Round Spermatid Injection

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KEYWORDS

• Azoospermia • Assisted reproductive technology • In vitro fertilization • Male factor infertility
• Round spermatid injection • Testicular sperm extraction

KEY POINTS

- The likelihood of successful identification of mature spermatozoa during a microdissection testicular sperm extraction procedure performed for azoospermia is between 40% and 60%.
- Round spermatids, which are immature precursors to mature spermatozoa, are seen in approximately 30% of men with nonobstructive azoospermia without sperm seen at the time of microdissection testicular sperm extraction.
- A recent publication from 2018 reported that successful births could be achieved through the use of round spermatid injection (ROSI) and that children born from ROSI were not at an increased risk for congenital malformations.
- Concerns regarding the potential risk of abnormal epigenetic patterns following ROSI remain.
- Overall low success rates have limited the clinical application of ROSI, although improvements in the identification of round spermatids and the technique itself may lead to higher utilization in the future.

INTRODUCTION

Azoospermia affects 10% to 15% of infertile men and is defined as no sperm seen in the ejaculate in a centrifuged sample.[1] Although patients with obstructive azoospermia are likely to have sperm retrieved with a procedure[2] such as a testicular sperm aspiration (TESA), around 60% of men with azoospermia have nonobstructive azoospermia (NOA) and thus lower rates of successful sperm retrieval.[3] NOA is due to defects in spermatogenesis, usually from primary testicular dysfunction.[4] Studies have shown that the likelihood of retrieval of sperm in NOA patients during microdissection testicular sperm extraction (microTESE), the standard of care for sperm extraction in men with NOA, is between 40% and 60%.[5,6] Y-chromosome microdeletion is present in 3% to 15% of men with severe oligozoospermia as well as in men with NOA.[7] In a sizable portion of azoospermic men, there is no sperm seen after microTESE, making it impossible for these men to father biologic offspring. Round spermatids are precursors of mature spermatozoa and are seen in about 30% of NOA men with no spermatozoa seen on microTESE[8] (**Fig. 1**). These are immature sperm cells that still contain a haploid genome, similar to the genetic composition of mature spermatozoa. Round spermatid injection (ROSI) uses this fact to inject these sperm precursors directly into an oocyte in hopes of fertilization and pregnancy.

SPERMATOGENESIS AND SPERM FUNCTION

Spermatogenesis is the process by which diploid spermatogonia become haploid spermatozoa (**Fig. 2**).[9] The spermatogonia increase in number via mitosis, and in the first stage of spermatogenesis, mitotic division results in diploid primary spermatocytes.[10] These primary spermatocytes undergo meiosis I to form secondary

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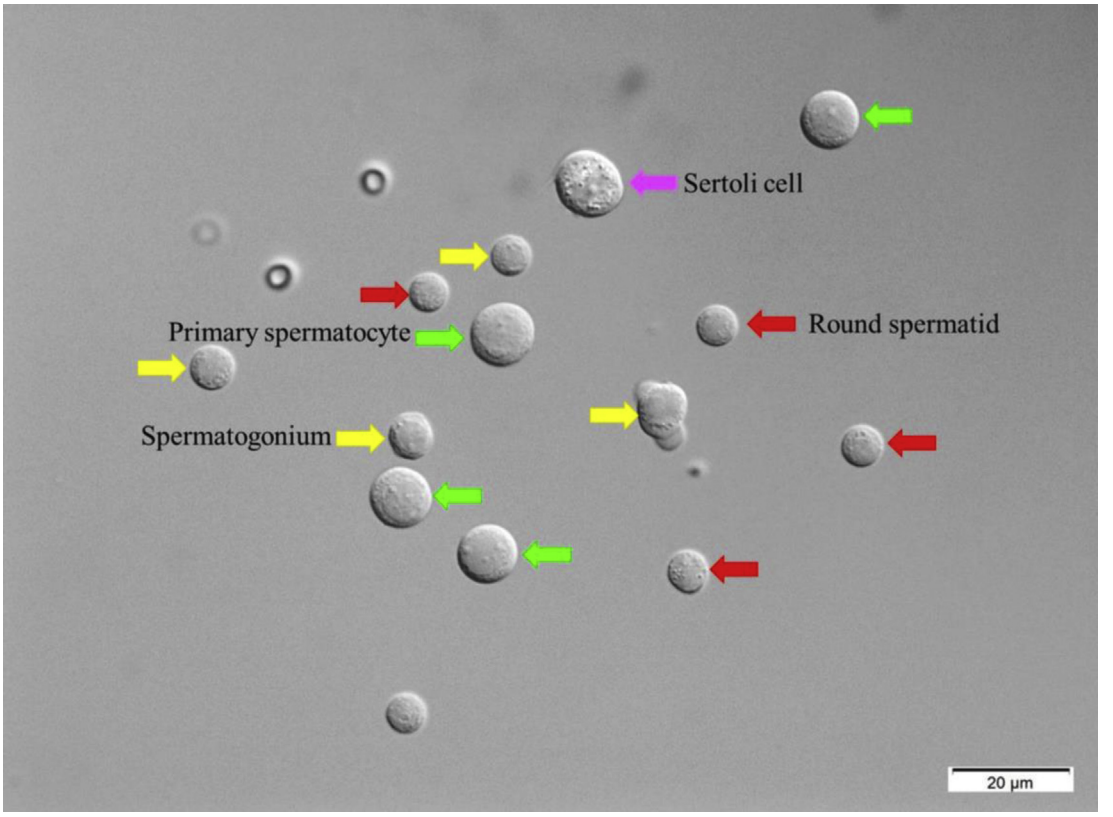

