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Uncommon but devastating event: total fertilisation failure following intracytoplasmic sperm injection

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Summary

Fertilisation with intracytoplasmic sperm injection (ICSI) is a consequence of complex molecular interactions between spermatozoon and oocyte. Disruption of the process obviously prompts a frustrating event called total fertilisation failure (TFF). Up to 3% of ICSI cycles may result in TFF, and brief counselling for subsequent cycle management is indispensable. Within this perspective, ICSI cycles of a centre over a 10-year period were analysed to document TFF cases. Initial TFF after ICSI and subsequent ICSI cycle of the same cases were documented to clarify predictive factors of successful outcomes after initial TFF. In subsequent cycles, assisted oocyte activation (AOA) with calcium ionophore and Hypo-osmotic swelling test (HOST)/pentoxifilline for sperm selection was used. In the current analysis, successful fertilisation was achieved in 85% of the cases with previous TFF. The significant contributing factors for successful fertilisation in the latter cycle were: improved oocyte quantity and better sperm morphology. In conclusion, sporadic TFF event in the first and only cycle is usually a technically modifiable condition, but repeated TFF could indicate possible gamete defects, which might not be overcomed in the next modified ICSI cycle.

Introduction

After achieving successful pregnancies by injecting a single spermatozoon into the cytoplasm of an oocyte by micromanipulation in the early 1990s, indications of intracytoplasmic sperm injection (ICSI) have been broadened. In general, the decision to treat a patient with *in vitro* fertilisation (IVF) or ICSI is mainly based on the evaluation and assessment of semen parameters. Majority of clinicians also refer couples to ICSI when the female partner has diminished ovarian reserve. Other proposed indications for ICSI are unexplained infertility, advanced maternal age, prior fertilisation failure with conventional insemination, pre-implantation genetic testing, after *in vitro* maturation, and fertilisation of cryopreserved oocytes (Practice Committee of the American Society for Reproductive Medicine & Society for Assisted Reproductive Technology, 2012).

Routine use of ICSI has greatly improved fertility outcomes of the cases complicated with oligoasthenoteratozoospermia (OAT) and has become the standard treatment for severe male factor infertility in assisted reproductive technology (ART) cycles. Main rationale of ICSI is to overcome possible adverse events related with spermatozoon during the fertilisation process and to avoid fertilisation failure in early spermatozoon–oocyte interaction period. Using this technique, up to 80% fertilisation rate can be achieved in the presence of mature oocytes in all age groups (Palermo *et al.*, 2000). On the other hand, failure of this fertilisation process obviously results in a frustrating event called total fertilisation failure (TFF). TFF may occur in up to 3% of ICSI cycles and is defined as the failure of all available oocytes to fertilise (Flaherty *et al.*, 1998). TFF may occur due to the defects in the oocyte, spermatozoon or the technique itself. Sperm related disturbances that may prompt TFF are nonviability, altered chromatin status, inability to activate the oocyte and decondensation failure (Nasr-Esfahani *et al.*, 2010). The main oocyte-related factor contributing to TFF is failed activation. Nevertheless, no such test can entirely exclude the possibility of TFF.

As TFF is frustrating both for the couples and clinicians, an improved understanding is of importance for counselling and for future management. Despite quite high

fertilisation rates that are achieved in subsequent attempts (Kinzer *et al.*, 2008), factors associated with TFF should be carefully assessed and well documented to avoid recurrent TFF. The main objective of this study is to evaluate the dynamics and outcomes of cycles that have ended with TFF and latter cycles that have ended with a successful embryo transfer (ET). Secondary objective is to identify the most predictive factors contributing to successful fertilisation in cases with previous TFF history despite ICSI.

Materials and methods

Computerised data of a single private ART centre between 2004 and 2014 were retrospectively analysed. Out of 7100 ICSI cycles over a 10-year period, 78 TFF cases were detected during initial ICSI attempt. Among these cases, 44 couples further underwent a new ICSI cycle. Repeated TFF occurred in 15.9% (7/44) of the cases where successful fertilisation and ET were performed in 84.1% (37/44). Demographic data and cycle outcome measures were compared between initial TFF cycles and subsequent ICSI cycles with successful ET. Exclusion criteria were; cases with previous ovarian and testicular surgery, azoospermia cases, testicular sperm extraction (TESE) cycles, cases with chemotherapy or radiotherapy history and cases with BMI >30 kg m^{-2}. Following TFF in initial cycle, none of the male partners have had any medical or surgical intervention before subsequent ICSI cycle.

