absence of elongated spermatids or spermatozoa. Thus, Silber *et al.* have objections to the ROSNI/ROSI techniques since they support the thesis that when spermatozoa are absent in the therapeutic testicular biopsy specimen, round spermatids are also absent. However, the results of the latter study cannot be unequivocally adopted because: (i) a limited number of participants was evaluated, (ii) the authors attempted to identify round spermatids via Nomarski or Hoffman lens although they admit that they have difficulties in identifying round spermatids using this methodology, and (iii) the most reliable methodology for round spermatid identification [i.e. transmission electron microscopy (TEM)] or another objective method [i.e. confocal scanning laser microscopy (CSLM) or fluorescent in-situ hybridization (FISH) methods] were not applied. To exclude the presence of round spermatids in testicular tissue, a great number of droplets of minced testicular tissue should be processed for the above microscopical techniques and the vast majority of the round germ cells should be examined. To the best of our knowledge the above techniques were not employed by Silber *et al.* (1997). In contrast, as we mentioned above, several studies by independent groups clearly indicate that complete arrest in spermiogenesis is not rare in non-obstructed azoospermic men. In the latter men a large or small number of round spermatids represent the most advanced germ cells.

Several biochemical mechanisms may be responsible for the inability of the round spermatids to undergo the elongation process. O'Donnell *et al.* (1996) have shown that intratesticular testosterone concentration (ITC) suppression maybe one of these mechanisms. Additional studies are necessary to clarify whether values of ITC

below a threshold cause failure of elongation of round spermatids. If this hypothesis is correct, testicular pathophysiology affecting optimal ITC may result in complete spermiogenetic failure. It should be emphasized that varicocele, the most frequent cause of male infertility, known to cause azoospermia occasionally, is accompanied by reduced ITC (Rajfer *et al.*, 1987). A diagnostic testicular biopsy negative for round spermatids does not rule out the probability that few or many round spermatids will be found in the therapeutic testicular biopsy material. Furthermore, peripheral serum FSH levels and testicular size do not predict the presence/absence of round spermatids in the therapeutic testicular biopsy material. It should be emphasized that the diagnostic testicular biopsy refers to the evaluation of a limited amount of tissue recovered from one testicular location. Diagnostic testicular biopsy material is exposed to various detergents during fixation/staining and subsequently a number of cells are degenerated and their identity cannot be defined. In contrast, therapeutic testicular biopsy refers to the isolation of a larger amount of tissue from several areas of testicular tissue. Therapeutic testicular biopsy specimens are minced for hours into isoosmotic solutions containing energy sources and isolated, single cells can be observed. Therefore, the value of the diagnostic testicular biopsy in the management of non-obstructed azoospermic men should be questioned. The Tottori University International Research Group does not request diagnostic testicular biopsy in non-obstructed azoospermic men, except if there is a need to differentiate between obstructive and non-obstructive azoospermia.

Combined analysis of our studies in the management of non-obstructed azoospermic men (Sofikitis *et al.*, 1995a, 1997b, 1998a; Yamanaka *et al.*, 1997) showed that among the men who participate for first time in an assisted reproduction programme (regardless of positive or negative results for spermatozoa in the diagnostic testicular biopsy), a percentage of 39% have spermatozoa in their therapeutic testicular biopsy material. For a percentage of 43% the most advanced spermatogenic cells are spermatids in the therapeutic testicular biopsy specimen. These men are candidates for ROSNI/ROSI techniques. It appears that nowadays a small percentage only of non-obstructed azoospermic men (~ 18%) are excluded from assisted reproductive techniques. Among the men who are positive for spermatids and negative for spermatozoa in the therapeutic testicular biopsy, a percentage of ~25% expose a large number of round spermatids, whereas the biopsy specimen of the remaining men should be prepared for an extended period (occasionally longer than 5 h) to identify few spermatids.

Round spermatids are occasionally present in the seminal plasma of non-obstructed azoospermic men. These ejaculated round spermatids can be used for ooplasmic injections (Tesarik *et al.*, 1996). Mendoza and Tesarik (1996) reported that 69% of non-obstructed azoospermic men have round spermatids in the ejaculate. However, the interesting results of that study may be criticized because the authors did not use standard, reliable methods for round spermatid identification [i.e. transmission electron microscopy (TEM), confocal scanning laser microscopy (CSLM) or fluorescent in-situ hybridization (FISH) techniques]. A current study by Tottori University International Research Group applying CSLM, FISH and TEM in semen samples of >200 non-obstructed azoospermic men indicates that round spermatids are present in >20% of the latter men (Y.Yamamoto *et al.*, unpublished observations). A landmark study by O'Donnell (1997) provides strong evidence that testosterone withdrawal promotes stage-specific detachment of round spermatids from the seminiferous epithelium. It should be emphasized that even if round spermatids are present in the ejaculate of a non-obstructed azoospermic man, therapeutic testicular biopsy is indicated for the following reasons: (i) if spermatids are present in the ejaculate, spermatozoa may be found in the biopsy specimen, (ii) the percentage of alive spermatids in the biopsy specimen is significantly larger than that in the ejaculate of the same individual (Y.Yamamoto, unpublished observations), (iii) round spermatids from testicular biopsy have larger fertilizing capacity than round spermatids from the respective ejaculate (Fishel *et al.*, 1997), and (iv) primary testicular damage is often progressive. Therefore, collection of a large number of spermatids from a testicular biopsy specimen and subsequent cryopreservation offers the possibility of performing ROSNI/ROSI techniques in the future even if testicular function deteriorates and the spermatogenic arrest at the round spermatid stage is replaced by spermatogenic arrest at the primary spermatocyte stage.

