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Round Spermatid Injection (ROSI): A review of biological potential, isolation methodologies and clinical applications

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Abstract

Round Spermatids (RS) represent the earliest haploid stage of male germ cells, possessing the essential chromosomal imprint required for fertilization despite lacking the structural attributes of mature spermatozoa. This review explores the biological potential and clinical application of Round Spermatid Injection (ROSI) as a therapeutic strategy for severe male infertility caused by spermiogenic arrest. Experimental evidence from animal models, including mice and rabbits, has demonstrated that RS nuclei can support full-term embryonic development, a finding subsequently translated to successful human pregnancies. Beyond reproduction, RS are also highlighted as effective vectors for transgenesis due to their accessible chromatin structure prior to protamine condensation. However, the clinical efficacy of ROSI is challenged by the difficulty in distinguishing viable spermatids from somatic contaminants like lymphocytes under standard optics. Consequently, this review critically examines isolation methodologies, emphasizing the superior purity achieved through Fluorescence-Activated Cell Sorting (FACS) compared to traditional sedimentation. Furthermore, it details molecular validation techniques using specific markers such as PRM2 and SP-10, alongside non-invasive mitochondrial selection protocols, which are pivotal for ensuring the accuracy and safety of using immature germ cells in assisted reproductive technologies.

Keywords: ROSI, biological potential, isolation methodologies, technologies, clinical applications

Introduction

Round spermatids represent the initial haploid phase of male germ cells, existing just prior to their morphological transformation into tailed spermatozoa. While these precursor cells are naturally incapable of fertilizing a mature oocyte, experimental trials with animal models have demonstrated that fusing them with oocytes can result in the birth of healthy, fertile offspring of both sexes. These results indicate that the nuclei of round spermatids already possess the necessary paternally imprinted chromosomes for complete embryonic development. Furthermore, this suggests that the intricate post-meiotic structural changes that usually occur within the testes are not strictly required for the genetic contribution to fertilization (Ogura *et al.*, 1994) ^[21].

Round spermatids (RS) represent a potential therapeutic resource for treating male infertility caused by failures in spermiogenesis. Offspring following transfer of the embryos produced by round spermatid injection (ROSI) into oocyte have been reported in mammals. Ogura *et al.* (1994) ^[21] reported that when mouse spermatids were fused with oocytes, some of the resulting zygotes developed into normal mice. In addition, Sofikitis *et al.* (1994) ^[30] reported that the intracytoplasmic injection of a round spermatid nucleus resulted in embryonic and fetal development in the rabbit in the human, Vanderzwalmen *et al.* (1995) ^[39] reported the successful fertilization of a human oocyte by the intracytoplasmic injection of a late-stage spermatid and Fishel *et al.* (1995) ^[11] and Tesarik *et al.* (1995) ^[36] reported pregnancy and birth after spermatid injection into oocytes. These reports demonstrated that the nuclei of spermatids can provide the paternally-imprinted chromosomes needed for full embryonic development.

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The RS have been proposed as substitute cells for transfection with foreign DNA to produce transgenic cattle (Camaioni *et al.*, 1992; Perry *et al.*, 1999) [8, 26]. During spermiogenesis, spermatozoa undergo a serial modification such as the change of protein molecules of DNA from histones to protamines as evidenced by transition proteins (TP1 and TP2), (Hecht, 1998) [13]. Formation of protamines in spermatozoa leads to the complex compaction of the DNA and protein resulting in difficulty of exogenous DNA insertion into spermatozoa. Therefore, it has been suggested that round spermatid prior to formation of protamine can be used as a nuclear donor for producing transgenic animals (Huang *et al.*, 2000) [14].

