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REVIEW

How to improve the clinical outcome of round spermatid injection (ROSI) into the oocyte: Correction of epigenetic abnormalities

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Abstract

Background: First successful human round spermatid injection (ROSI) was conducted by Tesarik et al. in 1996 for the sole treatment of nonobstructive azoospermic men whose most advanced spermatogenic cells were elongating round spermatids. Nine offsprings from ROSI were reported between 1996 and 2000. No successful deliveries were reported for 15 years after that. Tanaka et al. reported 90 babies born after ROSI and their follow-up studies in 2015 and 2018 showed no significant differences in comparison with those born after natural conception in terms of physical and cognitive abilities. However, clinical outcomes remain low.

Method: Clinical and laboratory data of successful cases in the precursor ROSI groups and those of Tanaka et al. were reviewed.

Results: Differences were found between the two groups in terms of identification of characteristics of round spermatid and oocyte activation. Additionally, epigenetic abnormalities were identified as underlying causes for poor ROSI results, besides correct identification of round spermatid and adequate oocyte activation. Correction of epigenetic errors could lead to optimal embryonic development.

Conclusion: Correction of epigenetic abnormalities has a probability to improve the clinical outcome of ROSI.

KEYWORDS
epigenetic abnormality, oocyte activation, round spermatid injection into oocyte (ROSI)

1 | INTRODUCTION

It is said that 1 out of 100 healthy men is azoospermic[1] and that about 70–80% of those are nonobstructive azoospermia cases.[2] Micro-TESE is the sole treatment to find spermatozoa. The incidence of detecting testicular spermatozoa is about 30–60%,[3–6] but about half of those spermatozoa found are immotile or have deformities. When no intact testicular spermatozoa could be found, the patients were considered as unable to become biological fathers and sperm donation was recommended. Ogura and Yanagimachi reported the capability of fertilization with round spermatid (R-ST) using hamster R-ST in 1993 for the first time.[7] The rationale of ROSI is that R-STs develop after two times of meiosis and have the same number of chromosomes and same contents of DNA as those of matured spermatozoa. After injection into the oocyte and with proper oocyte activation, R-STs have the same ability to fertilize the oocyte as spermatozoa. (Figure 1) They reported the birth of normal offspring of mice in 1994.[8,9] There have been many successful reports in mammals except for human beings. Edwards et al. presented the idea of using spermatids isolated from men with spermatogenesis

arrest at any stage of spermatogenesis as substitutes for spermatozoa in 1994.[10] Tesarik et al. reported the birth of normal babies following round spermatid injection (ROSI) into human oocytes in 1996.[11,12] Eight pregnancies were reported after that, and seven deliveries were reported.[12–18] Doctors became gradually skeptical about its clinical usefulness and ROSI disappeared from clinical application. However, in 2015, our clinic reported in PNAS[19] 14 healthy babies born following ROSI from azoospermic patients whose first Micro-TESE conducted by urologists at other institutions, had not found any spermatozoa but a second Micro-TESE at our clinic had found round spermatids. The clinical data of 90 babies born after ROSI whose physical and cognitive abilities proved to be no significant different in comparison with normal conception was reported in Fertility and Sterility in 2018,[20] However, clinical outcome following ROSI is still low. Our studies have elucidated the main cause of poor clinical outcome as derived from epigenetic abnormalities by differences of nuclear protein in round spermatid (histone) and mature spermatozoa (protamine).[21–23]

In 2017, Kong et al. reported the beneficial effects on the correction of epigenetic abnormalities. I would like to review the history of ROSI and refer to how to correct the epigenetic abnormalities consulting recent papers[22,24–27] without being against the guidelines on genetic editing.[22,28]

2 | RATIONALE FOR ROSI

Palermo et al. developed intracytoplasmic sperm injection (ICSI) in 1992.[29] This novel technique has greatly changed the concept of fertilization. Fertilization is completed by fusion of the oocyte with the spermatozoa, and it triggered the oocyte activation. Interaction between presumptive complementary receptors on the spermatozoa and oocyte plasma membranes triggers the activation of the G-protein that activates the production of inositol triphosphate that releases Ca^{2+} from the endoplasmic reticulum.

Meanwhile, ICSI proved that the release of Ca^{2+} oscillation-releasing factor from the injected spermatozoa triggered the oocyte activation cascade.[30,31]

In either way, the release of Ca^{2+} starts to spread into all the cytoplasm and resumes the second meiotic cell division, this resulted in the extrusion of the second polar body and both pronuclei.

Round spermatid has a haploid set of chromosomes 23 and 1N DNA content just as a mature spermatozoa. So, if R-ST can be injected directly into the oocyte with the same technique as ICSI, R-ST, which has no flagellum, can fertilize the oocyte and deliver a baby.

3 | THE REASON WHY USEFULNESS OF ROSI HAS BEEN RECONSIDERED

The generally accepted theory for nonobstructed azoospermia is that whenever R-ST exists in the human testis, there are also matured spermatozoa present.[32,33] When no spermatozoa or late-stage spermatids could be found, R-ST could still be found. So, it was concluded that ROSI was not necessary clinically, or should not be conducted. However, the new facts that mouse and human male with cyclic AMP-responsive element modulator (CREM) gene[27,34–38] mutation showed the maturation arrest at R-ST

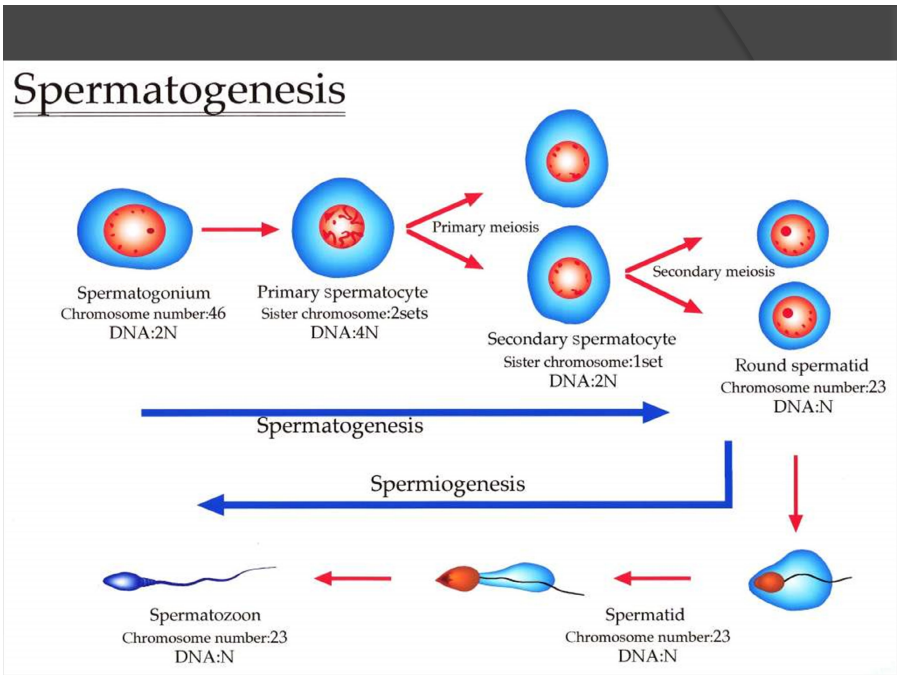

FIGURE 1 The rationale of ROSI is that R-STs develop after two times of meiosis and have the same number of chromosomes and same contents of DNA as those of matured spermatozoa. After injection into the oocyte and with proper oocyte activation, R-STs have the same ability to fertilize the oocyte as spermatozoa.

followed by the report that men with mutation genes, TAF4B and ZMYND15, showed the same maturation arrest. These facts suggest the reconsideration of the usefulness of ROSI. ROSI is now being reappraised.

4 | WHY IS ROSI NECESSARY?