Criteria for identification/isolation of human round spermatids

The gold standard for identification of round spermatids is TEM (Sofikitis *et al.*, 1994a). Recently, Mendoza and Tesarik (1996) attempted to identify round spermatids by selective staining of the acrosin contained in the acrosomal granules. Another approach is to visualize proacrosin with the use of a monoclonal antibody (Mendoza *et al.*, 1996). A drawback to all the above techniques is that application of these methods results in cell death. Therefore, observed spermatids cannot be used in assisted reproduction pro-

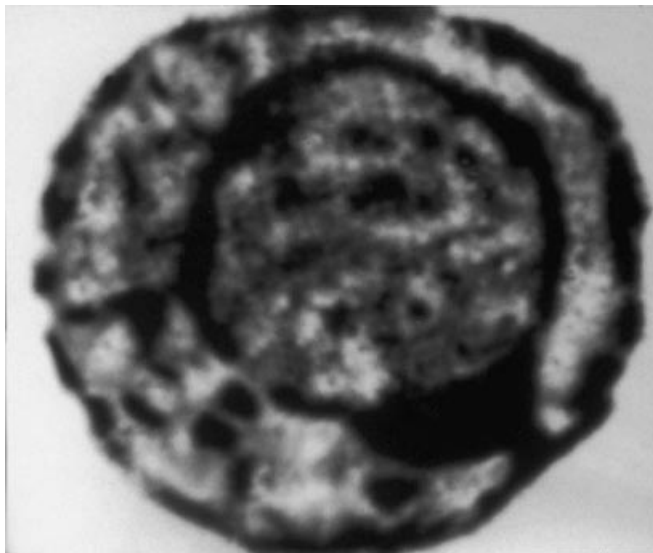


Figure 1. Observation of a human round spermatid via a confocal scanning laser microscope computer-assisted system.

grammes. Indeed, one of the most perplexing problems in the laboratories applying human ROSNI/ROSI procedures is the identification of alive, undisturbed, non-fixed, non-stained round spermatids. The following approaches are suggested for identification of undisturbed round spermatids in therapeutic testicular biopsy material or in cellular populations isolated from semen samples.

Observation of samples by confocal scanning laser microscopy

Observation of testicular or semen samples via CSLM allows identification of round spermatids (Figure 1). The CSLM is a relatively new instrument in the field of microscopy (Sofikitis *et al.*, 1994b). Unlike the conventional light microscope the CSLM produces sharp images free of out-of-focus artefacts that can be observed on a television monitor. It provides an automatic system for instantaneous measurement of distance. This technique has the capacity to provide three-dimensional images of cells up to $\times 10\,000$ magnification without requiring staining of materials. Therefore, the observed cells can be processed for assisted reproduction techniques. Human and rabbit round spermatids are easily identified via laser microscopy by the presence of multiple or a single, large (acrosomic) granule adjacent to the nucleus (Sofikitis *et al.*, 1994a, 1996b; Yamanaka *et al.*, 1997).

New models of CSLM computer-assisted systems (CAS) allow identification of round spermatids of stage 1 of spermiogenesis. These cells are negative for acrosomal granules.

CAS analysis of images provided by CSLM differentiates between round spermatids of stage 1 and secondary spermatocytes. The size of round spermatids of stage 1 is $<75\%$ of the size of secondary spermatocytes (Sofikitis *et al.*, 1997d). In addition, nuclei of stage 1 round spermatids show a finely granular texture as opposed to a cloud-like texture exhibited by secondary spermatocytes. Another advantage of the CSLM–CAS is that it allows identification and isolation of specific types of undisturbed primary spermatocytes. CSLM–CAS recognizes specific characteristics of leptotene primary spermatocytes (i.e. chromatin threads), zygotene primary spermatocytes (i.e. synaptonemal complex), and pachytene primary spermatocytes (i.e. sex vesicles). Diplotene primary spermatocytes can be easily identified because they are the largest germ cells.

Inverted microscope–computer assisted system (IM–CAS)

Application of quantitative criteria based on computer-assisted image analysis allows identification of round spermatids. ‘Round cells’ with minimum diameter between 6 and 10 μm that satisfy additional specific quantitative and qualitative criteria are considered to be spermatids (Yamanaka *et al.*, 1997). The minimal cellular diameter of round spermatids is approximately equal to its average diameter (Yamanaka *et al.*, 1997). In preliminary experiments human cadaveric germ cells with morphometric parameters satisfying Yamanaka’s quantitative criteria were recovered and processed for TEM. It was found that 63 and 16% of the cells isolated were round spermatids of stages 2–5 and stages 6–8 of spermiogenesis, respectively (Yamanaka *et al.*, 1997). These results were further confirmed by another study suggesting that cells $<7.5\ \mu\text{m}$ in diameter should be selected in assisted reproduction programmes using round spermatids (Angelopoulos *et al.*, 1997). However, the last approach excludes round spermatids of average diameter of $>7.5\ \mu\text{m}$.

Qualitative criteria

IM–CAS and CSLM–CAS are not available in most IVF centres. Human round spermatids (Mansour *et al.*, 1996a; Tesarik *et al.*, 1996; Antinori *et al.*, 1997a; Vanderzwalmen *et al.*, 1997; Yamanaka *et al.*, 1997) can be distinguished from other cell types according to the cellular shape, size, and the form of the nucleus. A developing acrosomal granule can be recognized in the round spermatid as a bright/dark spot adjacent to the cell nucleus. Nomarski lens

is preferable to Hoffman lens when qualitative criteria are applied for identification of round spermatids.