Spermatogenesis and sperm function

Spermatogenesis is defined as the developmental pathway through which diploid spermatogonia differentiate into haploid spermatozoa (Tanaka *et al.*, 2018) [34]. This process begins with the mitotic proliferation of spermatogonia, generating diploid primary spermatocytes (Neto *et al.*, 2016) [20]. These primary cells then enter meiosis I to produce secondary spermatocytes, followed by meiosis II, which yields haploid round spermatids. Although these spermatids possess the necessary genetic payload found in mature sperm, they lack motility and the functional capacity to fertilize an oocyte. Consequently, they must undergo spermiogenesis, a transformation phase where round spermatids elongate and develop tails to become mature spermatozoa. For fertilization to be successful, the resulting spermatozoon must not only transfer its genetic material but also supply the centrosome required to trigger oocyte activation (Aslam *et al.*, 1998) [3]. It has been proposed that differentiation of spermatids into mature spermatozoa serves only to provide the means to transport paternal genetic information to the oocyte. Therefore, in theory, the injected haploid genome of the round spermatid nucleus may be sufficient for fertilization and subsequent embryonic development in some species.

Earliest reports of round spermatid injection in animal models

In vitro fertilization combined with the assisted fertilization technique of ICSI has enabled the highly successful treatment of severe male factor infertility (Palermo *et al.*, 1992). In this procedure, only one viable sperm is needed for each oocyte retrieved. According to Van Steirteghem *et al.* (1993) [38] fertilization rates are in the range of 60 to 70% and delivery rates per retrieval are 38.5% in the first 560 couples. Ogura *et al.* (1994) [21] first reported that the nuclei of mouse round spermatids could initiate normal embryo development when mature oocytes were electrofused with spermatids and that the offspring were born normal and fertile. In rabbits, intracytoplasmic injection of round spermatid nuclei can participate in complete fertilization and subsequent embryonic and fetal development (Sofikitis *et al.*, 1994) [30]. However, the incidence of normal fertilization was very low using these methods.

The incidence of normal fertilization is improved first by electrical stimulation of the oocytes and then by injecting denuded spermatid nuclei (Kimura and Yanagimachi, 1995) [15]. In mice, the fertilization rate with spermatid injection is now almost the same as that with epididymal or testicular sperm injection. Sasagawa and Yanagimachi (1997) [28] reported that mouse round spermatids collected from cryptorchid testes, as well as those collected from the testes after reversal operations, can be used as substitutes for mature spermatozoa in *in vitro* fertilization to produce normal

offspring. Additionally, Sasagawa *et al.* (1998) [29] demonstrated that successful fertilization and the development of healthy offspring are possible when oocytes are injected with round spermatids harvested from prepubertal testes at the onset of their appearance.

Reference	Year	Animal	Embryo transferred	Pregnancies
Ogura <i>et al.</i>	1994	Mouse	346	6
Sofikitis <i>et al.</i>	1994	Rabbit	121	3
Kimura and Yanagimachi	1995	Mouse	131	37

Tesarik *et al.* (1995) [36] recovered round spermatids from semen of men with unexplained azoospermia and injected them into mature oocytes. One healthy baby was delivered by cesarian section at 39 weeks of gestation. Antinori *et al.* (1997) [2] also reported two successful pregnancies using intracytoplasmic injection of round spermatids. Thus, even with men who have no sperm production in their testes, round spermatids might be used in micromanipulation procedures with some chance of contributing to pregnancies.

Identification of spermatids

With the usual ICSI set-up (with Hoffman or Nomarski optics) it is not easy to identify and distinguish immature spermatogenic cells, especially early round spermatids, with absolute certainty. There is definitely a problem of identification of round spermatids by many practitioners, and we believe small lymphocytes can mistakenly be identified as round spermatids. Different identification methods are employed by different groups but the most commonly used method is based on the morphological characteristics of the round spermatid (Tesarik and Mendoza, 1996; Angelopoulos *et al.*, 1997) [35, 1]. Briefly, an early round spermatid (phases 1-3) was identified as a 7-8 µm diameter spherical cell with smooth outline. During the Golgi and cap phases of spermiogenesis, the acrosomal vesicle or cap manifests as a granular or crescent-like projection positioned on the cell's periphery. Initially, the nucleus is characterized by a smooth texture and a central, spherical placement. As the cell progresses toward maturity, the nucleus undergoes a morphological shift, becoming slightly oval as it establishes contact with the cytoplasmic membrane at the cell's edge. In early stages a small smooth rim of cytoplasm was present all around the nucleus. Phase 1 spermatids had no flagellum, but in phase 2 spermatids developing flagellum was seen protruding from the cell.