One out of hundred men suffers from azoospermia and about 70% of azoospermia cases are nonobstructive. The sole way for these patients to become biological fathers is to obtain testicular spermatozoa through Micro-TESE. However, the success rate of obtaining viable testicular spermatozoa has been reported to be low. About half of collected spermatozoa are immotile or have abnormally shaped heads.[39] When intact sperm could not be found, the patients are declared to be completely infertile. However, R-ST could be found in about 30–40% of those cases. They still have a chance to become biological fathers using their own R-ST.

Sperm donation is available around the world. However, many patients continue to desire to have children with their own gamete and not with donated sperm.

5 | CLINICAL ANALYSIS OF 22 MANUSCRIPTS THAT HAD ENOUGH INFORMATION TO PROPERLY EVALUATE ROSI

Twenty-two manuscripts about ROSI with information complete enough to perform a clinical analysis were reviewed. Reports that included information about number of injected oocytes, fertilization rate, pregnancy rate, miscarriage rate, type of oocyte activation, R-ST collection method, optics used and whether the R-ST used was fresh or frozen were included.

5.1 | Identification of ROSI

The manuscripts first published on ROSI reported methods that could be questioned in terms of morphological identification or oocyte activation. However, they were correct at identifying the genetic, epigenetic, and chromosomal risks that ROSI could pose. They predicted genetic imprinting, changes in DNA association with nuclear protein, cell cycle synchronization, and DNA methylation. None of the early manuscripts addressed the mechanism of how epigenetic abnormalities may occur or offered solutions to treat them.[1]

5.2 | Actual oocyte activation

Vigorous oocyte aspiration,[12,14,15,40–47] calcium ionophore, or ionomycin,[13,48] or no,[17,49–51] electric stimulation[19,20] were reported as oocyte activation methods. Most authors used the vigorous oocyte aspiration method reported by Tesarik et al,[11] 81.8% (18/22), electric activation 9.1% (2/22), ionophore or ionomycin 9.1% (2/22), none 18.2% (4/22), and not reported 4.5% (1/22). In 2004, T. Ebner et al.[52] reported a mechanical activation with deeper insertion of injection pipette to the opposite membrane with a slight invagination. However, benefits for clinical outcome could not be recognized.

Electric stimulation is now considered to be the most effective for ROSI. The effect of each oocyte activation was examined by Ca^{2+} oscillation.

It has become clear that Tesarik's oocyte aspiration was not sufficient for full oocyte activation.

5.3 | R-ST in ejaculate or testis

Round spermatid is found in the ejaculate or testis. R-ST in ejaculate is considered to be an inadequate sample for ROSI in comparison with testicular one due to the high possibility of apoptotic change of R-ST[53] and high probability of spermatozoa existence in the same ejaculate. It has been revealed that more apoptosis occurs in the R-ST than in the testis, so the reasons that led Tesarik to use R-ST in ejaculate are still unclear. There are two assumed reasons. First was an easier collection. Second was the difficulty of the procedure of testicular biopsy at the time. So far, four cases performed ROSI with R-ST in ejaculate and the remaining 20 cases used testicular R-ST.

5.4 | Fresh or frozen–thawed R-ST

Tanaka et al conducted ROSI using cryopreserved R-ST, because there is no guarantee of 100% collection of R-ST at the Micro-TESE. However, in the initial ROSI reports only four cases used cryopreserved R-ST,[14,18,43,49] the remaining 16 cases used fresh spermatozoa. These results suggest that the cryopreservation method had not been established yet in the early days or the first researchers were not confident about the correct identification of R-ST. Two cases of delivery[14,18] were reported. Freezing method was vitrification. Now it has been proved that in terms of recovery rate, the thawing slow-freezing method is superior to vitrification (Figure 2).

5.5 | Most advanced spermatogenic cell in ROSI

The three kinds of most advanced spermatogenic cells (spermatozoa, elongated, elongating spermatids) reported in Tanaka's cases were all arrested at the stage of R-ST,[19,20] but in 20 early reported groups they were mixed. Nine cases[13,14,43–47,49,50] of R-ST, 5 cases[12,15,48,51,54] of elongating spermatids, 5 cases[16–18,41,42] of spermatozoa. The strict definition of ROSI describes it as the method that uses only

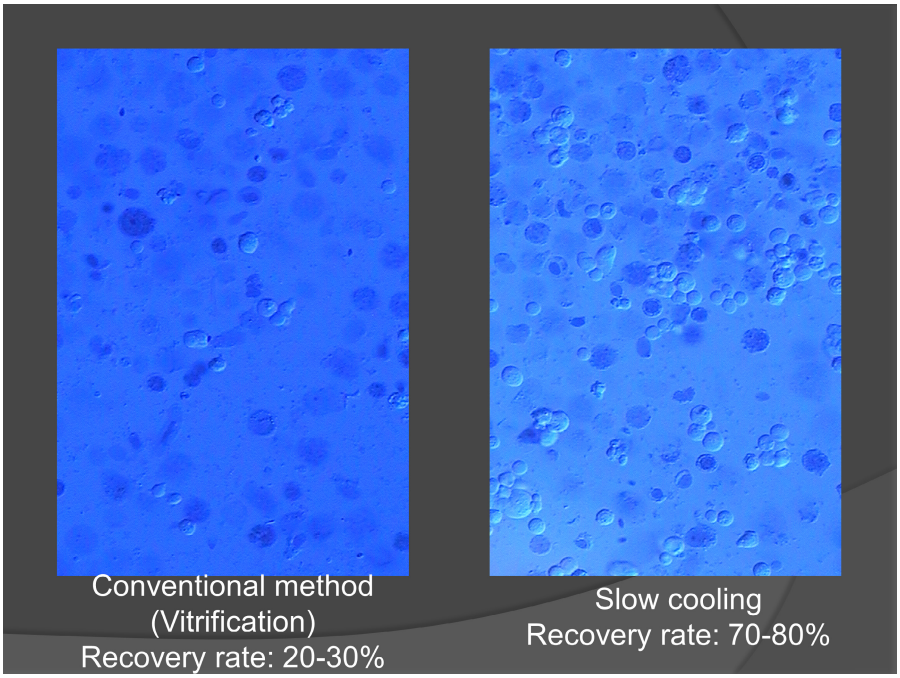

FIGURE 2 Recovery rate after thawing is significantly higher with slow cooling method than with vitrification method (Magnification: 200×).

R-ST obtained from cases of complete maturation arrest at the stage of R-ST. It has been well-known that the clinical outcome of R-ST differs greatly whether maturation arrest was complete or incomplete. ROSI results using R-ST obtained from complete maturation arrest was significantly lower than that of incomplete one. This difference could be caused by degree of transitions of nuclear protein, histone to protamine. This result made R-ST develop into elongating or elongated spermatids for the starting group of researchers doing in vitro culture.

5.6 | Clinical outcome (Table 1)

Average fertilization rate (%), clinical pregnancy rate (%), miscarriage rate (%), number of live offspring and birth rate in 20 initial ROSI reports, the precursors group,[12–18,40–51,53,55] and Tanaka group,[19,20] was (36.60 ± 15.53%) and (58.15 ± 1.95%), (18.47 ± 15.91%) and Fresh-T: 10.98 ± 7.84%, Frozen-T: 19.8 ± 5.7%, (4.76 ± 12.59%) and Fresh-T: 54.38 ± 15.02%, Frozen -T: 40.52 ± 13.41%, (9, 12 and 84) and (6.12% (9/147)), Fresh-T: 3.27% (52/1592) Frozen -T: 10.76% (44/409). There was a significant difference between Fertilization rate (%) in the precursors group and Tanaka group, and Tanaka group obtained a significantly higher fertilization rate (%) than that of the first researchers group.