ROSI versus ROSNI

ROSI procedures ensure the transfer of all the cytoplasmic components of the male gamete into the maternal gamete and are less time-consuming than round spermatid nucleus injection. Furthermore, manipulations of the nuclear matrix and envelope are avoided when ROSI techniques are applied. In contrast, ROSI procedures have two disadvantages: (i) injecting micropipettes of larger diameter are necessary, and consequently the probability of injuring oocytes during injections is larger, and (ii) persistence of a large amount of cytoplasm around the round spermatid nucleus may impede its transformation into male pronucleus. In the mouse (Ogura *et al.*, 1993; Kimura and Yanagimachi, 1995a; Kimura and Yanagimachi, 1995b) and the rabbit (Yamamoto *et al.*, 1997), transferring the round spermatid nucleus into the oocyte is a far more efficient procedure in achieving fertilization and embryonic development than transferring the intact round spermatid cell. Embryonic development is faster after ROSNI than ROSI techniques (Yamamoto *et al.*, 1997). The Tottori University International Research Group applies ROSNI rather than ROSI because occasional inability of the ooplasm to digest the cytoplasm of the round spermatid and subsequently lack of exposure of the round spermatid nuclear membrane to ooplasmic factors have been considered as causes of failure of fertilization after ROSI techniques (Ogura *et al.*, 1993).

The number of pregnancies achieved via ROSI techniques (Tesarik *et al.*, 1995; Mansour *et al.*, 1996a; Antinori *et al.*, 1997a,b; Vanderzwalmen *et al.*, 1997) is larger than the number of ROSNI-pregnancies (Hannay *et al.*, 1995; Sofikitis *et al.*, 1995a, 1997b). This difference in favour of ROSI techniques is false, however, for two reasons: (i) ROSI techniques are relatively simple and applied by a large number of centres internationally. In contrast, ROSNI techniques are applied by Japanese centres only; (ii) most of the Japanese centres applying ROSNI techniques cannot publish achieved ROSNI pregnancies because of recommendations by Japanese ethical committees.

Contributions of the round spermatid to the zygote

The male gamete contributes several components important for the fertilization process and early embryo development to the zygote: the genetic material, the reproducing element of the centrosome (Schatten *et al.*, 1986; Schatten, 1994;

Simerly *et al.*, 1995), the microtubule organizer component of centrosome (Schatten, 1994), the oocyte-activating substance in spermatozoon/spermatid (OASIS; Swan, 1990; Parrington *et al.*, 1996), nuclear proteins (Ogura and Yanagimachi, 1993), and factors affecting early embryonic development and capacity for implantation.

Genetic material

The deliveries of normal mouse and rabbit offspring (Sofikitis *et al.*, 1994a; Ogura *et al.*, 1994; Kimura and Yanagimachi, 1995a) and healthy human newborns (Tesarik *et al.*, 1995; Mansour *et al.*, 1996a; Vanderzwalmen *et al.*, 1997; Antinori *et al.*, 1997; Sofikitis *et al.*, 1997b) after ROSNI/ROSI indicate the maturity of the genetic material of the early haploid male gamete (i.e. the chromosomes of the round spermatid are capable of pairing with those of the oocyte and participate in syngamy, fertilization, and subsequent embryonic and fetal development).

OASIS

The male gamete-induced cascade of biochemical ooplasmic events that results in resumption of meiosis of the female gamete is referred to as oocyte activation. Oocyte activation is a prerequisite for male pronucleus development and fertilization. Therefore, an anatomical or functional defect of the OASIS may cause fertilization failure ICSI/ROSNI/ROSI procedures. It is generally agreed that the spermatozoon or spermatid triggers the embryonic development by increasing the Ca^{2+} ion concentration in the oocyte cytoplasm (Vitullo and Ozil, 1992; Sousa *et al.*, 1996; Yamanaka *et al.*, 1997; Sofikitis *et al.*, 1998a). These transient oscillatory or wave-form increases in Ca^{2+} ion concentration have been observed both after normal fertilization (Taylor *et al.*, 1993) and ICSI/ROSI techniques (Tesarik *et al.*, 1994; Sousa *et al.*, 1996).

Injections of mouse round spermatids into oocytes do not result in oocyte activation suggesting that the mouse OASIS has not been expressed at the round spermatid stage (Kimura and Yanagimachi, 1995a,b). In contrast, ooplasmic injections of rabbit round spermatids lead to oocyte activation in a significant percentage (Sofikitis *et al.*, 1994a). Electrical stimulation of the rabbit oocyte enhances the OASIS and benefits the activation process (Sofikitis *et al.*, 1996a). Although electrical stimulation usually results in a monophasic ooplasmic Ca^{2+} response, it appears that there is a synergistic action of electrical stimulation and round spermatid OASIS which eventually produces Ca^{2+} oscillations. There is strong evidence that the human OASIS is activated at/before the round spermatid stage (Yamanaka *et al.*, 1997; Sousa *et al.*, 1996,

1998a). The achievement of human pregnancies via ROSNI/ROSI without application of an exogenous electrical or chemical stimulation supports the above thesis. The human oocyte activation after ROSI/ROSNI may not be attributed to parthenogenetic activation of the oocytes since ooplasmic injections of medium only have not resulted in activation (Fishel *et al.*, 1996b; Yamanaka *et al.*, 1997; Sofikitis *et al.*, 1998a). Dozortsev *et al.* (1995) and Meng and Wolf (1997) have emphasized that human or monkey mechanical ooplasmic stimulation and/or oocyte exposure to a low or a relatively high extracellular calcium concentration of medium can alter intracellular Ca^{2+} but not, alone, cause activation. The human round spermatid OASIS should be nucleus-associated since nuclear injections are sufficient to cause activation (Yamanaka *et al.*, 1997).

Human oocyte activation is faster after ROSNI techniques than ROSI procedures (Yamamoto *et al.*, 1997). The faster speed of oocyte activation after ROSNI techniques may be due to the presence of a smaller amount of male gamete cytoplasm facilitating the closer contact of the male gamete OASIS–nucleus complex with the cytoplasm of the oocyte.