Elongated spermatids (phases 4-6) were asymmetric, with an elongated nucleus at one side of the cell and with a large cytoplasmic region at the other side surrounding the flagellum. Although it was difficult to differentiate very immature spermatids from secondary spermatocytes the remainder of spermatogenic cells (primary spermatocytes, spermatogonia), Sertoli cells and Leydig cells were distinguishable from the round spermatids because of their relatively larger size. Lymphocytes had roughly the same diameter as spermatids but distinguishable from them mainly by a larger nucleus/cytoplasm ratio. Lymphocyte nuclei usually appeared slightly kidney-shaped or round and eccentric, and were dense. Lymphocytes had a very thin and discontinuous pink cytoplasmic zone. Neutrophils were distinguished from spermatids by the nuclei with inter connected lobes and the granular cytoplasm.

In the cellular suspension of such testicular tissue digestion we identified primary spermatocytes as the largest cells with a

diameter of 16-18 μm . Round spermatids were spherical cells and had a diameter of 6-8 μm with clear cytoplasm and smaller but distinctive round nucleus. This was centrally situated in the early round spermatids and peripheral in maturing spermatids. The acrosomal cap could be visualized only in the cap phase spermatids but frequently this required manual rotation under visual inspection. Elongating spermatids had a diameter of 4-6 μm and were asymmetric cells. Spermatids at the onset of nuclear condensation and elongation exhibit distinct morphological features, including a protruding nucleus with a developing acrosome at one pole and a prominent cytoplasmic lobe at the opposite end. In terms of size, these early-stage cells are noticeably larger than late-stage spermatids. The latter are more fully condensed and typically shed their cytoplasmic lobes, resulting in a morphology that closely resembles the head of a mature spermatozoon (elongated spermatid).

Methods of Isolation

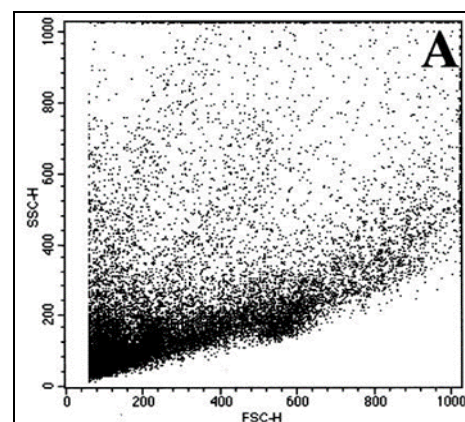
Isolation of RS to purity is frequently complicated by contamination with various cell types that are present in germ cell suspensions recovered after dissociation of testicular tissues. Numerous studies have been performed to improve the procedure in isolating pure RS populations. These various methods include velocity sedimentation (Wolgemuth *et al.*, 1985; Willison *et al.*, 1990) [41, 40], centrifugal elution (Bucci *et al.*, 1986; Blanchard *et al.*, 1991) [7, 4], immunoselection panning technique (Pelengaris and Moore, 1995) and flow cytometry followed by cell sorting (Petit *et al.*, 1995; Suter *et al.*, 1997; Lassalle *et al.*, 1999) [27, 31, 16]. Although the latter technique has proved to be an effective method for isolating pure populations of RS in mice and human (Lassalle *et al.*, 1999; Ziyyat *et al.*, 1999) [16], more studies with the use of donor cell for production of reconstructed embryos and offspring are needed to assess cell survivability and DNA fragmentation.