Fig. 1. Testicular cells after processing. *(Reprinted* by permission from the American Society for Reproductive Medicine [Tanaka, A., Suzuki, K., Nagayoshi, M. et al.: Ninety babies born after round spermatid injection into oocytes: survey of their development from fertilization to 2 years of age. Fertility and Sterility. 2018;110:443.])

spermatocytes and meiosis II to form spermatids,[11] such as round spermatids. At this point, spermatids have the haploid genetic material that spermatozoa contain, but the spermatids are not yet motile and are not yet able to fertilize an oocyte. In the next phase, also called spermiogenesis, the round spermatids become elongated and eventually develop a tail as they progress to become mature spermatozoa. For normal fertilization to occur, the spermatozoa must provide genetic material to the oocyte by means of the centrosome and initiate oocyte activation.[12]

HISTORY OF ASSISTED REPRODUCTION IN AZOOSPERMIA

Intracytoplasmic sperm injection (ICSI) was developed in the 1990s and has been revolutionary in allowing paternity for men with severe male factor infertility.[13–15] In this procedure, a single spermatozoon is directly injected into the oocyte. This allows for testicular sperm extraction as an assisted reproductive technology, because sperm retrieved by these methods have not fully matured and do not yet have the ability to swim or fertilize an egg. Despite initial theoretic concerns about the long-term outcomes of children born by ICSI, any negative effects appear to be minimal, and ICSI has seen widespread use in recent years.[16,17] The use of testicular sperm with ICSI has allowed many men with NOA as well as men with obstructive azoospermia to achieve fatherhood and have biological offspring. Before the advent of ICSI, there were limited options for patients with severe male factor infertility. In patients without male factor infertility, the live birth rate was 36.5% with ICSI compared with 39.3% with conventional in vitro fertilization (IVF) alone.[18] This 2015 study also found that the use of ICSI increased from 76.3% to 93.3% from 1996 to 2012 in cycles with male factor infertility present. Not only that, ICSI use increased in cycles without male factor infertility from 15.4% to 66.9% during the same time period.

ROUND SPERMATID INJECTION IN ANIMAL MODELS

In the 1990s, there were several animal studies that reported successful births and healthy offspring via ROSI. Kimura and Yanagimachi[19] in