Similar levels of OASIS activity have been demonstrated in human round spermatids and testicular spermatozoa using a quantitative assay (Sofikitis *et al.*, 1997c). Whether the technique applied for ooplasmic injections of spermatids or spermatozoa influences the oocyte activation process is controversial. Remarkably high oocyte activation rates after ROSNI/ROSI techniques have been demonstrated after injections with minimal (Yamanaka *et al.*, 1997; Sofikitis *et al.*, 1998a) or vigorous ooplasmic stimulation (Tesarik *et al.*, 1996). Tesarik *et al.* (1994) have supported the idea of a positive role for vigorous ooplasmic stimulation during ICSI techniques in the oocyte activation process. In contrast, Mansour *et al.* (1996b) have recommended a minimal ooplasmic stimulation. Recent studies have suggested that there may be an OASIS deficiency in selected subpopulations of non-obstructed azoospermic men (Sofikitis *et al.*, 1996c). In the latter men application of electrical (Sofikitis *et al.*, 1995a) or chemical (Vanderzwalmen *et al.*, 1997) stimulation prior, during, or immediately after ROSNI/ROSI techniques may (i) support the action of OASIS, or (ii) act synergistically with OASIS.

Mouse OASIS has not been expressed at the secondary spermatocyte stage (Kimura and Yanagimachi, 1995b). We have recently shown that ooplasmic injections of human secondary spermatocytes with minimal ooplasmic stimulation do not activate oocytes and result in premature condensation of the chromosomes of the male gamete (Sofikitis *et al.*, 1998b). However, when a second vigorous

mechanical ooplasmic stimulation is applied 1–2 h after the secondary spermatocyte injection, human oocytes are activated in a significant percentage and both the oocyte and the secondary spermatocyte complete the second meiotic division (Sofikitis *et al.*, 1998b).

Centrosomic components: a challenge to the theory of centrosomes

The zygote's centrosome is a blend of paternal and maternal components. The restoration of the zygotic centrosome at fertilization requires the attraction of maternal centrosomal components to the paternal reproducing element (Schatten *et al.*, 1994; Simerly *et al.*, 1995). The male gamete contributes to the zygote centrosome by transferring the reproducing element of the centrosome, the microtubule organizing centre and a γ -tubulin binding protein. However, the maternal γ -tubulin is necessary for the function of the zygote centrosome (Schatten *et al.*, 1994).

The delivery of healthy babies after human ROSI/ROSNI tends to suggest that the centrosomic components of the human round spermatid are normal, functional, and mature. Additional studies are necessary on the development of aster and the ooplasmic microtubule organization after ROSNI/ROSI procedures. Several studies have suggested that mammalian oocytes lose their centrosomes when they mature and that centrosomic material is introduced into oocytes by the spermatozoa (see for review Palermo *et al.*, 1994; Schatten, 1994). However, the normal embryonic and fetal development after ROSNI plus embryo transfer procedures in the rabbit (nuclei were proven to be free of cytoplasmic and subsequently centrosomic material; Sofikitis *et al.*, 1994a, 1996a,b), the artificial parthenogenesis in several mammalian female gametes (Schatten *et al.*, 1994), and the development of parthenogenetic rabbit fetuses up to day 10 of pregnancy (Ozil, 1990) can be interpreted as a challenge to the theory of centrosomes and raise the probability that, when paternal centrosomic material is absent, novel maternal spindle organizing centres can develop and previously denatured/non-functional/inactive female centrosomic material can undergo renaturation/activation. However, the probability that paternal centrosomic material is transferred during spermatid nuclear injections cannot be excluded (Navara *et al.*, 1994) since the centrosomic material is tightly anchored to the nuclear envelope in most of the cells (Schatten, 1994).

The consideration of the centrosome as a cellular organ may be out of date (Mazia, 1984). Observations of Mazia (1984) on the centrosomic cycle refute any notion of the centrosome as an entity that is either present or non-present

and is always the same when it is present, and suggest that the centrosomes should be considered as flexible cyclical structures altering their shape and form. The flexible centrosome hypothesis has been further supported by Schatten *et al.* (1986). Studies suggesting the absence of centrosomic material within mammalian oocytes depend on immunological recognition studies or TEM studies. However, when female gamete centrosomic material cannot be identified by immunological antibody studies, these negative results may be due to the lower concentration of the antigen (Schatten *et al.*, 1986). If unitary centrosomes can exist in a linear form, auto-immune methods might only detect nodes of higher concentration of the antigen. Furthermore, negative results on oocyte centrosomic material obtained by TEM do not exclude the presence of centrosomic material within mature oocytes, because of difficulties locating centrioles and centrosomes at spindle poles by this method (Sathananthan *et al.*, 1991) and the ability of centrosomic material to change shape. In conclusion, negative results on oocyte centrosomic material cannot be unequivocally accepted. As has been previously suggested by Sathananthan *et al.* (1991), a maternal contribution of centrosomic material still needs to be considered and further investigation of centrosomes in both human oocytes and zygotes is clearly warranted.

Nuclear proteins

Spermiogenesis is characterized by alterations in the protein composition of the nucleus. Testis-specific histones are replaced by spermatid-specific basic proteins. The latter are gradually replaced by protamines (Perreault *et al.*, 1987). Following ROSNI/ROSI and disintegration of the round spermatid nuclear membrane within the ooplasm, the round spermatid DNA–nuclear protein complex is exposed to ooplasmic factors. Since the histones are proteins containing a reduced number of disulphide bonds, questions may be raised as to (i) how the round spermatid DNA that is not associated with disulphide bond proteins can survive within the ooplasm and how it is protected against an immediate action of ooplasmic factors, and (ii) how a male gamete nucleus that has not undergone removal of spermatid-specific histones has the capacity to undergo the cascade of events that leads to normal male pronucleus development.