Fluorescence-activated cell sorting

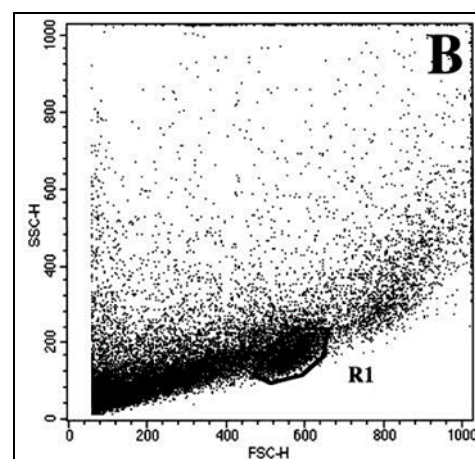
Research into male germ cell biology is complicated by the high degree of cellular heterogeneity within the testis and the absence of an *in vitro* model that accurately mimics spermatogenesis. Consequently, robust isolation protocols are essential for obtaining enriched populations of specific cell types. Fluorescence-activated cell sorting (FACS) has become a standard tool for this task (Gaysinskaya and Bortvin, 2015; Lima *et al.*, 2016) [12, 17], as it outperforms other isolation techniques in terms of purity, yield, and the variety of germ cell types it can distinguish (Bryant *et al.*, 2013; Margolin *et al.*, 2014) [6, 18]. The underlying principle of flow cytometry involves exciting single cells with a laser beam to detect specific light patterns. As cells traverse the laser path, they scatter light in a manner proportional to their physical characteristics: forward scatter (FSC) indicates cell size, while side scatter (SSC) reflects intracellular complexity.

Lassalle *et al.* (1999) [16] demonstrated the efficacy of flow cytometry and cell sorting in isolating pure populations of round spermatids from mouse testes. The protocol began with enriching germ cells via discontinuous Percoll gradient centrifugation, followed by analysis on a FACScalibur cytometer based on cell size and density. This process identified a distinct, well-defined cell population designated as R1. Post-sorting analysis revealed that the R1 cells were morphologically homogeneous with a size of approximately 11 μm , consistent with round spermatid cytology. To validate the isolation at a molecular level, RT-PCR analysis was

performed. The cells tested positive for spermatid-specific markers, specifically the protamine 2 gene (PRM2) and SP-10, with the latter being detectable even in single cells. Crucially, the purity of the sample was confirmed by the absence of c-kit transcripts (a marker for spermatogonia), effectively ruling out contamination. These findings definitively establish flow cytometry as a robust method for isolating high-purity round spermatids.



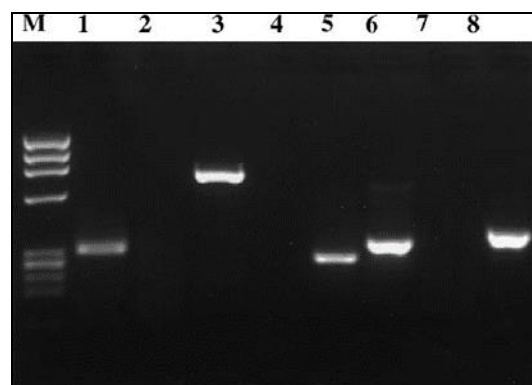
Without Percoll density Gradient



With Percoll density Gradient

Characterization of sorted cell by RT-PCR

The molecular characterization of the sorted R1 fraction (3,000 cells) revealed distinct gene expression patterns. The cells tested positive for the spermatid markers PRM2 and SP-10. As anticipated, the stem cell marker c-kit was absent; this negative result was validated using nested PCR, which failed to produce a signal. Specific to the c-kit transcript. HPRT expression was minimal, yielding only a weak signal restricted to the nested PCR conditions.