Ovarian stimulation and embryo transfer procedure

All subjects have undergone controlled ovarian hyperstimulation either with agonist (long luteal or microdose flare protocol) or short antagonist protocol. All subjects received 150–450 IU of daily gonadotrophin using recombinant FSH (Gonal F, Serono) with or without hMG (Menogon, Ferring, or Merional, IBSA) at the discretion of the physician. Gonadotrophin regimen was maintained daily and adjusted individually according to serum estradiol (E2) concentrations and ovarian response as noted by ultrasound. Recombinant human chorionic gonadotropin (rhCG) (250 μg/subcutaneously, Ovitrelle, Merck-Serono) or urinary hCG (10 000 IU/intramuscularly, Pregnyl, MSD) was administered to all subjects for the final oocyte maturation when at least three follicles >18 mm in diameter were detected. Transvaginal oocyte retrieval process was performed at the 35th–36th hours of triggering ovulation for final oocyte maturation.

Following retrieval, cumulus oophorus was removed from oocytes by incubation in a solution containing hyaluronidase (Vitrolife, Frölunda, Sweden). The remaining cells were removed mechanically using commercial denuding pipettes in g-mops (Vitrolife) drops (10 μl). Denuded oocytes were cultured in G-IVF (Vitrolife) medium at 37 °C in a humidified atmosphere of 5% CO_2–95% air, until used for ICSI.

Semen samples were collected by masturbation 2–5 days after the last ejaculation, for ICSI. The seminal analysis of sperm concentration, sperm morphology and volume of ejaculate was performed according to the World Health Organization criteria (WHO, 2010). Seminal processing was performed using the discontinuous gradient technique with the use of the sperm grad (upper layer, lower layer and sperm rinse) (Vitrolife) and followed by the evaluation of total seminal count in the sample retrieved. Sperm morphology was evaluated in a single sample at the initial assessment of patients, counting 100 cells, according to the strict criteria proposed by Kruger/Tygerberg and adopted by the World Health Organization (World Health Organization, 2010), using SpermMac™. In cases with total immotility, vitality of spermatozoa was estimated by hypo-osmotic swelling test (HOST) (between 2004 and 2006), pentoxifilline solution (between 2006 and 2010) and Sperm Mobile GM 501 (Gynemed, Lensahn, Germany) (between 2010 and 2014). As for the HOST test, sperm samples and distilled water (1 : 1 ratio) were put into 50 μ sperm droplet for 15 min. Subsequently, spermatozoa with curved tail were picked up and collected in 5 μl G-Mops dish. After 15 min, spermatozoa with properly formed tail were selected to perform ICSI. There was no frozen sperm usage. ICSI procedure was routinely performed in all cases by the same embryology team. Fertilisation was confirmed in the presence of two pronuclei and two polar bodies 16–18 h following ICSI. After 8–10 h of fertilisation control, early cleavage zygotes are controlled again for discarding of early syngamy possibilities.

In the subsequent ICSI cycle, Ca++ Ionophore (AOA) was the method to activate oocytes, which was performed routinely for all subjects with previous TFF history. Briefly, all metaphase II (MII) oocytes were exposed to a commercially available ready-to-use ionophore (GM508 Cult-Active; Gynemed) for 15 min following removal of HEPES immediately after ICSI. Then, oocytes were rinsed again with G-IVF (Vitrolife), were transferred into G-1 (Vitrolife) droplets (which contain 5% HSA) and were equilibrated at 37 °C, 5% O_2, and 6% CO_2 (O/N). As ready-to-use ionophore GM508 Cult-Active has been available in the local market since 2009, another ionophore was the method for AOA before that date (10 micro mol/l A23187).

Embryo transfer was performed on day 3 or 5 after oocyte retrieval. Embryo transfer procedure and standard luteal support were performed as described elsewhere (Pabuccu *et al.*, 2014). A biochemical pregnancy was defined as βhCG concentration >10 IU l^{-1} on the 12th day after transfer. A clinical pregnancy was defined as the

presence of an intrauterine gestational sac with a heartbeat 3 weeks after a positive βhCG test.

Statistical analysis

Data analysis was performed using SPSS for WINDOWS, version 11.5 (SPSS Inc., Chicago, IL, USA). Whether the distribution of continuous variables were normal or not was determined by Kolmogorov–Smirnov test. Data were shown as mean ± SD (95% CI) or median (min–max), where applicable. The mean differences between groups were compared by Student's *t*-test; otherwise, Mann–Whitney *U*-test was applied for comparison of the median values. The differences in median values between more than two independent groups were analysed by Kruskal–Wallis test. When the *P* value from Kruskal–Wallis test statistics were statistically significant, Conover's nonparametric multiple comparison test was used to know which group differs from others. Categorical data were analysed by Pearson's chi-square or Fisher's exact test, where applicable. Degrees of association between continuous variables were evaluated by Spearman's rank correlation analyses. The best predictor(s), which affects the number of fertilised oocytes, was evaluated by multiple linear regression analysis backward procedure. Any variable whose univariable test had a *P* value <0.05 was accepted as a candidate for the multivariable model along with all variables of known clinical importance. Coefficient of regression and 95% confidence intervals for each independent variable were also calculated. Because distribution was not normal, logarithmic transformation was used for the number of 2PN in regression analyses. A *P* value <0.05 was considered statistically significant.