The answer to the first question is that the activation of the oocyte can rescue the chromosomes of the round spermatid from premature condensation (Kimura and Yanagimachi, 1995a,b). Therefore, in non-obstructed azoospermic men whose spermatids expose an impaired capacity for oocyte activation, application of an exogenous stimulus for oo-

plasmic activation is of paramount importance. In addition, it may possible that spermatid-specific histone removal is not a prerequisite for the formation of the male pronucleus.

Factors affecting early development and capacity for implantation of ROSNI/ROSI embryos

Janny and Ménéz (1994) have shown that the mission of the male gamete is not only to activate and fertilize the oocyte but also to contribute to the zygote potential to undergo the first mitotic divisions. It appears that there is a paternal effect on early embryonic development. This thesis has been further supported by Sofikitis *et al.* (1996b) and Ono and *et al.* (1997). The latter studies have shown a defect in the capacity for early development and implantation of embryos generated from the fertilization of oocytes by round spermatids or spermatozoa isolated from animals with varicoceles. Thus, embryos derived from the fertilization of human oocytes by spermatids recovered from men with primary testicular damage may have an impaired potential for further development and implantation. In addition, the round spermatid/elongating spermatid factors mediating the paternal influence on the embryonic development may be deficient since the round or elongating spermatid represents an immature stage of the male gamete. The above speculation may explain why ROSNI/ROSI techniques result in low pregnancy rates, although fertilization rates are relatively high (Antinori *et al.*, 1997a; Yamanaka *et al.*, 1997; Sofikitis *et al.*, 1998a). Therefore, we may recommend transfer of all embryos generated from the fertilization of oocytes by spermatids (Sofikitis *et al.*, 1998a).

Guidelines/prerequisites for ROSNI/ROSI techniques

The Tottori University International Research Group achieved the first spermless pregnancies in 1994 (Hanay, 1995; Sofikitis *et al.*, 1995a). Later a number of other investigators achieved additional pregnancies. However, it is obvious that there is limited experience on human ROSNI/ROSI techniques. We consider the following issues important for successful performance of ROSNI/ROSI techniques.

Quality control for identification of round spermatids

Several methods for identification of round spermatids were discussed above. Furthermore, it should be emphasized that training is necessary for the staff of assisted reproduction centres applying ROSNI/ROSI. Even if a centre has an excellent ICSI programme, ROSNI/ROSI will result in poor outcome if the staff of that centre have

not spent many hours observing animal testicular tissue specimens attempting to identify round spermatids via an inverted microscope. Technicians/embryologists/physicians performing ROSNI/ROSI should also confirm via TEM, FISH or CSLM that the cells that are considered as human round spermatids are indeed round spermatids.

Quality control for viability of round spermatids

An occasional finding in ROSNI/ROSI programmes is the absence or a reduced number of live spermatids. Fractions of round spermatids retrieved from testicular tissue should be processed for assessment of viability (Sofikitis *et al.*, 1996a). Men with a percentage of live round spermatids <10% have a poor ROSNI outcome. Preliminary studies have shown that spermatids from these men cannot fertilize oocytes (N.Sofikitis *et al.*, unpublished observations).

It should be mentioned that the Trypan Blue stain assesses the plasma membrane and cytoplasmic viability but it does not evaluate the nuclear viability. Theoretically, a live nucleus of a round spermatid with partially degenerated cytoplasmic content may have the capacity to fertilize oocytes. Therefore, nuclear staining techniques are recommended for assessment of round spermatid viability.

Quality control for the capacity of round spermatids to activate oocytes

A previous study has shown that ICSI or ROSNI failure in a selected subpopulation of infertile men is attributable to subnormal OASIS profiles (Sofikitis *et al.*, 1997c). Application of a recently reported quantitative assay to appreciate OASIS activity is recommended (Sofikitis *et al.*, 1997c; two round spermatids are injected into a hamster oocyte). If the percentage of activated hamster oocytes in the latter assay is <8%, fertilization is not anticipated after human ROSNI/ROSI. Alternatively, when OASIS deficiency is suspected, an exogenous stimulus (i.e. chemical or electrical) may be applied to support human oocyte activation and subsequently facilitate fertilization.

Stage of the round spermatid and fertilization

When round spermatids are observed via an inverted microscope the cells with the larger acrosomal (Golgi) bright/dark spots should be preferred for ooplasmic injections because they represent the most mature forms of the male gamete. A recent study has clearly indicated that round spermatids of stages 1 and 2 have smaller reproductive potential than round spermatids of stages 3–5 (Sofikitis *et al.*, 1997d).

When both elongating and round spermatids are present in the testicular biopsy specimen, ooplasmic injections of

elongating spermatids are considered preferable because they result in a higher fertilization rate (Fishel *et al.*, 1997).

Media for maintenance of round spermatids

Most of the popular media in assisted reproduction programmes have been devised to maintain spermatozoa rather than spermatids. However, there are several anatomical and biochemical differences between the round spermatid and the spermatozoon. A medium (SOF medium) has been developed by the first author to prolong the viability of round and elongating spermatids (Sofikitis *et al.*, 1998a). It has been already used for maintenance of human and rabbit round spermatids (Sofikitis *et al.*, 1997d; Yamanaka *et al.*, 1997). It contains lactate and glucose as energy substrates. Previous studies have demonstrated that lactate is the preferable energy substrate for round spermatids (Nakamura *et al.*, 1978). Round spermatids have a larger amount of cytoplasm than spermatozoa. To protect round spermatids against environmental shock and to stabilize the spermatid membrane, cholesterol has been added to the SOF medium in a small concentration. We have also demonstrated that iron and vitamins influence spermatid viability. Therefore vitamins and ferric nitrate have been chosen as components of the SOF medium (Sofikitis *et al.*, 1998a).

Media for culture of oocytes injected with round spermatids

Previous studies have shown that the addition of antioxidants to media used for culture of embryos generated from the fertilization of oocytes by spermatids has beneficial effects on embryonic development (Sofikitis *et al.*, 1996a, 1997d).