Gene expression analysis of the R1 cell population using RT-PCR to detect *PRM2*, *SP-10*, *c-kit*, and *HPRT*. Lanes 1 and 2 display the results for *PRM2* using standard and negative (RT-) controls. Similarly, Lanes 3 and 4 represent the *SP-10* amplification results. Nested RT-PCR using inner primers was performed for *HPRT* (Lane 5) and *c-kit* (Lane 7). For comparison, Lanes 6 and 8 serve as positive controls, showing *HPRT* and *c-kit* amplification from whole testis polyA+ mRNA using outer primers.

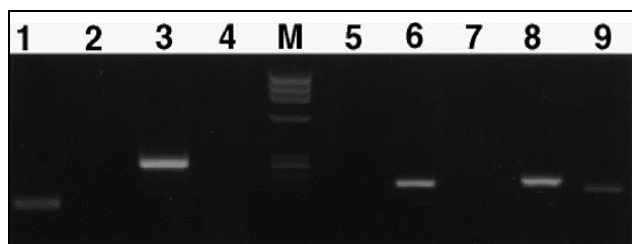
Ziyyat *et al.* (1999) [16] employed flow cytometry combined with RT-PCR to isolate and characterize round spermatids from men with infertility. The biological material consisted of testicular tissue harvested during biopsy procedures for Intracytoplasmic Sperm Injection (ICSI). Sorting was conducted using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The protocol involved the simultaneous measurement of cell size via forward scatter (FSC) and cell density via side scatter (SSC). Instrument calibration followed the specific settings previously established by Lassalle *et al.* (1999) [16], with an acquisition of 10,000 events per sample. To ensure recovery, sorted cells were collected into 50 ml culture tubes pre-coated with PBS containing either 4% bovine serum albumin (BSA) or fetal calf serum (FCS) for 24 hours at 4 °C. To prevent spermatids from adhering to the walls of the plastic collection tubes, it is essential to maintain a concentration of approximately 4% BSA or FCS throughout the sorting process. Following cell collection, PolyA+ mRNA was extracted using an mRNA capture kit. The extraction was performed on two distinct sample types: total cells from ejaculate and round spermatids purified from testicular biopsies via FACScalibur.

Testicular biopsies from obstructive azoospermia

Analysis of the 22% Percoll fraction via flow cytometry and cell sorting successfully isolated round spermatids. These cells appeared as a uniform cluster located between 400 and 600 arbitrary units on the Forward Scatter (FSC) axis. While the abundance of these cells varied among patients, the overall dot plot patterns were consistent. The protocol effectively distinguished round spermatids from lymphocytes based on size differences. The resulting spermatid population was highly homogeneous, with a mean diameter of 10.6 ± 0.5 μ m. Furthermore, post-sort viability was exceptionally high (>99%), as verified by the Trypan Blue exclusion test.

RT-PCR characterization of the sorted cells

mRNA corresponding to 3000 cells of the purified round spermatid population were analysed for the presence of *PRM1* and *PRM2*, *MAGE1* and *CD3-d* transcripts.



Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *PRM1*, *PRM2*, *MAGE1* and *CD3-d* expression. Lane 1 and 2, RT1 and RT-round spermatid samples with *PRM1* primers. Lanes 3 and 4, RT1 and RT-round spermatid samples with *PRM2* primers. Lanes 5, RT1 round spermatid sample with *MAGE1* nested primers. Lane 6, RT1 whole

testis sample with *MAGE1* nested primers. Lane 7, RT1 round spermatid sample with *CD3-d* nested primers. Lanes 8 and 9, RT1 lymphocyte samples with *CD3-d* outer and nested primers respectively. M, f *Hae* III marker. *PRM1* PCR product was 150 bp; *PRM2* PCR product was 301 bp; *MAGE1* nested PCR product was 213 bp; *CD3-d* product was 225 bp; *CD3-d* nested PCR product was 189 bp.