The reason why there were so big differences between the two groups, the reports between 1996 to 2015 and the Tanaka's reports in 2015,[19] 2018[20] could be attributed to the following points: (1) microscopic observation of R-ST by Hoffman phase contrast microscope which has lower resolution than Nomarski differential interference contrast microscope.[56] (2) Different preparation methods for removed seminiferous tissues, simple procedures versus with or without enzymatic preparation. Preparation medium containing DNase and collagenase made it easier to isolate the spermatogenic cell in the Beginning group. (3) Almost all reports of precursors group used ooplasm aspiration which has almost no effect as oocyte activator.

6 | IN VITRO CULTURE OF HUMAN SPERMATOGENIC CELLS

A great number of studies tried in vitro culture of spermatogenic cells into mature spermatozoa but none of them was completely successful.

6.1 | In vitro culture of spermatogenic cells with Sertoli cells in culture system with follicle-stimulating hormone and testosterone

In 1998, Tesarik et al[57] reported the successful in vitro human spermatogenesis and spermiogenesis in a simple culture system (GAMATE-100) supplemented with FSH concentration of 50–100 IU/L and testosterone at concentrations of 1 μmol/L. He showed that the combination of FSH and T resumed the second meiotic cell divisions and subsequent spermiogenesis. In 2000,[53] he reported the first case of human pregnancy (twin babies, 36 weeks). The reason why he started the clinical application with some unsolved problems about development of abnormal shaped elongating/elongated spermatids remains unclear. If he had been confident on the procedure's safety he would have continued the treatment and tried to spread the method. No report indicating that the maturation arrest

TABLE 1 Clinical outcome in reviewed paper.

Year	Author	Number of injected oocytes Fresh/frozen–thawed (FT)	Fertilization rate (%)	Pregnancy rate (%) (fresh-T/frozen-T)	Miscarriage rate (%) (fresh-T/frozen-T)	Number of live offspring (fresh-T/frozen-T)	Most advanced spermatogenic cell	Oocyte activation	R-ST in ejaculate or micro-TESE	Optics		Fresh or frozen–thawed R-ST		
													Frozen	
										Hoffman	Nomarski	Fresh	Vitrification	Slow cooling
1996	Tesarik et al.	47	42.55	16.66 (2/12)	0	2	Elongated ST	Vigorous	Ejaculate	○		○		
1997	Vanderzwalmen et al.	260	21.92	14.28 (1/7)	0	1	R-ST	Ionophore	Micro-TESE	○		○		
1997	Antinori et al.	15	46.66	16.66 (1/6)	0	1	R-ST	Vigorous	Micro-TESE	○			○	
1997	Antinori et al.	135	55.55	3.57 (3/84)	33.3 (1/3)	2	Elongating ST	Vigorous	Micro-TESE	○		○		
1997	Amer et al.	251	25.09	0	0	0	Sa ejaculate Sa testis	Vigorous	Ejaculate/ Micro-TESE	○		○		
1997	Yamanaka et al.	49	69.38	0	0	0	R-ST	None	Micro-TESE	○		○		
1998	Kahraman et al.	199	25.62	3.12 (1/32)	0	1	Spermatozoa	Vigorous	Micro-TESE	○		○		
1998	Barak et al.	37	27.02	25 (1/4)	0	1	Spermatozoa	None	Micro-TESE		○	○		
1998	Bernabeu et al.	7	42.85	0	0	0	Elongating ST	Not reported	Ejaculate($n = 6$), Micro-TESE ($n = 2$)	○		○		
1999	Ghazzawi et al.	574	21.95	0	0	0	Spermatozoa	Vigorous	Micro-TESE	○		○		
1999	Al-Hasani et al.	49	18.36	0	0	0	R-ST	None	Micro-TESE	○			○	
1999	Gianaroli et al.	5	40	50 (1/2)	0	1	Spermatozoa	Vigorous	Micro-TESE	○			○	
2000	Balaban et al.	356	56.17	0	0	0	Spermatozoa	Vigorous	Micro-TESE	○		○		
2000	Levran et al	178	45.5	0	0	0	R-ST	Vigorous	Micro-TESE		○		○	
2001	Vicdan et al.	60	28.33	0	0	0	R-ST	Vigorous	Micro-TESE	○		○		
2002	Urman et al.	1021	40.54	0	0	0	R-ST	Vigorous	Micro-TESE	○		○		
2002	Sousa et al.	98/28	17.4/19.2	0/0	0	0/0	R-ST	Vigorous	Micro-TESE	○		○		
2002	Khalili et al.	42	21.42	0	0	0	Elongating ST	None	Micro-TESE	○		○		
2003	Ulug et al.	36	41.66	0	0	0	R-ST	Vigorous	Micro-TESE	○		○		
2015	Goswami et al.	13	61.53	0	0	0	Elongating ST	Ionomycin	Micro-TESE	○		○		
2015	Tanaka et al.	734	59.54	16.5 (20/121)/ 23.8 (10/42)	65.0 (13/20)/ 50.0 (5/10)	9/5 (14)	R-ST	Electrical	Micro-TESE		○			○
2018	Tanaka et al.	14 324	56.77	5.4 (80/1471)/ 15.8 (58/367)	43.8 (35/80)/ 31.0 (18/58)	48/42 (90)	R-ST	Electrical	Micro-TESE		○			○

at the stage of primary spermatocytes (Pr-Sc) resumed spermatogenesis accompanied first and second meiosis and almost complete spermiogenesis could be found.

6.2 | Developmental potential of human spermatogenic cells co-cultured with Sertoli cells

In 2002, Sousa et al.[46] reported the new co-culture system using Vero cell conditioned medium with FSH and T for 2–3 weeks. Fertilization rate (%) and blastocyst rate (%) of R-ST and elongating spermatid were (37.5, 28.6) and (30.5, 42.9). However, most of the embryos did not reach the morula stage and showed major sex chromosome abnormalities.

6.3 | In vitro culture of human Pr-Sc with Vero cells

In 2003 Tanaka et al.[58] reported that human Pr-Sc which were collected from five nonobstructive azoospermic men whose spermatogenesis were arrested completed the meiosis through in vitro coculture with Vero cells. They were cultured on the feeder layer of Vero cells in the medium of MEM + 50% human synthetic oviduct fluid +10% human serum for a week. Chromosomal analyses were performed in all cleaved cells (two cells, four cells). The generation rate of round spermatids in various types of culture medium with Vero cells was 4.4% (5/120). However, no spermatid could be developed in the group without co-culturing with Vero cells.

6.4 | Developmental potential of elongating and elongated spermatids obtained after in vitro maturation of isolated round spermatids using co-culture on Vero cells

In 2001, Cremades et al.[59] reported the results of in vitro maturation of isolated R-ST in obstructive and nonobstructive azoospermia groups. Maturation rates of elongating spermatids (%), early elongated spermatids (%), and late elongated spermatids (%) in both groups were (31.5, 13, 9.3) and (23.4, 9.8, 4.3). Normal fertilization rate (%) and blastocyst rate (%) were (40.9 and 42.9). They did not seem to transfer these developed embryos into uterus after considering the potential risks of prion transmission and low entire replicability. The newly developed gametes have not completed spermiogenesis and stopped before becoming mature spermatozoa.

In 2009, Tanaka et al.[60] reported the first differentiation of human round spermatids into motile spermatozoa through in vitro coculture with Vero cells. Coculture condition in Tanaka et al. was almost the same as that of Cremades. Maturation rate into elongating spermatids (36.0%), elongated spermatids (14.0%), spermatozoa with an immotile flagellum (0%), and spermatozoa with a motile flagellum (0%) in both groups are shown. We could confirm two spermatozoa with normal shape of head and motile flagellum with intact midpiece for the first time in the world (Video S1). Our institutional Review Board did not allow clinical application of this treatment for azoospermia due to poor clinical outcome.

