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Round Spermatid Injection (ROSI) - A Challenging Way of Fertility Treatment of Azoospermic Men

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Abstract

Intracytoplasmic sperm injection (ICSI) is a revolutionary technique of treatment of azoospermia where natural pregnancy is impossible. For this technique sperm must be available in testes. In case of maturation arrest, Sertoli-cell-only syndrome (SCOS) and significant hypospermia, where mature sperm is not available ICSI is not possible. If immature spermatozoa or round spermatids can be injected in the same way, fertilization is possible in these cases. Though identification of round spermatids is difficult, different techniques are adopted for correct identification and isolation from other similar cells. Oocyte activation is an essential part to overcome the low fertilization rate. In-spite of that, in general fertilization and pregnancy rate is much lower than conventional ICSI by mature spermatozoa. Scientists proved that congenital anomaly rate is not higher than conventional ICSI or naturally born babies. There is no significant differences of physical and mental growth and development between ROSI babies and naturally born babies.

Introduction

Injection of round spermatid in oocyte (ROSI) is a type of assisted reproductive technology. When there is no sperm in ejaculate of a man is called azoospermia. So, there is no scope of natural pregnancy in case of azoospermia. Before 1992 azoospermic men were unfortunate, and they remained childless as there was no treatment before that period. Intracytoplasmic sperm injection (ICSI), the revolutionary treatment, "the state of the art" in assisted reproductive technology made it possible to become a father of an azoospermic man. But there are still some cases of azoospermia where in spite of micro TESE sperms are not available. In such cases, round spermatid or immature spermatozoa can be injected as an alternative. During the normal process of spermatogenesis, spermatogonia divide to produce primary spermatocytes. These spermatocytes enter the first meiotic division to produce secondary spermatocytes. Secondary spermatocytes divide again by a second meiotic division that results in the formation of round spermatids, which contain a complete haploid set of chromosomes. These round spermatids start to elongate and to lose their cytoplasm until they resume the typical appearance of mature spermatozoa. This final complex stage of maturation is known as spermiogenesis.

In cases of arrest of maturation at the spermatocyte stage (spermatogenesis), the germ cells are still in a diploid state and therefore cannot be considered for injection into the egg cytoplasm. In the case of maturation arrest at the stage of round spermatids (arrest of spermiogenesis), the germ cells have already accomplished the haploid state and fertilization of egg is possible if spermatid can be injected into the oocyte.

History of ROSI

The first successful fertilization of hamster oocyte by injecting spermatid nuclei was demonstrated by Ogura and Yanagimachi in 1993[1]. Successive experiments on animals brought success getting pregnancies after injection of round spermatids in 1994 and 1995 [2,3]. In 1994 Edwards et al. suggested the clinical use of round spermatids for the management of nonobstructive azoospermia[4]. First human ROSI birth was reported by Tesarik and Mendoza and Tesarik et al in 1996 [5,6]. Out of 39 cases of ROSI they got two pregnancies. Subsequently a number of investigators reported on-going pregnancies and live birth babies after ROSI[7-10]. There were many unsuccessful attempts too[11-13]. As ROSI was considered for experimental rather than clinical practice, investigators lost their interest. It is Atushi Tanaka from Japan who continued treating azoospermic men by ROSI. He reported 90 baby's outcome including

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their two years follow up of physical, mental and epigenetic condition[15]. He shown that there was no physician, mental and epigenetic problems and there was no significant difference between babies of ROSI compared to babies from spontaneous pregnancy. So, this can be an excellent alternative for patients who do not want donor sperm or adoption where no sperm is available for ICSI.

Who are the candidates for ROSI?