The importance of preserving a cytoplasmic blanket around the round spermatid nucleus

During human ROSNI techniques round spermatids are treated with a variety of detergents to isolate nuclei surrounded by a thin cytoplasmic layer (cytoplasmic blanket; Yamanaka *et al.*, 1997). Although ooplasmic injections of rabbit nude nuclei have resulted in delivery of healthy offspring, when human ROSNI techniques are scheduled, maintenance of a cytoplasmic blanket around the nucleus is preferred to avoid exposure of the male gamete nuclear material to chemical and mechanical stimuli.

Time to observe pronuclei

Pronuclei should be observed at 9 h after human ROSNI techniques (Sofikitis *et al.*, 1995b; Yamanaka *et al.*, 1997). When human ooplasmic injections of elongating spermatids are performed, appropriate time for pronuclei observation is 13 h post-injection (Sofikitis *et al.*, 1998a).

Table I. Clinical pregnancies achieved via ooplasmic injections of spermatids or secondary spermatocytes

	Ooplasmic injection of	Clinical pregnancies	Abortions/fetal losses	No. of offspring
Hannay <i>et al.</i> (1995)	Round spermatid nuclei	4	4	0
Tesarik <i>et al.</i> (1995)	Round spermatids	2	0	2
Fishel <i>et al.</i> (1995)	Elongated spermatid	1	0	1
Mansour <i>et al.</i> (1996a)	Round spermatids	1	0	1
Tanaka <i>et al.</i> (1996)	Round spermatids	1	1	0
Araki <i>et al.</i> (1997)	Elongated spermatids	3	0	4
Antinori <i>et al.</i> (1997a)	Round spermatids	2	0	2
Antinori <i>et al.</i> (1997a)	Elongating/elongated spermatids	3	1	2
Antinori <i>et al.</i> (1997b)	Round spermatids	1	0	1
Vanderzwalmen <i>et al.</i> (1997)	Elongating/elongated spermatids	3	0	3
Vanderzwalmen <i>et al.</i> (1997)	Round spermatids	1	0	1
Sofikitis <i>et al.</i> (1997b)	Round spermatid nuclei	3	0	3
Amer <i>et al.</i> (1997)	Elongating/elongated spermatids	2	0	No information
Sofikitis <i>et al.</i> (1998a)	Elongated spermatids	2	0	2
Sofikitis <i>et al.</i> (1998b)	Secondary spermatocyte nuclei	1	0	1

How many ROSNI/ROSI embryos to transfer

As we discussed in a previous paragraph the implantation potential of human ROSNI/ROSI embryos is small and, therefore, we recommend transfer of all the normally fertilized oocytes that subsequently cleave (Yamanaka *et al.*, 1997; Sofikitis *et al.*, 1998a).

Cryopreservation of round spermatids

Antinori *et al.* (1997b) achieved the first human pregnancy via ooplasmic injections of frozen–thawed round spermatids. That study indicates the importance of cryopreserving round spermatids in all ROSNI/ROSI cycles.

Time to perform the second ROSNI/ROSI trial

Testicular damage in non-obstructed azoospermic men with complete or incomplete arrest in spermiogenesis (Amer *et al.*, 1997) may be progressive. Therefore, it is uncertain whether a man with a limited number of round spermatids in the therapeutic testicular biopsy material will still have round spermatids in his biopsy material 1 year later. Therefore, the second ROSNI/ROSI trial should be performed relatively soon after the first therapeutic testicular biopsy–ROSNI/ROSI cycle (4–9 months) regardless of the partner's (female) age. In contrast, a theoretical risk of damaging the testis exists if a second testicular biopsy is applied early after the first biopsy. However, we believe that most clinical studies indicating testicular damage after a testicular biopsy are due to inappropriate technique, usage of electrocautery, or damage of arteries by a needle during (i) administration of

local anaesthesia or (ii) tissue recovery and cannot be unequivocally attributed to the testicular biopsy *per se* (N.Sofikitis, unpublished observations).

Adequate counselling (see below)

Genetic implications of ROSNI/ROSI procedures

To evaluate the genetic risk of assisted reproductive technologies, one has to consider the genetic risk inherent to the treatment population and the genetic risk inherent to the procedure performed (Baschat *et al.*, 1996). Considering the limited number of full-term pregnancies achieved by ROSNI/ROSI procedures to date, we can only speculate on the safety/risks of these procedures.

Genetic risks inherent to ROSNI/ROSI procedures may involve (i) centrosomic abnormalities resulting in aberrant spindle formation and subsequently in an increased risk of mosaicism, (ii) injection of disomic/diploid genetic material which could give rise at fertilization to a trisomic/triploid embryo and fetus, (iii) genomic imprinting abnormalities (see below), and (iv) abnormalities due to the out-of-phase cycles of the round spermatid and the oocyte. The round spermatid is at the G1 stage, whereas the oocyte in the metaphase of the second meiotic division is in its M phase. However, the results of the studies of Kimura and Yanagimachi (1995a and 1995b), Sofikitis *et al.* (1994a, 1996a, 1997b), Fishel *et al.* (1996), and Tesarik *et al.* (1996) indicate that the cell cycle imbalance between

the oocyte and the round spermatid does not affect fertilization, embryonic development, and fetal development. It must also be emphasized that the cell cycles of the spermatozoon and the oocyte are out of phase (Fishel *et al.*, 1996). When spermatids are injected into oocytes the metaphase promoting factor which maintains the oocyte in the metaphase of the second meiotic division may also drive the spermatid nuclei to the metaphase (Fishel *et al.*, 1996). Genetic risks of ROSNI/ROSI inherent to a population of men with primary testicular damage are the same with the genetic risks of ICSI procedures (transferring sex chromosomal abnormalities or reciprocal translocations associated with spermatogenic impairment). Inheritance of gene mutations/deletions of DNA sequences in specific regions of the Y-chromosome long arm represent additional risks.