7 | THE CAUSES OF POOR CLINICAL OUTCOME OF ROSI IN THE PRECURSORS GROUP

7.1 | Insufficient identification of R-ST

The most accurate identification of R-ST cannot be made by morphological findings but by chromosomal analysis. R-ST is the only round cell among the spermatogenic cell which has finished two meiotic cell divisions. So, R-ST has the haploid set of 23 chromosomes. Spermatogonia (SG) which is very difficult to be differentiated from R-ST is a somatic cell with a diploid set of 46 chromosomes. In the beginning groups, identification of R-ST was made by cytologic characters; cell and nuclear size, round shape and smooth outline, acrosome granule, crescent acrosomal vesicle. Another identification method reported by Tesarik is the aspiration of presumptive R-ST into the injection pipette and if the cell is R-ST, it is not deformed and it never separates into the nucleus and cytoplasm. Almost all of cell findings described in early reports are correct but morphological change of aspirated presumptive round cell in a pipette was not correct (Figure 3).[20] Tesarik's description is characteristic of a somatic cell. The most difficult differentiation is between R-ST and spermatogonium, but few articles have reported about it.

Acrosomal vesicles or granules are found in about 20–30% (Figure 4).[19] of R-ST and these findings are very helpful to identify them.

SG are defined as the primordial germ cells which have migrated from the gut endoderm early in the fourth week toward the gonadal ridge via the dorsal mesentery by means of amoeba like movement and ceased its movement when they reached the seminiferous tubules. However, some of them were found to be continuing amoeba like movement in the seminiferous tubules. These cells which protrude active pseudopodia were identified as SG by the immunohistochemistry with alkaline phosphatase staining and γ-H2AX conjugated with fluorescein.[61] These cells were 8–10 μm in diameter, had a high N/C ratio and had one to two prominent nucleoli that were close to a distinct nuclear membrane (Figure 5).[19,20] These morphological characteristics became the conclusive evidence of SG. However, there is a limit to make a perfect differentiation morphologically. FISH analysis is useful to differentiate round cells whether it is diploid or haploid cell. FISH does not necessarily represent the whole chromosomal picture. Whole chromosomal analyses were conducted by injection of a presumptive R-ST into metaphase-2 oocyte derived from in vitro cultured immature cell after confirming

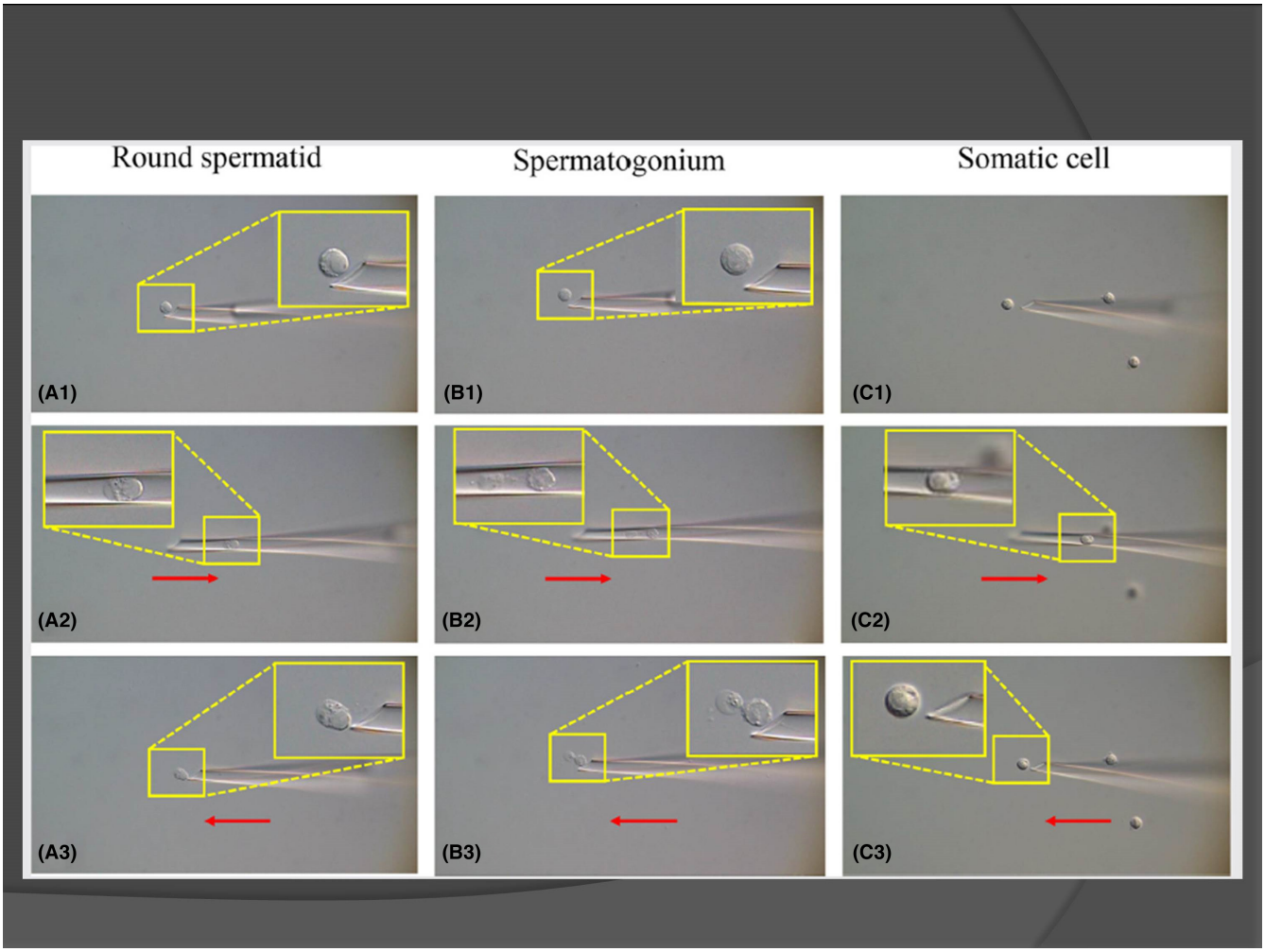

FIGURE 3 How to distinguish spermatids and spermatogonia from somatic cells. (A) When a spermatid is sucked in and out of a micropipette, its plasma membrane is readily broken and the nucleus and cytoplasm are separated. Its nucleus appears as a clear sphere. (B) A spermatogonium also can be separated into the nucleus and cytoplasm by pipetting. Its nucleus, unlike spermatid nucleus, contains clearly visible one or, more commonly, a few nucleoli. (C) Somatic cells (such as interstitial cells, fibroblasts, blood cells, and Leydig cells) released in the medium during maceration of seminiferous tubules have flexible plasma membranes which cannot be readily broken by pipetting (Magnification: 200× → zoom Magnification: 400×).

the patient's informed consent (GV,M-I staged oocyte at collection) by the effect of metaphase-promoting factor (MPF). Decisive identification of spermatogenic cells of each stage is made only by chromosomal analysis.

Spermatogonia and Pr-Sc have a diploid set of 46 chromosomes. In Pr-Sc, the crossing over that is peculiar only to Pr-Sc is found. When you could find the crossing over, you can say these cells are in the meiosis process, that is this cell is Pr-Sc. ST has a haploid set of 23 (Figure 6).[19]

There is a significant difference in the resolution of the optics used by the two groups. A Nomarski differential interference microscope has a much higher optical resolution than a Hoffman phase contrast microscope (Figure 7).[19]

One of the causes of lower clinical outcome of ROSI by the precursors group was that they used Hoffman phase contrast microscope for morphological observation, not a Nomarski differential interference contrast microscope which has much higher definition and can help obtain a more accurate identification of R-ST than when using a Hoffman phase contrast microscope.

7.2 | Insufficient oocyte activation

7.2.1 | What is oocyte activation?

The start of life begins at the encounter of the sperm and the oocyte. They fuse and develop into a new creature with the help of oocyte activation. Oocyte activation is the mother of our life.