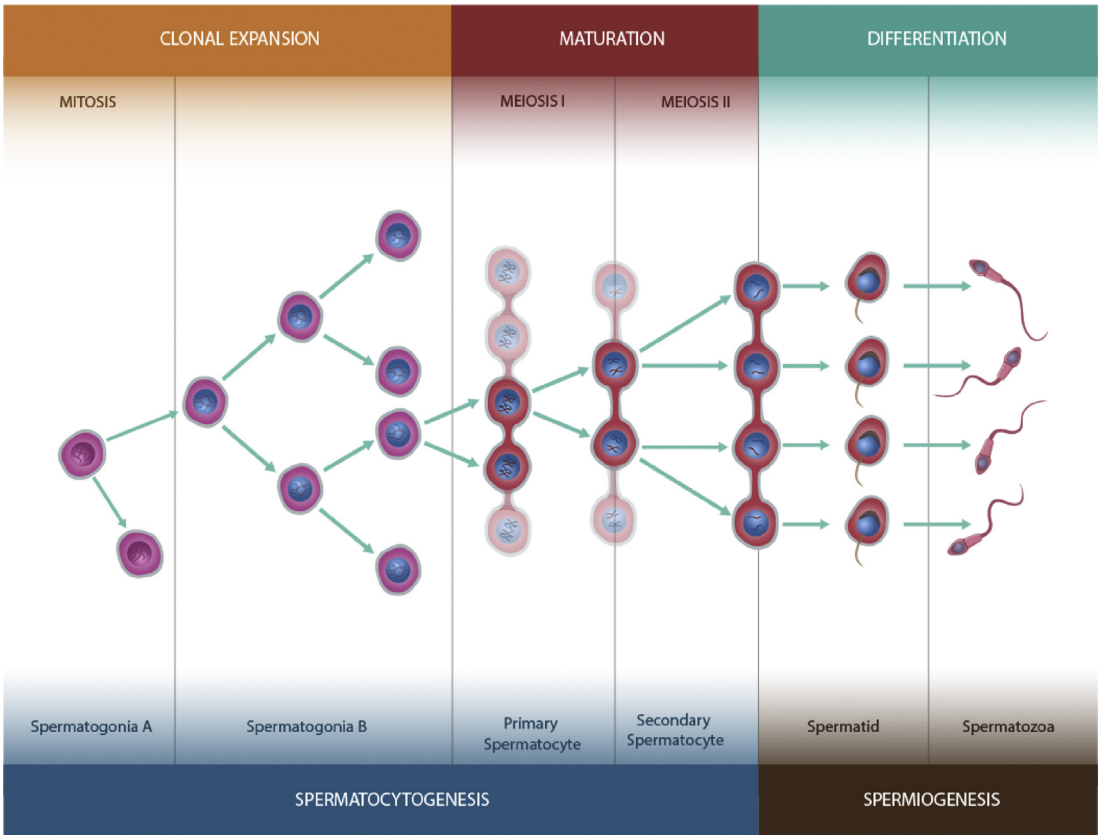

Fig. 2. Timeline of spermatogenesis.

1995 reported a fertilization rate of 77% and a pregnancy rate of 28.2% with healthy offspring in mice. They found that in the mouse, gamete imprinting happened before spermiogenesis. However, oocyte activation could not be triggered by spermatids, so this was done by electric current. Oocyte activation requires a soluble sperm factor, which is thought to be contained in spermatozoa's cytoplasm; it enables oocytes to develop a characteristic series of calcium spikes that round spermatids seem to lack, but it was found that round spermatids could be treated with a calcium ionophore.

In 2011, Ogonuki and colleagues[20] looked at fertilization of mouse oocytes using round spermatids without using artificial oocyte activation. Round spermatids in mice lack the capacity to activate an oocyte at this stage, but the investigators found when the round spermatids were frozen and thawed before microinjection, a proportion of them still developed into 2-cell embryos without artificial activation. Using frozen-thawed spermatids was thought to help with the oocyte-activating capacity in this study.

Ogonuki and colleagues[21] in 2017 studied spermatid injection in the common marmoset using immature male marmosets. The spermatids were found to acquire the ability to activate an oocyte at the late round spermatid stage. Marmoset oocytes were then microinjected with frozen-thawed late round spermatids and were able to develop to the 8-cell stage.

Despite the feasibility of this procedure, the broad adoption of ROSI has been limited because of controversy surrounding using this beyond research purposes. In addition, it must be noted that physiologic differences in the oocyte activation process between animal models and humans may exist. Therefore, certain oocyte activation protocols and fertilization techniques, which demonstrate success in animals, may not result in successful results in humans. The issue of potentially increased rates of embryonic aneuploidy and epigenetic aberrations must also be considered in humans, whereas, in animals, these issues may have a lesser role.