In non-obstructive azoospermia three types of tissue identified in histopathology of biopsied material. i) Reduced spermatogenesis with all stages of germ cells (hypospermia), ii) arrest of final stage of spermatogenesis (maturation arrest), and iii) complete absence of germ cells (Sertoli-cell-only syndrome, SCOS)[16]. Maturation arrest is defined as an absence of mature spermatozoa due to an arrest of development of spermatozoa. It may be early maturation arrest (MA) at the spermatogonia, or spermatocyte stage or late MA occurs at spermatid stage[17-19]. Spermatids are the male germ cells with single set of chromosomes after completion of meiosis. At this stage all structural elements are present except morphological changes. These spermatids undergo a complex cellular differentiation and maturation process known as spermiogenesis to form a complete motile spermatozoa with head, body and tail. The most important changes that take place in this procedure is nuclear DNA packaging or condensation, replacing of lysin-rich histone by arginine-rich protamine and formation of disulphide bonds that stabilize the chromatin structure.[20-22]. Other changes are genomic imprinting, disappearance of the distal centriole and the formation of the acrosome. Without these changes though spermatid can not go into the stage of fully formed spermatozoa, fertilization of ovum by injecting round spermatid is possible. In men with nonobstructive azoospermia where neither mature spermatozoa nor late-stage spermatids were isolated from testicular samples, in 30% cases round spermatids were retrieved from surgical samples[5]. These patients are the candidates for ROSI.

How to identify round cells?

It is not easy to identify round cells from ejaculate or extracted testicular tissue. Main problem is identification and differentiation from other cells. Although karyotyping and fluorescence in situ hybridization (FISH) can identify exactly the haploid spermatids, it is not ideal way to use this invasive procedure for ROSI. Tesarik and Mendoza identified round spermatids on the basis of morphologic characteristics of small size of the cell with clear rim of cytoplasm all around the nucleus, and a centrally located, smooth, uncondensed nucleus [5]. Subsequently Tanaka et al clearly described the identifying points for correct identification of round cells which is the key to success in ROSI[14,15]. To identify round cells testicular tissue enzymatically dissociated and macerated. He identified round spermatids from other cells based on their physical properties. There may be confusion of identifying round spermatid from other spermatogenic cells like spermatogonium and Sertoli cells and also blood cell lymphocytes.

Fig 1: Testicular cells after treatment Sertoli cell (green arrows); round spermatids (yellow arrows); spermatogonia with or without pseudopodia (red arrows), and primary spermatocytes (blue arrows), erythrocytes (purple arrow).