Oocyte activation is the process by which the oocyte resumes the second meiotic division. During this process, a sperm cell triggers a series of calcium (Ca^{2+}) oscillations within the ooplasm which are involved in crucial events, such as the exocytosis of cortical granules, extrusion of the second polar body, regulation of gene expression, and the initiation of embryogenesis.[25]

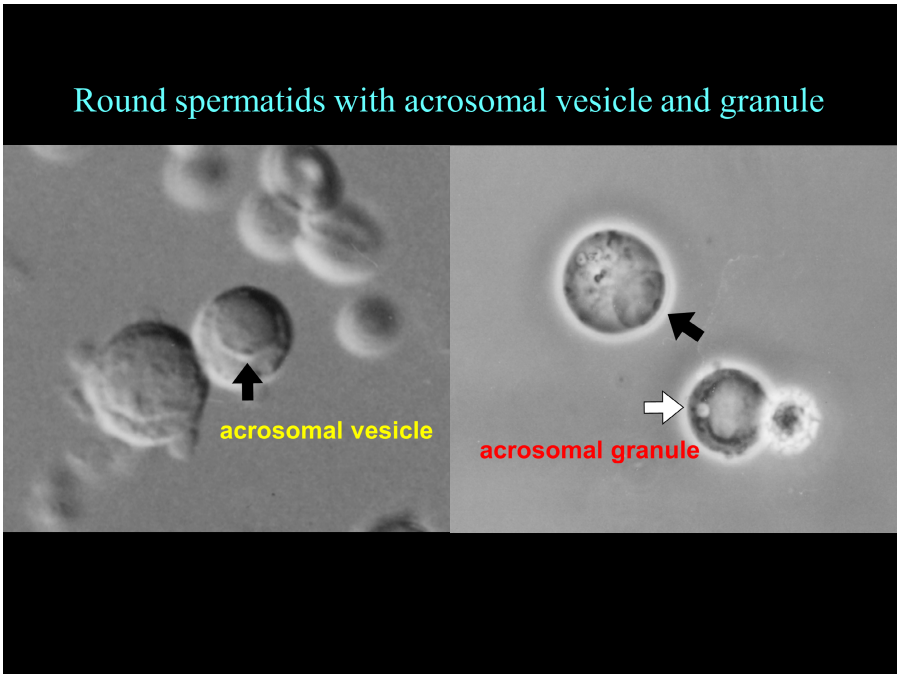

FIGURE 4 Acrosomal vesicle or granule is found in about 20–30% of round spermatids and these findings are very helpful to identify it (Magnification: 400×).

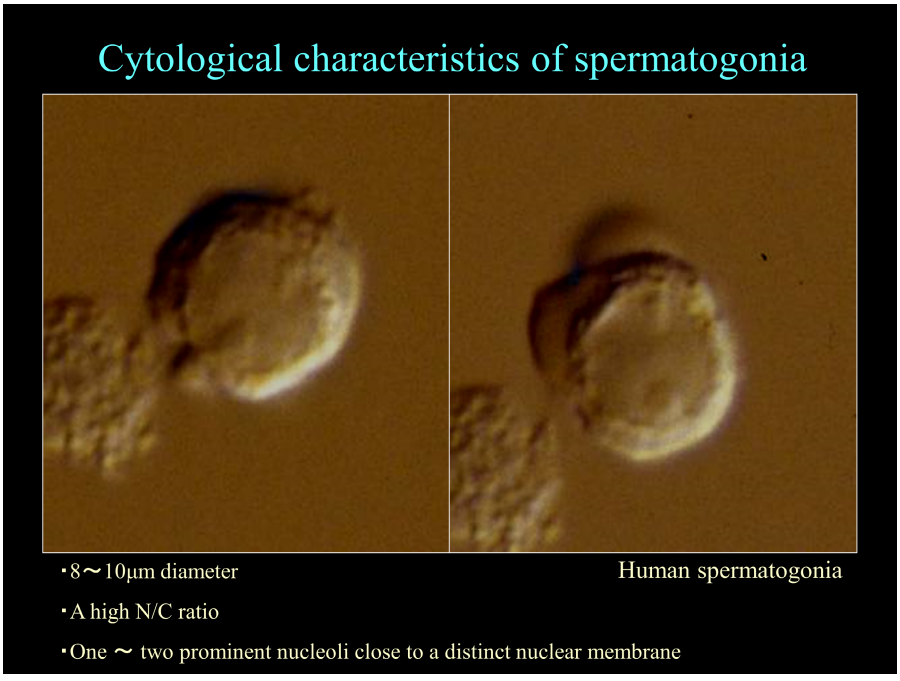

FIGURE 5 The cytological characteristics of SG is 8–10 mm diameter, a high N/C ratio one is two prominent nucleoli, close to a distinct nuclear membrane (Magnification: 400×).

7.2.2 | The mechanism of Ca^{2+} oscillation

Fusion of the oocyte with the spermatozoa is the physiological trigger of oocyte activation. Interaction between presumptive complementary receptors on the spermatozoa and oocyte plasma membranes triggers the activation of the G-protein that activates the production of inositol triphosphate (IP3) that releases Ca^{2+} from the endoplasmic reticulum. At fertilization, mammalian eggs show repetitive transient Ca rises each of which is due to Ca^{2+} release from the endoplasmic reticulum through IP3receptors. During fertilization, a factor from the sperm, the sperm factor, is released into the oocyte and induces series of Ca^{2+} spikes that are required for oocyte activation. They are known as Ca^{2+} oscillations.

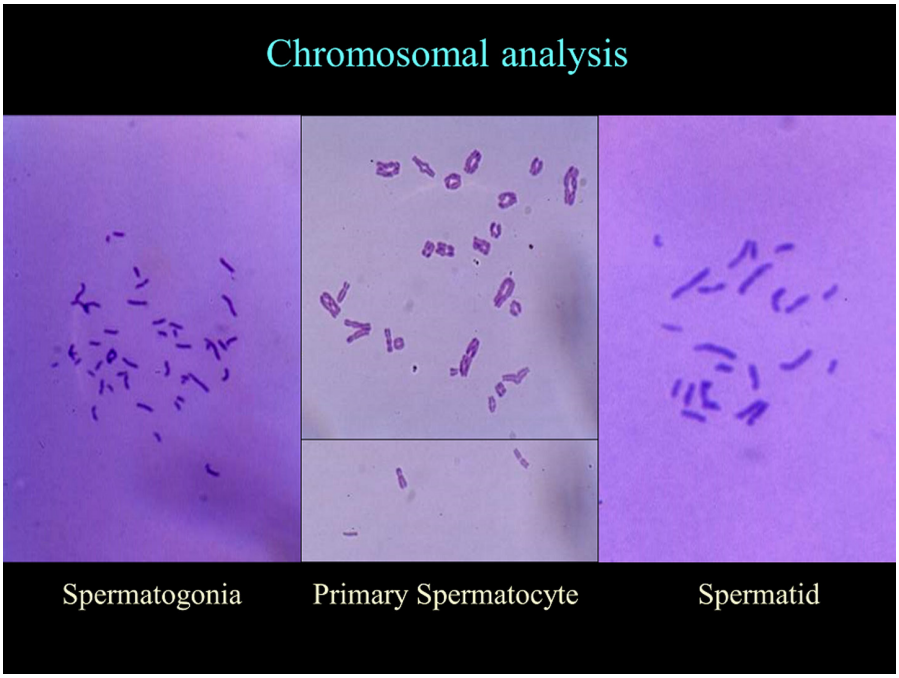

FIGURE 6 The result of chromosomal analysis. SG and Pr-Sc have a diploid set of 46 chromosomes. In Pr-Sc, the crossover that is peculiar only to Pr-Sc is found. When you could find the crossing over, you can say these cells are during the meiosis, that is, this cell is primary spermatocyte. ST has haploid set of 23 (Magnification: 400×). The center part of this figure is a composite photo put together for clarity and to keep the same level of magnification of the adjacent pictures.