Genomic imprinting abnormalities

Most genes are expressed equally from the two parental alleles, but a small subgroup of mammalian genes are differentially expressed depending on whether they have been inherited from the mother or the father. The process which differentially marks the DNA in the parental gametes is termed genomic imprinting. Genes whose expression is inhibited after passage through the mother's germline are called maternally imprinted, whereas genes whose expression is inhibited when transmitted by fathers are called paternally imprinted.

Imprinted genes have been identified in mice and humans. Mouse insulin-like growth factor-II (IGF-II) gene is expressed only from the paternal allele. In contrast, the gene encoding a differentiation-related fetal RNA (H19) is expressed only from the maternal alleles. IGF-II and H19 are also monoallelically expressed in the human. Additional imprinted genes have been characterized (Tycko, 1997).

Several studies have shown that imprinted genes regulate the development of the embryo/fetus. It has been also suggested that DNA methylation maintains the imprinting of some genes. Abnormalities in genomic imprinting are associated with genetic diseases. Prader-Willi syndrome and Angelman syndrome are two examples of abnormal functional imprinting. Furthermore, abnormal functional imprinting is implicated in tumorigenesis. Although previous studies (Ogura *et al.*, 1994; Sofikitis *et al.*, 1994a; Kimura and Yanagimachi, 1995a) suggest that genomic imprinting is complete at the rabbit and mouse round spermatid stage, additional studies are necessary in the human. If genomic imprinting is incomplete in subpopulations of men with primary testicular damage, abnormalities may not become manifest at the early embryonic development

but they may be detectable in the fetus or during the postnatal life. A question of great clinical importance is whether genomic imprinting has been completed at the human round spermatid stage. To attempt to answer this question the imprinting of a gene should be divided into three stages: (i) erasure of the previous imprint, (ii) re-imprinting, and (iii) consolidation of the new imprint. There is strong evidence that erasure of the previous imprint occurs prior to meiosis and that re-establishment of the new imprint begins prior to the pachytene stage of meiosis (see for review Tycko, 1997). In contrast, the fact that DNA methyltransferase enzyme is present in spermatids may be an argument against the thesis that genomic imprinting is complete at the round spermatid stage. However, it should be emphasized that waves of DNA methylation have been demonstrated during early embryonic development, the blastocyst stage, and the time of implantation (Fishel *et al.*, 1996). These observations tend to suggest that even if genomic imprinting is not complete at the round spermatid stage, genomic imprinting may be completed after the transfer of the round spermatid within the ooplasm. The work of Kimura and Yanagimachi (1995a,b) supports the latter thesis. Fishel *et al.* (1996) claim that the genomic imprinting of mouse spermatogenic cells is complete in the testis prior to the male second meiotic division.

Is it early to perform ROSNI/ROSI procedures? Ethical issues

The first successful fertilization/pregnancy via ICSI was achieved accidentally (Palermo *et al.*, 1992). Furthermore, prior to the first human ICSI pregnancy, there was a lack of studies in experimental animals evaluating the health/chromosomes/genes of offspring born after ICSI techniques. In contrast, (i) prior to performance of human ROSNI/ROSI these techniques had been applied in rabbits (Sofikitis *et al.*, 1994a) and mice (Ogura *et al.*, 1994) and resulted in delivery of healthy offspring, and (ii) human ROSNI/ROSI had been carefully scheduled before they were initially performed (Sofikitis *et al.*, 1995a; Tesarik *et al.*, 1995; Vanderzwalmen *et al.*, 1995). Thus, it appears that ROSNI/ROSI procedures had been designed more carefully than ICSI techniques had been prior to their initial clinical application.

ROSNI/ROSI techniques have been criticized by a number of scientists because of (i) genetic risks, (ii) low pregnancy rates, and (iii) inherent technical difficulties (mainly regarding the identification of live round spermatids) that do not allow the majority of the assisted reproduction centres to perform these techniques. We feel that theoretical genetic risks should not be used to exclude men

from appropriate infertility treatment. Rather, genetic risks should be extensively discussed with the ROSNI/ROSI or ICSI candidate. To date there has been no evidence of a major or minor abnormality in all ROSNI/ROSI human newborns and animal offspring. In addition, it is a fundamental human right for every couple to obtain therapy for relief of the disease of infertility. Thus, non-obstructed azoospermic men have the right to choose their treatment after adequate information. Furthermore, although specialized staff are necessary to perform ROSNI/ROSI, these techniques should be inexpensive. If fertilization is achieved, embryonic biopsy (preimplantation diagnosis) is recommended. Alternatively, couples should be advised to undergo prenatal control after achievement of pregnancy. ROSNI/ROSI may not be criticized because of their low pregnancy rates since these techniques represent the only hope for non-obstructed azoospermic men to father their own children. During the 4 years that have passed since the first application of ROSNI/ROSI in the human, >20 pregnancies have been achieved worldwide via ooplasmic injections of spermatids by seven different groups working independently (Fishel *et al.*, 1995; Sofikitis *et al.*, 1995a, 1997b, 1998a; Tesarik *et al.*, 1995; Mansour *et al.*, 1996a; Tanaka *et al.*, 1996; Amer *et al.*, 1997; Antinori *et al.*, 1997a,b; Araki *et al.*, 1997; Vanderzwalmen *et al.*, 1997). Additional research efforts are necessary to improve the outcome of ROSNI/ROSI. These efforts should be directed to the development/discovery of (i) criteria for identification of round spermatids, (ii) biochemical media prolonging the viability of spermatids, (iii) exogenous stimuli to support oocyte activation in men with OASIS deficiency, (iv) methodology to identify and purify human OASIS, and (v) methodology to study the metabolism and the implantation process of embryos generated by ooplasmic injections of spermatids. Finally, criticism of ROSNI/ROSI techniques based on their technical difficulties is not justified. Training and basic research in experimental animals is necessary for the staff of assisted reproduction centres applying ooplasmic injections of spermatids. The latter suggestion is supported by the fact that occasionally groups with excellent ICSI results but without active basic research programmes on testicular physiology have failed to demonstrate acceptable ROSNI/ROSI outcomes.