CLINICAL USE OF ROUND SPERMATID INJECTION

The first report of human fertilization with spermatid injection was by Vanderzwalmen and

colleagues[22] in 1995. Tesarik and colleagues[23] then published a case series in 1996 of 11 cases of spermatid injection, 6 with round spermatids (Table 1). Fertilization occurred in 10 of 11 treatment cycles, and a pregnancy was achieved in 2 ROSI cycles, which then proceeded to live birth. However, these results were not replicated at fertility centers across the world when first

Table 1
Outcomes of clinical studies of round spermatid injection

Author, Year	Fertilization Rate, %	Pregnancy Rate, %	Live Birth Rate, %	Oocytes Injected	Oocytes Fertilized	Embryos Transferred
Tesarik et al,[23] 1996	35.9	16.7	16.7	39	14	12
Vanderzwalmen et al,[25] 1997	21.9	14.3	14.3	260	57	7
Antinori et al,[26] 1997	55.6	3.6	—	135	75	56
Antinori et al,[27] 1997	46.7	16.7	—	15	7	6
Yamanaka et al,[28] 1997	69.4	0.0	0.0	49	34	24
Kahraman et al,[40] 1998	25.6	3.1	0.0	199	51	32
Barak et al,[41] 1998	62.2	4.3	4.3	37	23	23
Bernabeu et al,[29] 1998	44.9	0.0	0.0	69	31	31
Ghazzawi et al,[30] 1999	22.0	0.0	0.0	574	126	40
Al-Hasani et al,[31] 1999	18.4	0.0	0.0	49	9	9
Gianaroli et al,[56] 1999	40.0	50.0	50.0	5	2	2
Balaban et al,[57] 2000	56.2	—	—	356	200	—
Tesarik et al,[58] 2000	53.8	—	—	26	14	—
Levran et al,[32] 2000	45.5	0.0	0.0	178	81	48
Vicdan et al,[33] 2001	28.3	0.0	0.0	69	17	5
Urman et al,[34] 2002	40.5	0.0	0.0	1021	414	16
Sousa et al,[35] 2002	15.9	0.0	0.0	126	20	9
Khalili et al,[36] 2002	21.4	0.0	0.0	42	9	6
Sousa et al,[39] 2002	34.6	—	—	26	9	—
Ulug et al,[37] 2003	41.7	0.0	0.0	36	15	10
Tanaka et al,[8] 2015	59.5	14.4	5.8	734	437	208
Tanaka et al,[9] 2018	56.8	3.6	2.2	14,324	8132	3882

Data from Refs.[8,9,23,25–37,39–41,56–58]

attempted.[24] Tesarik and colleagues stressed the importance of using the whole round spermatid, avoiding the use of just the nucleus. Vanderzwalmen and colleagues[25] published a series in 1997 of 73 azoospermic men in which 260 oocytes were injected with round spermatids. Of a total of 39 transfers, 5 pregnancies were achieved with a total of 3 term births, 1 miscarriage, and 1 ongoing pregnancy. The implantation rate was 5.5%.

Antinori and colleagues[26] published 2 studies in 1997. One study looked at 2 azoospermic men with only round spermatids. Of the thawed spermatids, 70% were found to be viable for injection. Of 15 oocytes that were injected, 7 fertilized normally. There were 6 embryos at the 4- to 6-cell stage and 1 ongoing clinical pregnancy. The second study looked at 36 patients with NOA, 19 of which only had round spermatids present.[27] Another 17 patients had elongated spermatids. Of 135 oocytes from 19 partners that were injected with round spermatids, a fertilization rate of 55.6% was found as well as a pregnancy rate of 3.6%.