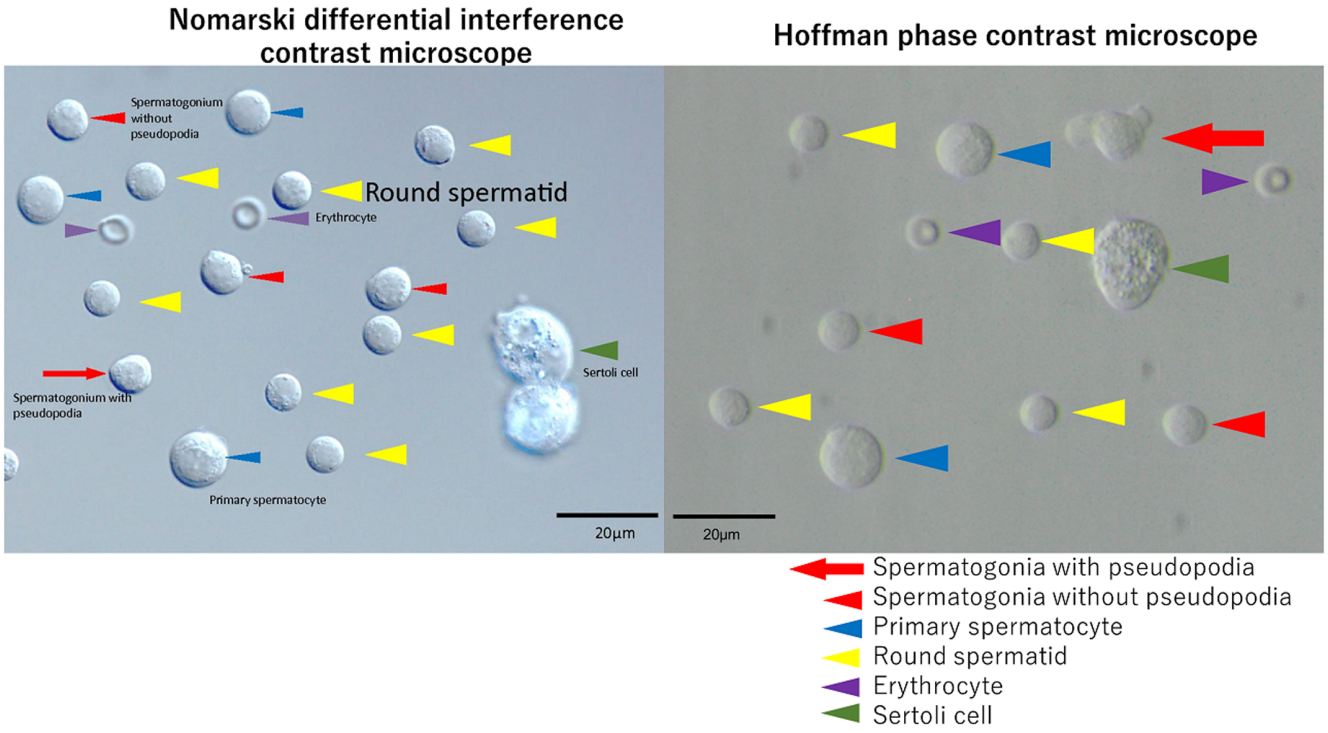

FIGURE 7 There is a big difference in the resolution of the optics used by the two groups. A Nomarski differential interference microscope has a much higher optical resolution than a Hoffman phase contrast microscope (Magnification: 400×).

7.2.3 | Which is the most effective oocyte activation?

We examined vigorous cytosolic aspiration, ionomycin, only ROSI, electric stimulation, and electric stimulation plus ROSI to find the most effective oocyte activation method. The oocytes were incubated in Ca^{2+}-sensitive fluorescent dye, Fluo8 first, and activated them in various stimulation and measured intracellular Ca^{2+} concentration using Ca^{2+} imaging. In the cytosolic aspiration and injection activation method, Ca^{2+} oscillations did not occur (Figure 8). In the ionocycin activation method, fluorescent intensity gradually decreased 5 min after treatment.(Figure 9) In ROSI alone no Ca^{2+} oscillation could be observed (Figure 10). Electrical stimulation showed the Ca^{2+} oscillation clearly about 15 min after the stimulation (Figure 11). However, height

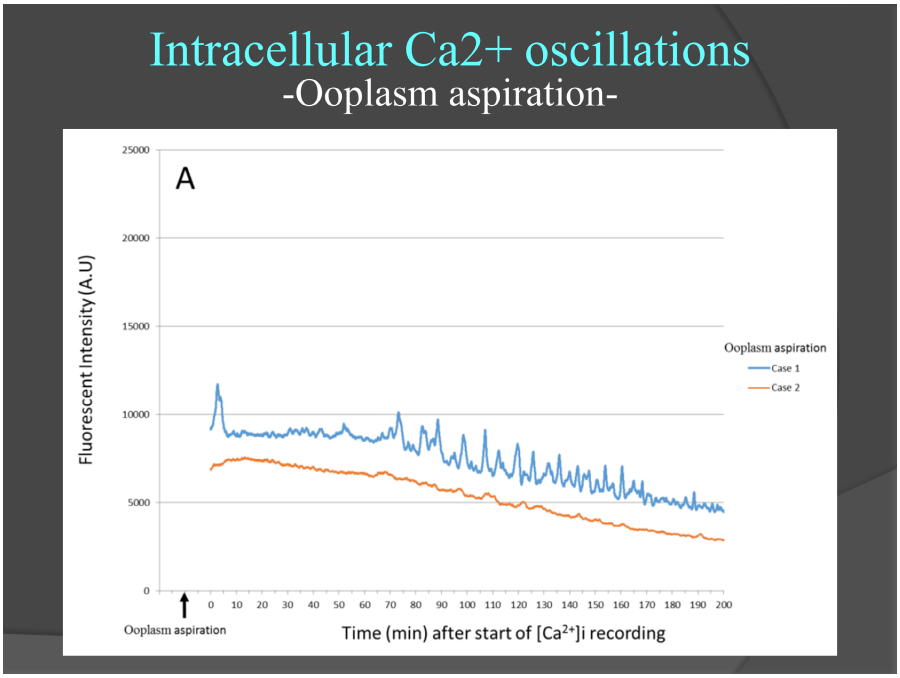

FIGURE 8 In the cytosolic aspiration and injection activation method, Ca oscillations did not occur.

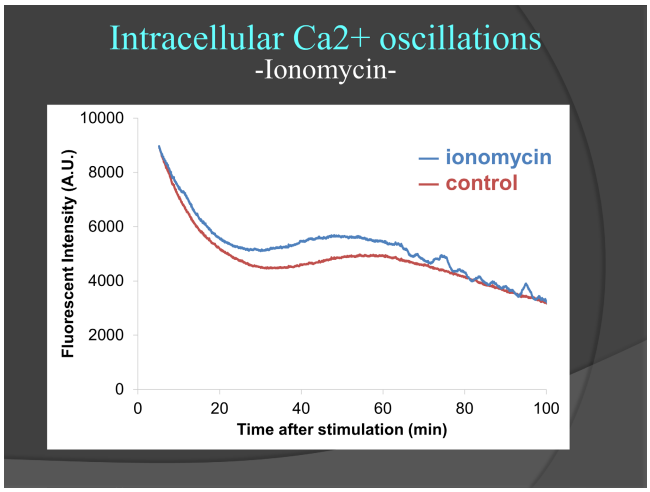

FIGURE 9 In the ionocycin activation method, fluorescent intensity gradually decreased 5 min after treatment.

interval and duration of spikes were irregular. Ca^{2+} oscillation of electric stimulation plus ROSI (Figure 12). Induced consistent large, repetitive Ca^{2+} oscillations. It was concluded that the best oocyte activation, so far, is electrical stimulation. The poor clinical outcome of precursor ROSI group could be caused by the lack of adequate aspiration oocyte activation. However, this conclusion might not always be true. A recent study reported that mouse ROSI was successful without pretreatment with SrCl2. This phenomenon might suggest the possibility that ROSI can be successful without oocyte activation. However, its likelihood might be very low. Yamaguchi et al, reported the establishment of appropriate methods for egg activation by human PLCZ1RNA injection into human oocyte in 2017,[28] PLCZ is expected to become a strong candidate for oocyte activation.