Importance of ROSNI/ROSI techniques

The majority of clinicians worldwide may consider the ooplasmic injections of spermatids as important techniques because they have resulted in delivery of human newborns fathered by non-obstructed azoospermic men. Although a baby is the target in assisted reproduction programmes we

feel that ROSNI/ROSI techniques are important for another reason: they can serve as a tool to investigate alterations in the viability, physiology, DNA integrity, and reproductive capacity of the early haploid male gamete as a response to a certain pathophysiology/toxic factor (Sofikitis *et al.*, 1996b). Thus, various laboratories of different orientations and interests such as cellular biology, cellular metabolism, molecular biology, developmental physiology, and testicular physiology have a novel assay to investigate the influence of a physiological or non-physiological stimulus on the early haploid male gamete (Sofikitis *et al.*, 1996b).

Considerations for the future: the post-ROSI era

ICSI or ROSI/ROSI procedures offer alternative solutions for men with non-obstructive azoospermia and testicular foci of spermatogenesis up to the spermatozoon or round spermatid stage, respectively. In addition, preliminary trials of human ooplasmic secondary spermatocyte injections (SECSI techniques) recently resulted in delivery of a healthy boy (Sofikitis *et al.*, 1998b). In that study the human second meiotic division was completed within the ooplasm. However, clinical application of SECSI procedures may be limited since most of the non-obstructed azoospermic men with secondary spermatocytes in the therapeutic testicular biopsy material have spermatozoa and/or spermatids. In contrast, men in whom the most advanced spermatogenic cells are primary spermatocytes cannot nowadays be candidates in assisted reproduction programmes. For the latter men three recent achievements in basic research may offer new possibilities in assisted reproduction programmes in the future.

Artificial testis/in-vitro culture of germ cells

An artificial testis may be considered an in-vitro culture system where primary spermatocytes or round spermatids will be cultured under biochemical conditions similar to testicular microenvironment, aiming to induce the human meiosis *in vitro* or to achieve generation of the spermatid flagella. The haploid products of an artificial testis may be used in assisted reproduction programmes. To create a functional artificial testis more research efforts are necessary on the factors/second messenger systems regulating meiosis in the testis. Few studies support the idea that utilization of an in-vitro culture system in assisted reproduction may be possible in the future. Early studies of Gerton and Millette (1984) demonstrated generation of spermatid flagella *in vitro*. Furthermore, Gritsch *et al.* (1997) showed that humanspermatogenic cells can survive long term in in-vitro culture conditions. In a landmark study, Weiss *et*

al. (1997) demonstrated generation of round spermatids from primary spermatocytes *in vitro*. Goto *et al.* (1996) achieved induction of the second meiotic division *in vitro*.

Transplantation of human spermatogenic cells into a host testis

Results from transplantation of mouse spermatogenic cells into mouse seminiferous tubules and rat spermatogenic cells into mouse seminiferous tubules indicate that the donor germ cells are capable of differentiating to form spermatozoa morphologically characteristic of the donor species (Russell *et al.*, 1996; Russell and Brinster, 1996). Furthermore, recent findings in our laboratory (Tanaka *et al.*, 1997) have shown that (i) spermatogenic cells isolated from hamsters with primary testicular damage are capable of transforming to hamster spermatozoa within the seminiferous tubules of host non-immunosuppressed animals, indicating that the transluminal compartment of the seminiferous tubules is immunologically privileged; and (ii) the above cellular transformations of the donor cells are also inducible within the seminiferous tubules of immunosuppressed animals after macroscopic transfer techniques. If the above studies are applied successfully in the human, primary spermatocytes of non-obstructed azoospermic men may be transformed into human spermatids or spermatozoa within a host testis, giving the opportunity to these men to be candidates for ROSNI/ROSI or ICSI.

However, even if induction of human meiosis becomes possible within a host testis, application of human ROSNI or ICSI using human haploid male gametes generated in an animal testis is susceptible to genetic and immunological risks.

Gene transfer for the treatment non-obstructive azoospermia

Gene therapy is an exciting and powerful technique capable of introducing novel genetic sequences to alter the cell phenotype. Recent data reported by Werthman *et al.* (1997) confirm successful gene transfer of a reporter gene to murine testicular tissue. This technique may have the potential to reverse the effects of genetic mutations which lead to non-obstructive azoospermia by reconstitution of the wild-type gene (Werthman *et al.*, 1997).

Acknowledgements

The authors would like to express their gratitude to Toshiko Toda, MD, PhD for participating in the development of ROSNI techniques, Manami Takenaka, BS for significantly improving the technique of ooplasmic injections of round spermatids, and Ira Sharlip, MD and Krinos Trokoudes, MD for their contribution to

the performance of the first ROSNI trials in USA and Cyprus respectively. Furthermore, we are thankful to our co-workers Panayiotis Zavos, PhD; Yasuyuki Mio, MD, PhD; Atsushi Tanaka, MD; Keiko Yamanaka, PhD; Katsuhiko Takahashi, MD, PhD; Martin Neil, PhD; George Mekras, MD; Jim Stain, PhD; Hiroshi Kawamura, PhD; Spyros Antypas, MD; Emmanouel Agapitos, MD; Konstadinos Kalianidis, MD; Nikolaos Kanakas, MD; Fotini Dimitriadou, BS; Sanae Tsukamoto, BS and Ritsa Bletsas, BS for assisting us in improving ROSNI techniques.

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Received on February 3, 1998; accepted on April 17, 1998