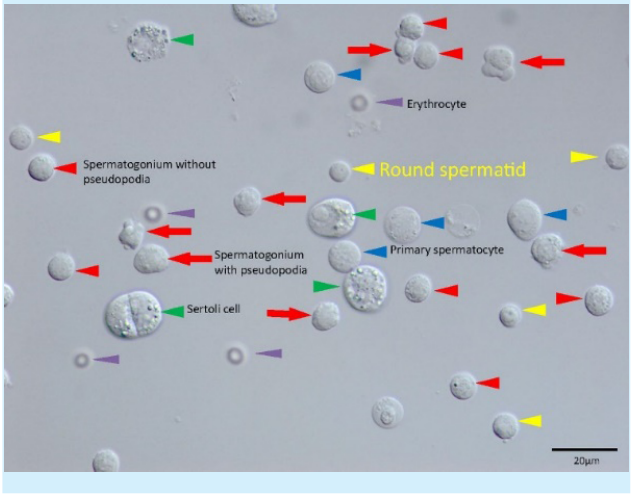

Fig 1 shows different types of cells under microscope. Large Sertoli cells and large primary spermatocytes could be readily identified. Smaller spermatogonia and round spermatids are similar in size, may be difficult to distinguish and can be distinguished by examination under interface-contrast microscope. Nuclei of spermatogonia contains two or more nucleoli whereas spermatid does not. In addition, cytoplasm surrounding the nucleus of spermatid is thinner, so slightly higher nucleus-cytoplasm ratio than that of spermatogonium. Another differentiating point of round spermatid is that it's cytoplasm can be readily separated from the nucleus when the cell is drawn back and forth within the injection pipette. Nucleus looks like transparent, round and "bouncy". But in some cases, it did not happen may be due to apoptosis of the cell as described by author. On the other hand, somatic cells and blood cells e.g., lymphocytes, having similar size of round spermatids have flexible and tough plasma membrane, which can not be readily broken even by vigorous pipetting. Though spermatogonium also disrupted and nucleus can be separated from cytoplasm like round cells, it can be differentiated by presence of nucleoli in spermatogonium. Spermatogonia shows active pseudopodia which is not seen in other cells (Fig 1) [23]. Another identifying point of round cell is presence of acrosome vesicles or acrosome cap on the nucleus of small round cells[14,15].

Procedure of injection

Round spermatid injection can be done by similar mechanism of intracytoplasmic sperm injection. Difference is that it needs non spike needle having 8-10 µm in diameter (PIAZO) to inject spermatid. Another important thing is activation of oocyte. Though human round spermatids may contain some oocyte-activating ability, complete activation of oocyte may not occur[24]. Ogonuki et al reported that fresh round spermatids when injected into mouse oocyte were unable to activate oocytes. They noticed that when frozen and thawed spermatids were injected oocytes were activated properly[25]. It is thought that some endogenous oocyte-activating factors may mobilize during freezing thawing process[26]. Tanaka et al mentioned that electrical stimulation

was the most effective in inducing repetitive large Ca2+ oscillations and oocyte activation till exploring the best method to activate human ROSI oocyte[14]. Phosphatidyl inositol[26, 27], phospholipase[28-30], Ca2 ionophore[31-33], 4-dimethyle amino-pyridine[34], and post acrosomal protein (PAWP) can be considered as oocyte activating factor[35-37].

Fertilization and pregnancy rate in ROSI

Fertilization rate varies widely in different studies by different scientist. Till publication of 2018 fertilization rate after ROSI was 0-56.77% [6, 8-10, 15, 35-51]. Highest fertilization rate shown by Tanaka et al[15]. Pregnancy rate was 0-28.57% (2/7) [6,8-10,15, 38-54]. But largest series performed by Tanaka et al shows pregnancy rate 19.14% (138/721). Though pregnancy rate per embryo transfer was 3.6%, average pregnancy rate was 13.37% and live birth rate was 8.9%. among 22 studies. Only one case report shows 1 pregnancy from 1 couple[46]. A large number of studies reported no pregnancies from ROSI[11-13, 38,39,41, 43-45, 50-52,54].

Fertilization rate increased if elongated spermatid can be injected. Al- Hasami et al shown higher fertilization rate when elongated spermatid was injected (ELSI). Fertilization rate was 56% in ELSI in comparison to only 18% in ROSI. In ELSI cycle two pregnancy were achieved from two patients, whereas no pregnancy was achieved in 4 patients from ROSI cycle[45]. Elongated spermatids can be easily identified than round cells as they acquire head shape and becomes elongated.

Probable reasons of low fertilization rate in ROSI

As mentioned earlier that identification of round cells from other cells is difficult. This difficult identification of round cells and incomplete activation of oocyte are the factors of low fertilization rate of ROSI. In addition, immaturity of the spermatids may be the main factor to lower fertilizing capacity of round cells. In immature cell there is no transition of histone to protamine, which might lead to chromatin instability and sensitivity. It makes spermatids more vulnerable to denaturing stress leads to DNA fragmentation and apoptosis. Protamine is essential substance for transformation of spermatid nucleus into male pronucleus. This event can not take place due to premature chromatin condensation due to absence of protamine[55,56]. Genetic abnormality and aneuploidy may be other factors for consideration. Different studies reported that men with maturation arrest have an increased frequency of detectable genetic abnormalities[57-60]. This genetic abnormality may be due to defective genomic imprinting. Genomic imprinting may occur during late gametogenesis, plays an important role in the regulation of fertilization and embryo development.