8 | EPIGENETIC MODIFICATION OF MALE GAMETE DNA

The risk of epigenetic abnormalities had been pointed out since the first successful report by Tesarik and some of following papers.

The conclusive disadvantage of ROSI seems to be incomplete transition edition of nuclear protein of histone in R-ST to protamine in matured spermatozoa.

DNA methylation and histone modification patterns, which are designed for normal embryonic development, are completely altered in male gamete chromatin before and after fertilization. Recently, abnormality of such an epigenetic modification has been a source of concern in children born after artificial reproductive technology (ART) interventions, including ROSI. Since no proven abnormality has been reported in children delivered after ROSI,[19,20] ROSI embryos with serious epigenetic abnormalities may be eliminated. However, the risk of epigenetic abnormality must be discussed when the establishment level of epigenetic modification in the round spermatids and the low implantation rate in ROSI embryos is considered. The process of epigenetic modification in mammalian male gamete is summarized as follows.

8.1 | DNA methylation

In vertebrate cell division, gene expression patterns are inherited to progeny cells with methylation of the cytosine nucleotides (5mC) in the CpG sequence, where a cytosine nucleotide is adjacent to a guanine nucleotide along 5′–3′ direction.[62] DNA methylation typically works to repress transcription by preventing the binding of transcription factors, which is required for initiation of gene

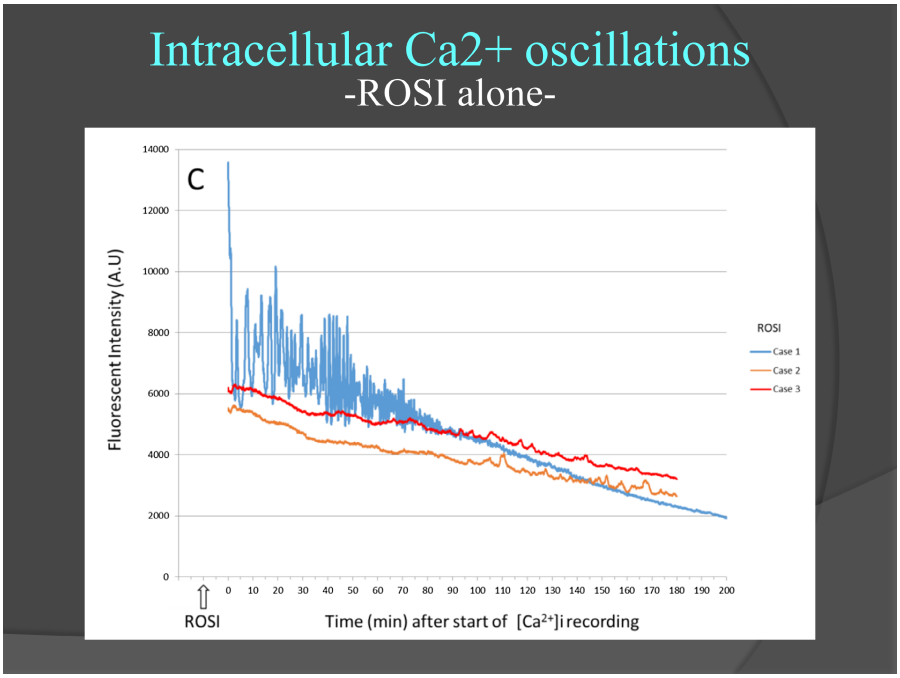

FIGURE 10 No Ca^{2+} oscillation could be observed after ROSI alone.

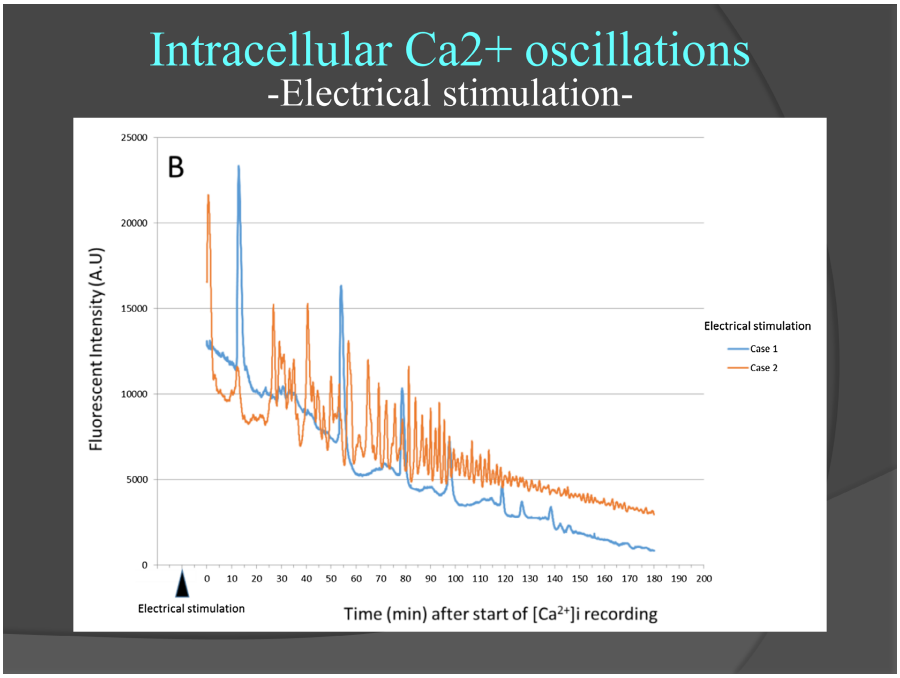

FIGURE 11 Clear Ca^{2+} oscillation after electrical stimulation. The first spike appeared about 15 min after the stimulation. However, height interval and duration of spikes were irregular.

expression.[63–65] In addition, DNA methylation is essential for X-inactivation and expression of parental imprinted genes.[66] Although the patterns of DNA methylation are maintained in proliferated and differentiated cells by maintenance methyl transferases during DNA replication and de novo methyl transferases, global demethylation and successive remodeling of methylation patterns occur in gamete genesis and postfertilization cell cleavage. In the paternal germ line, primordial germ cells undergo genome-wide demethylation during migration from yolk sack to the genital ridge[67] and then reestablishment of methylation patterns occurs through meiosis and spermiogenesis.[68] In rats, there are differential DNA methylation regions modified among the round spermatid, maturating sperm, and mature