In 1997, Yamanaka and colleagues[28] injected 49 mature oocytes with round spermatids from men with spermatid arrest at the round spermatid stage or primary spermatocyte stage. A total of 24 embryos were transferred, but no pregnancies were achieved. Similarly, a 0% pregnancy rate was found by Bernabeu and colleagues[29] in 1998, Ghazzawi and colleagues[30] in 1999, Al-Hasani and colleagues[31] in 1999, Levran and colleagues[32] in 2000, Vicdan and colleagues[33] in 2001, Urman and colleagues[34] in 2002, Sousa and colleagues[35] in 2002, Khalili and colleagues[36] in 2002, and Ulug and colleagues[37] in 2003, so there were clear difficulties nationwide in achieving the promising results that some centers were able to achieve with ROSI.[38]

Sousa and colleagues[35,39] in a retrospective study evaluating 159 treatment cycles in 148 azoospermic patients found injection of intact round spermatids resulted in very low rates of fertilization (17%) and no pregnancies achieved. Likewise, Levran and colleagues[32] studied the comparison of ICSI and ROSI from testicular sperm extraction samples for both and compared the results between frozen and fresh samples in a retrospective analysis of 18 infertile couples whereby the men had NOA. The fertilization and cleavage rates following ROSI with fresh versus frozen-thawed were comparable; however, the fertilization rate was 44%, which was significantly lower than ICSI (69%), and a surprisingly higher rate of cleavage arrest was found in ROSI (40%) compared with ICSI (8%). Also, no pregnancy was achieved through ROSI compared with a 50% clinical pregnancy rate by ICSI.[32] However, it is important to note that there was no method of oocyte activation being used.

In 1998, Kahraman and colleagues[40] described 20 men in whom only round spermatids were found. Of 51 oocytes fertilized, there was 1 clinical pregnancy, but unfortunately this ended in an early spontaneous abortion. Barak and colleagues[41] looked at 13 couples with male factor infertility and with 37 oocytes injected and found a 62.2% fertilization rate and a 4.3% live birth rate. Gianaroli and colleagues achieved a live birth in a single patient using frozen-thawed spermatids with 2 oocytes fertilized of 5 injected.

Similarly, in a prospective analysis, Benkhalifa and colleagues[42] assessed 14 couples who underwent ROSI and fluorescence in situ hybridization (FISH) and preimplantation genetic diagnostic. This resulted in a fertilization rate of 36% with no pregnancies achieved. Not surprisingly, only 11 out of 143 oocytes developed to have several blastomeres, and cytologic/cytogenetic abnormalities accounted for most of the blockage at oocyte, zygote, and early mitotic division stages, with only 4 biopsied embryos being normal, all of them being implanted without success.

Goswami and colleagues[43] attempted to use ROSI for treating 2 NOA patients. For the first patient, calcium chloride was used to activate the oocyte, ending in a 25% fertilization rate (2 out of 8). Using ionomycin gave a fertilization rate of 63% (8 out of 13), even though no pregnancy was achieved, and no abnormality was seen in the embryos.

Tanaka and colleagues[8] described in 2015 the birth of 14 babies from ROSI to human oocytes. All patients had undergone a microTESE, and seminiferous tubules were enzymatically dissociated and kept frozen until their use for ROSI. After thawing, through a differential interference microscope, the round spermatids were identified by their size and morphology and confirmed by FISH and karyotyping. ROSI combined with electric stimulation was used to induce oocyte activation; therefore, all oocytes were stimulated 10 minutes before ROSI. In total, 730 NOA patients that had undergone previous microTESE in other institutions participated in 163 transfer cycles. This resulted in 14 pregnancies, all of which were karyotypically normal, with average gestational age and normal birth weight. There were no developmental effects noted at 2 years. Cryopreserved and thawed spermatids yielded a better result than fresh with fertilization rates of 76.4% and 55.6%, respectively, and a pregnancy rate of 23.8% in the frozen group compared with 16.5% of the fresh sample group.[8]

Tanaka and colleagues[9] published a second study, with a total of 90 babies born by ROSI. From a total of 721 men who participated in ROSI, 90 babies were born and were followed for 2 years with repeated measures of physical and cognitive development. The fertilization rate was nearly the same as in the past study, with the frozen group performing better than the fresh sample group, 58% and 52.7%, respectively. Likewise, the pregnancy rate was higher in the frozen group with 15.8% in contrast to 5.4%. Only 3 children of the 90 had congenital malformations, all of them corrected through surgery (cleft lip and omphalocele) or spontaneously (ventricular septa). Although the fertilization and pregnancy rates are highly different between ROSI and ICSI, the 90 babies developed normally in both physical and cognitive spheres at their first 2 years after birth compared with the naturally conceived control group.