In-vitro maturation of round cells

Identification of round cells is difficult, which effect the ultimate outcome of the procedure. But investigators explored many different physical characteristics for successful identification of the round cells[5,14,15]. In-spite of that, success in terms of fertilization of injection of round cells are much lower that that of elongating or elongated spermatozoa45. That is why concept of in-vitro maturation came to overcome this barrier. According to report of investigators 22% round spermatids can grow flagella under in-vitro culturing in modified Eagle's medium with no hormonal supplementation[61]. Cremades et al reported that it is possible to mature the round cells up-to elongating/ elongated step and even to mature spermatozoa when co-culture on Vero cell monolayer at 320 C for seven days[62]. In-vitro maturation of germ cells may overcome the inadvertent use of apoptotic spermatids and other cells and increase the fertilization rate[63]. Initially some authors concluded that in-vitro culture offers no clinical benefit except improving fertilization rate[64,47]. But birth of babies reported by other authors after in-vitro culture of round cells in complete and incomplete maturation arrest[65-67]. Not only round cells can be grown to advance stage by co-culturing, but primary spermatocyte can also be grown to round spermatid using co-culture with a Vero cell line. Tanaka et al were the first team reported in-vitro development of four round spermatids from single primary spermatocytes using co-culture with a Vero cell line[68].

Safety of ROSI

ROSI by passes all the physiological steps of fertilization. It does not require the transformation of haploid spermatids into motile tadpole-like cells. So, safety of injection of immature haploid germ cells has been questioned. One of the important issues of these artificial technologies is the possibility of genetic and epigenetic risks to the offspring. Genomic imprinting essentially occurs during gametogenesis and there is possibility of incomplete or defective imprinting in immature gametes or in gametes those are matured under artificial environment e g in-vitro culture[69]. During spermatogenesis, the histone- to-protamine transition ensures protection from mutation of the spermatid DNA, which is missing in round spermatids. Absence of this histone-to-protamine transmission in round cells of animal model showed high level of DNA damage[70]. High level of DNA damage also found in round spermatid in patients with complete spermatogenesis failure in NOA[69]. Chromatin structure of the immature cells differ from mature cells, which might affect the epigenetic behaviour of the paternal genome[71]. There is incomplete male-specific DNA methylation imprinting in round spermatids. Therefore, strict control of DNA methylation in the preimplantation embryo is necessary for normal development. Moreover, immature cytoplasm of immature cell is concern factor. The centrosome and oocyte activating factors are two important factors for normal embryonic development. Abnormal, immature, defective or damaged centrosomes of immature spermatids may cause abnormal spindle formation, which leads to embryonic arrest, mosaicism and anomalies in spermatid injection[72]. In 2000 Zech et al reported two major congenital (Arnold-Chiari malformation and hydrocephaly combined with trisomy 9) malformations in four pregnancies achieved using ELSI[73].

In-spite of these short comings a number of healthy babies born after ROSI. In 2018 Tanaka et al[15] reported birth defect and the 2 years postnatal follow up result regarding physical and cognitive growth and development of 90 ROSI babies. This is the largest series in the world. Only 3.3% congenital abnormalities found in the form of cleft lip in one, ventricular septal defect in one and omphalocele in one case. The congenital anomaly rate is not more than normal population (3%)[74, 75]. There was no statistically significant differences of childhood growth and development between ROSI babies and natural pregnancy babies at 24 months of age[15]. There were no unusual physical or mental aberrations and was no significant differences in cognitive development.

Conclusion

Round spermatid injection is a challenging procedure for reproduction as success rate is very low till today. Both fertilization and implantation rates are low with round spermatids, though it is a bit higher with elongated spermatids. As identification is difficult in case of round spermatids, it can be grown to elongated spermatid in vitro. Whatever may be the success rate of ROSI, the success of individual patient, who has no other way of being a father must be accounted. Considering this, the challenge of ROSI should be taken both by patient and clinician.

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