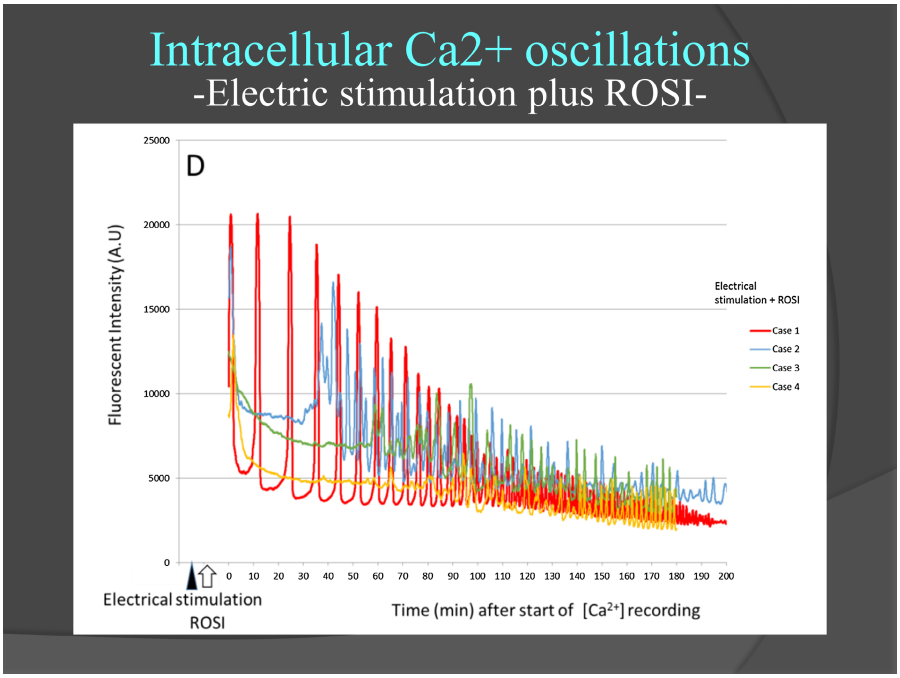

FIGURE 12 Ca^{2+} oscillation of electric stimulation plus ROSI. It induced consistent large, repetitive Ca^{2+} oscillations.

sperm stages, suggesting a possibility that epigenetic modification appears to be still incomplete in round spermatids, when meiosis has been completed.

Shortly after fertilization, another broad demethylation and successive reestablishment of methylation occurs in both sperm and oocyte nuclei. However, only paternal nuclear DNA is modified in a manner that is independent with DNA replication. 5mC in the paternal CpG sites, except for those of imprinted genes, is quickly converted to 5-hydroxymethylcytosine (5hmC) by enzymatic activity of TET proteins[69–72] before the first embryonic DNA replication. Subsequently, a gradual decrease in 5hmC is caused depending on DNA replication in the paternal DNA, although demethylation which is initiated by DNA methyltransferase 3 mainly occurs in the maternal DNA.[73–75] Zygotic DNA methylation patterns have been reestablished in the inner cell mass at the blastocyst stage.[62]

8.2 | Histone modification

In addition to DNA methylation, histone modification also regulates gene expression patterns in embryonic development. Four types of core histones (H2A, H2B, H3, and H4) form the nucleosome along with 147 base pair of double stranded DNA,[76] and 3′ terminals of each core histones extended from the histone cores are subjected to various modifications, including the acetylation or the mono-, di-, and trimethylation of lysines.[77] Histone acetylation, which is caused by histone acetyl transferases, is linked to transcriptional activation, changes the configuration of nucleosomes exposing DNA areas required for the transcription.[78] However, histone deacetylation, which is the opposite action of histone acetylation, is linked to transcriptional repression activity. The histone deacetylases are recruited to the histone tails of the nucleosomes with methylated CpG sites, resulting in chromatin condensation that diminishes accessibility for transcription factors.[77,79,80] At the deacetylated sites, histone methylation appears to follow demethylation.[79,81] Histone methylation which is catalyzed by histone methyl transferases on arginine or lysine residues of histone tails[81] controls either gene expression or repression. Methylation formed in the nucleosome tails of promotor or body regions of active genes (methylation of H3K4 or H3K36 residues) is linked to transcriptional activation. Methylation on H3K9 residues appears to play a role to suppress transcription interacting with DNA methyl transferases.[62,82]

In spermiogenesis, round spermatids replace nuclear proteins with protamines instead of the core histones to transform into mature sperm while completing DNA methylation patterning. After mature sperm with a tightly condensed nucleus penetrate oocytes, decondensation and chromatin remodeling (re-replacement of protamines into histones) of the sperm nucleus are promoted by nucleoplasmins contained in ooplasm in a few hours,[83–85] and then a male pronucleus is formed. During the specific chromatin remodeling, histone H3K9 trimethylation that is linked to gene silencing is maintained at a very low level in the male pronucleus, compared to the female pronucleus,[86,87] although high level of H3 and H4 acetylation is maintained in both male and female pronuclei.[86,88]

In ROSI embryos, the lack of methylation and histone rearrangement that must be established in spermatogenesis would have to be restored for normal embryonic development and successful delivery.

9 | EPIGENETIC ABNORMALITY IN ROSI EMBRYOS

Previous ROSI studies in mice reported that the low rate of the offspring delivery is owing to epigenetic abnormality at the early embryonic stage. In 1994, Ogura et al.[8] reported that in mice normal offspring was born with oocyte fusion of spermatids, when DNA methylation had been partially established, although the success rate was very low. A comparative study between ICSI and ROSI mouse embryos revealed that epigenetic errors are associated with the poor development of ROSI embryos.[86] In 90% of mouse ROSI embryos, significant hypermethylation was found in male pronucleus, as compared with one in ICSI embryos. DNA methylation patterns of injected spermatids appears to be directly copied by maintenance methyltransferase through DNA replication, since spermatid nucleus declines to skip demethylation process by transformation of 5mC to 5hmC which runs parallel to histone-protamine replacement. Consequently, the ROSI embryos of which male pronucleus DNA was normally demethylated by chance appears to be successfully delivered.[23] The distinct demethylation level of male pronuclear DNA was associated with body sizes of E11.5 ROSI fetuses. Zhu et al.[89] showed a possibility that decreased cell proliferation depending on hypermethylation of Rec 8 promoter regions may result in the smaller body sizes at the E11.5 stage.

However, genetic screening of ART children has revealed a possibility that similar imprinting disorders arise during in vitro culture of early cleavage embryos. In mice, serum components used for in vitro culture of early cleavage embryos decreased the expression of imprinting genes, which are generally methylated, in fetuses.[90] Many studies have also reported that demethylation of imprinted genes, such as H19, is associated with the operation of ART in mice and humans.[91–95] However, Novakovic et al.[91] showed with peripheral blood cells that ART-associated methylation disorder found in ART-neonatal children largely resolves by adulthood, suggesting no direct evidence of serious effects on their growth and health. This is a hopeful outcome that dispels a concern about abnormal methylation at the early cleavage stages. Further studies would be important to track the fate of embryonic methylation abnormalities.

10 | RECOVERY OF POOR ROSI EMBRYO DEVELOPMENT

In order to improve the success rate of ROSI, the application of several substances which inhibit abnormal DNA methylation of spermatid-derived pronucleus to the culture of mouse ROSI embryos has been attempted. Trichostatin A[96,97] and Scriptaid[21,98] inhibit histone deacetylation and successively enhance transcriptional activity and protein expression. Postfertilization treatment of mouse ROSI embryos with Trichostatin A for 20h reduced the hypermethylation level of the sperm-derived pronucleus to a level similar to that of the sperm-derived pronucleus.[86] Hosseini and Salehi[99] attempted to use mouse round spermatids exposed to Trichostatin A (100nM, 45min) prior to ROSI and confirmed similar effects that enhance blastocyst qualities (ICM number and ICM marker gene expression). Scriptaid treatment (250mM, 10h) of mouse ROSI embryos at the pronucleus stage more enhanced blastocyst formation and delivery rates restoring gene expression and abnormal DNA methylation.[21] Wang et al.[100] recently found that a compound "A366" which were selected by screening of the epigenetic modification-related small compound library was effective on normal development and delivery of mouse ROSI embryos improving epigenetic abnormalities (300nM, 20h). As Hosseini and Salehi,[99] confirmed, very brief exposure of round spermatids to these compounds may be equally effective and reduce predictable risks. In addition to such effectiveness, the studies used Scriptaid and A366 reported that the live offspring from mouse ROSI embryos were healthy and fertile. The use of these compounds must improve the success rate of ROSI in humans, although ethical concerns remain.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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