To ensure sample purity, we employed nested RT-PCR to screen for contamination by lymphocytes or diploid germ cells. The assay targeted CD3-delta, a ubiquitous lymphoid surface antigen (Van Den Elson *et al.*, 1986), and *MAGE1*, a tumor rejection antigen found in spermatogonia and primary spermatocytes but absent in haploid germ cells (Takahashi *et al.*, 1995) [33]. Regarding cell selection, Sutovsky *et al.* (1999) [32] established a non-invasive technique using rhesus monkey and bull models to isolate individual round spermatids for ROSI. This method utilizes MitoTracker™, a vital fluorescent probe, to visualize specific mitochondrial polarization patterns via epifluorescence microscopy. Identification is cross-referenced using Nomarski differential interference contrast (DIC) microscopy to confirm the presence of the acrosomal granule or cap. This polarization phenomenon was validated by co-labeling MitoTracker-stained cells with antibodies against nascent sperm accessory structures and nuclear pore complex components (which disappear at this developmental stage). Using an inverted epifluorescence microscope, researchers can pick specific spermatids from a mixed population. The method offers significant safety advantages: the MitoTracker dyes use wavelengths that minimize DNA photodamage, and the dye-loaded paternal mitochondria are naturally eliminated post-fertilization. Consequently, this approach is highly valuable for training clinical personnel prior to human application.

Isolation of spermatogenic cells and MitoTracker labelling

Adult bull testes, obtained from a slaughter house, were dissected and a small piece transferred to a Petri dish filled with TALP-HEPES (Boatman, 1987) [5] and minced using two fine forceps. Rhesus monkey testes were obtained from males undergoing necropsy, and testicular cells were prepared as described above. The minced tissue was filtered through a fine mesh to remove the tissue debris and the cell suspension was centrifuged for 5 min at 700 g. The pellet was resuspended in 12 ml of warm TALP-HEPES and centrifuged one more time for 5 min at 700 g. Twenty-five microlitres of the loose final pellet were then resuspended in 975 μ l of TALP-HEPES for MitoTracker labelling. A primary stock of 1 mmol/l MitoTracker CMTM Ros (Molecular Probes, Inc., Eugene, OR, USA) was prepared in dimethylsulphoxide (Sigma, St Louis, MO, USA).

A secondary stock of 100 μ mol/l MitoTracker was made from the primary stock in KMT medium (100 mmol/l KCl, 2 mmol/l MgCl₂, 10 mmol/l Tris-HCl; pH 7.0) warmed to 37°C. This stock was added to 1 ml suspension of testicular cells to obtain a final concentration of 200 nmol/l and incubated for 20 min at 37°C. In some experiments, the round spermatids were incubated for 2 h with a vital, lysosomespecific probe LysoTracker Green DND-26 (Molecular Probes) at the concentration of 10 μ mol/l, combined with 200 nmol/l MitoTracker CMTM Ros and 5 μ g/ml Hoechst 33342 at 37 °C. After incubation, the cells were collected by a 5 min centrifugation at 700 g, and washed by resuspension and centrifugation in 12 ml of TALP-HEPES medium. Peripheral blood was drawn from healthy rhesus males and stained as described above in order to demonstrate

the difference between MitoTracker staining patterns in testicular and blood cells.