Taken as a whole, it appears that early attempts to use ROSI in humans were unsuccessful. The lack of clinical success led to a subsequent decrease in the popularity of the procedure. However, given the recent reports of higher success rates and reassuring long-term developmental outcomes within ROSI offspring, a resurgence in interest surrounding ROSI may occur in the coming years. Because laboratory techniques, embryo culture protocols, and success rates with IVF and ICSI have improved over the last decade, success rates with ROSI in the setting of a modern IVF laboratory may also improve. When evaluating the potential utility of this technique, one must consider that the laboratory environment in the late 1990s and early 2000s when ROSI was first described was quite different than it is today.

CHALLENGES AND INNOVATION

Novel methods are being tried to solve core difficulties regarding the ROSI procedure. A key difficulty many centers had was in recognizing the round spermatid under the microscope.[44] It is not easy to recognize and discriminate immature spermatogenic cells, particularly round spermatids, with complete confidence.[12,24] The identification was mainly through morphology, although round spermatids do have a similar appearance to lymphocytes.[12] It is normally a cell of 7 to 8 μm with a visible nucleus, surrounded by continuous cytoplasm; an acrosomal granule, if it appears, is a bright spot adjacent to the nucleus.[45] Hayama and colleagues[46] developed a simple flow cytometry-based method to isolate round spermatids. Similarly, microfluidics, which is a technology that uses small volumes of fluids, has begun to be used in sperm selection and testing and conceivably could be implemented in helping to identify and separate round spermatids.[47] There have also been concurrent interesting advances in using microfluidics for sperm sorting, although this is beyond the scope of this review.[48]

Another conceivable technology that could be expanded to improve the identification of round spermatids is through single-cell sequencing.[49,50] This has been used to identify markers in human spermatogonial stem cells, and the technology could be used to identify and target markers for round spermatids that could improve the rate of identification and thus likely overall success with ROSI.

There are also concerns about epigenetic abnormalities associated with improper methylation patterns owing to immature spermatozoa. There have been concerns associated with the epigenetics in assisted reproductive techniques increasing the risk of imprinting disorders adversely affecting embryonic development owing to using immature spermatids.[51] Deregulation of imprinted regions has been associated with Angelman syndrome and possibly Beckwith-Wiedemann syndrome. Kishigami and colleagues[52] found distinct methylation patterns between injections of round spermatids versus spermatozoa. Men with impaired sperm production also more often had increased aneuploidy, which may also explain the increased risk of sex chromosome abnormalities in conceptions from ICSI. The spermatid is a haploid cell with a decondensed nucleus, which is mainly composed of histone proteins, in contrast to spermatozoa, whereby the predominance is of protamines. It was hypothesized that the lower fertility rate achieved by ROSI was due to such differences in the chromatin structure affecting the consequent reprogramming of the paternal genome. Kong and colleagues[53] used a histone deacetylase inhibitor named “Scriptaid” to inhibit the typical hypermethylation observed in the spermatid-oocyte interaction, assessing for blastocyst formation and birth rate.

Precise genome editing is a promising tool for analysis of gene function; the CRISPR-Cas9 system from bacteria has been used in numerous species for modifying the genome with high sensitivity and specificity. Protocols are being developed for using this system for transplantation of the gene-modified spermatogonial stem cells-derived round spermatids for producing healthy offspring.[54] Wu and colleagues[55] used CRISPR-Cas9 to mutate an EGFP transgene or the endogenous Cryqc gene in spermatogonial stem cells after transplantation to infertile mouse testes to

develop round spermatids, which were injected into mature oocytes.

SUMMARY

At this time, challenges still remain in ROSI becoming a widespread technology, and overall low success rates have limited its adoption. After initial trials in animal models, early studies of ROSI in humans had varied results and did not gain traction as a widespread procedure that could be used in azoospermic men who did not have mature spermatozoa on microTESE in large part because of difficulties many centers had in replicating the early outcomes. Recent studies have showed improvements in outcomes compared with the initial studies and on a larger scale. Broader adoption of the technology will likely need to be preceded by improvements in identification of round spermatids, although there are several possibilities that could be developed to improve the process. In addition, the possibilities are immense as to what can be done to take things beyond the current standards. There is still room for improvement in making this accessible and more successful, but feasibly allows azoospermic men to father biologic children where no sperm is seen on microTESE.

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