Immunofluorescence and cell imaging

Testicular cells were stained with MitoTracker CMTMRos as described above and attached to poly-L-lysine-coated coverslips in a drop of warm KMT medium. Coverslips were fixed for 40 min in 2% formaldehyde in 0.1 mol/l phosphate-buffered saline (PBS) and permeabilized overnight in 0.1% Triton X-100 in 0.1 mol/l PBS. Non-specific antibody binding was blocked by a 1 h incubation in 0.1 mol/l PBS containing 10% normal goat serum (NGS). Coverslips with rhesus cells were then incubated with a mixture of anti-outer dense fibre antibody pAb 4443 (Okou, 1988; diluted 1/200) [22] and a nucleoporin-specific antibody mAb 414 (Meier *et al.*, 1995; BabCo, Berkeley, CA, USA; diluted 1/200) [19] in 0.1 mol/l PBS containing 0.05% NaN₃, 2 mmol/l EGTA, 1% NGS and 0.1% Triton-X-100 (further referred to as labelling solution). After a short wash in labelling solution, the coverslips were incubated for 40 min with a mixture of fluorescein isothiocyanate (FITC)-conjugated goat-anti rabbit IgG and Cy5-conjugated goat anti-mouse IgG (Zymed Labs Inc., South San Francisco, CA, USA; diluted 1/40). Five micrograms per ml of 6-diamidino-2-phenylidone (DAPI; Molecular Probes Inc., Eugene, OR, USA) were added to the labelling solution 10 min before the end of incubation. At the end of incubation, the coverslips were washed in PBS and mounted on microscope slides in a VectaShield mounting medium (Vector Labs, Burlingame, CA, USA). Bull testicular cells were processed as described above except that the perinuclear theca-specific antibody pAb 427 (Okou and Maravei, 1994; diluted 1/200) [23] was used instead of pAb 4443. The antibody pAb 427 also cross-reacts with the perinuclear theca of rhesus monkey spermatids (data not shown).

In some experiments, the rhesus monkey testicular cells were double-labelled with a mixture of anti-syntaxin rabbit polyclonal antibody (Conner *et al.*, 1997) [9] diluted 1/200; kindly donated by Dr Garry Wessel) and mAb 414, or with a mixture of anti-syntaxin and mouse monoclonal anti-acrosin antibodies (De Ioannes *et al.*, 1990) [10]; diluted 1/50; kindly donated by Dr Claudio Barros), followed by appropriate secondary antibodies. Both antisyntaxin and anti-acrosin antibodies cross-reacted with the acrosome of rhesus monkey round spermatids. Slides were examined on a Zeiss Axiophot microscope and images were recorded by a cooled CCD camera (Princeton Instruments, Inc., Trenton, NJ, USA) operated by Metamorph software (Universal Imaging Corp., West Chester, PA, USA). Original data were archived on recordable compact disks.

Conclusions

The round spermatid represents a pivotal stage in the spermatogenic cycle, defined as the earliest form of haploid male germ cells situated immediately prior to their morphological differentiation into tail-bearing spermatozoa. As detailed in the progression of germ cells within the seminiferous epithelium, this stage marks the transition from meiosis to spermiogenesis, where the cell possesses a haploid set of chromosomes but lacks the motility apparatus of a mature sperm. Despite this structural immaturity, it is concluded that the round spermatid holds the intrinsic biological potential to fertilize an ovum and support embryonic development, leading to the birth of healthy offspring. This capability has established Round Spermatid

Injection (ROSI) as a transformative prospect for the treatment of male infertility, offering specific hope for patients diagnosed with non-obstructive azoospermia where mature spermatozoa are absent.

However, the clinical success of ROSI is intrinsically linked to the precision of the cell selection process. To increase fertilization and pregnancy rates, it is imperative that round spermatids are correctly evaluated and distinguished from somatic cells and other germ cell stages. The efficiency of this selection relies heavily on the isolation method employed. In the context of the Percoll density gradient method, findings indicate that a 35% Percoll fraction is the most suitable concentration for the enrichment of round spermatids before they are processed further. While density gradients offer a viable separation approach, Flow Cytometry-based cell sorting (FACS) has been identified as the more efficient method for isolation. By leveraging advanced sorting parameters, FACS ensures a higher purity of viable round spermatids, thereby optimizing the conditions for successful ROSI procedures.

Future Prospects

- Further comprehensive research studies on this field are clearly needed to improve the success and efficiency of the ROSI procedure.
- Further research is needed for the developing the parameters indicating the quality of the individual spermatid.

Conflict of Interest

Not available

Financial Support

Not available

Reference

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