Results

During the study period, the mean fertilisation rate after ICSI was 98% and TFF was observed in 1.09% of the cycles (78/7100). The mean age of patients was 39 ± 6 years. The characteristics of patients (age, basal ovarian reserve markers), cycles (type of gonadotropins, stimulation protocols) and andrology data are given in Table 1. Total immotility was recorded in 62% ($n = 49$), abnormal sperm morphology was detected in 90% (70/78) and low sperm count (<5 million ml^{-1}) was observed in 70.5% (55/78) of the cases. A combination of at least one abnormal sperm parameter (poor morphology, low count or immotility) and poor oocyte yield was present in 74% (58/78) of TFF cycles. Poor oocyte yield (number of retrieved oocytes <5) was recorded in 79% (62/78) of the cycles. The outcomes of cycles with TFF and subsequent cycles with ET are given in Table 2. When compared with TFF cycles, significantly higher number of retrieved oocytes (4 versus 2, $P < 0.001$) and mature oocytes (3 versus 1, $P < 0.001$) were obtained in subsequent cycles, with a mean fertilisation rate of 50% (25–100%). The biochemical and clinical pregnancy rates were 13.5% and 8.1% respectively. Among seven cycles that have resulted with repeated TFF, the mean age of women was 40.2 and mean number of available mature oocytes was 2. As for andrologic parameters, mean sperm concentration was 32×10^6 ml^{-1}. Five cases out of seven had total immotile spermatozoa, and all seven subjects had poor sperm morphology (normal morphology <4%). The number of repeated TFF cycles was considered as limited for further statistical analysis.

Table 1 Patient characteristics and treatment protocols of cycles with total fertilisation failure and subsequent cycles with embryo transfer

	Total fertilisation failure ($n = 78$)	Cycles with ET ($n = 37$)	*P* value
Age (year)	39.4 ± 6.0	38.4 ± 6.0	NS
Basal AFC	4 (1–16)	4 (1–16)	NS
Basal FSH	10 (3.6–39)	12 (3.9–21)	NS
Type of gonadotropin			
rFSH	42 (53.8)	21 (56.8)	NS
hMG	20 (25.6)	7 (18.9)	
rFSH+hMG	16 (20.5)	9 (24.3)	
Protocol			
Agonist	35 (44.9)	20 (54.1)	NS
Antagonist	43 (55.1)	17 (45.9)	
Sperm count (10^6 per ml)	28.5 (1–118)	41 (1–119)	NS
No of subjects with sperm morphology <4% (%)	70 (90)	30 (81)	NS
No of subjects with total immotility (%)	49 (62.8)	18 (48.6)	NS
Total Motile Sperm Count	15.4×10^6	18.3×10^6	NS

NS, not significant; AFC, antral follicle count; FSH, follicle-stimulating hormone; rFSH, recombinant FSH; hMG, human menopausal gonadotropin.

According to Spearman's rank correlation analyses, sperm motility, sperm morphology, peak E2 levels, number of retrieved and mature oocytes were independent variables that have been associated with fertilisation (Table 3). According to multiple linear regression analysis backward procedure, MII oocytes and sperm morphology were the best predictors, affecting the number of fertilised oocytes (for MII oocytes B = 0.246, 95% CI: 0.187–0.305, $P < 0.001$, and for morphology >4% B = 0.336, 95% CI: 0.019–0.654, $P = 0.039$).

Discussion

Based on the results of 10 years of data in this study, successful fertilisation is practicable in cases with TFF history

Table 2 Cycle outcomes of patients with total fertilisation failure and subsequent cycles with embryo transfer

Parameter	Total fertilisation failure (n = 78)	Cycle with ET (n = 37)	P value
Peak E2 (pg ml^{-1}) Median (min–max)	800.5 (111–5580)	1245 (132–5300)	NS
Duration of stimulation (days) Median (min–max)	11 (3–19)	11 (8–15)	NS
Endometrial echo (mm) Median (min–max)	9.5 (6–18)	10 (7–14)	NS
Total gonadotropin use (IU) Median (min–max)	3000 (150–11 250)	3600 (1350–9750)	NS
No of retrieved oocytes Median (min–max)	2 (1–9)	4 (2–15)	**<0.001***
No of MII oocytes Median (min–max)	1 (1–7)	3 (1–9)	**<0.001***
No of 2PN Median (min–max)	–	2 (1–8)	–
Fertilisation n (%)	–	50 (25–100)	–
No of transferred embryos Median (min–max)	–	1 (1–3)	–
Biochemical pregnancy n (%)	–	5 (13.5)	–
Clinical pregnancy n (%)	–	3 (8.1)	–

NS, not significant; E2, estradiol; MII, mature oocytes; 2PN, 2 pronuclei.
*$P < 0.001$ statistically significant cycle outcome parameters.

Table 3 Spearman's Rank Correlation of independent variables associated with fertilisation

Independent variables	Correlation coefficients	P
Age (years)	−0.178	NS
Sperm count ($\times 10^6$ ml^{-1})	0.064	NS
Normal sperm morphology	*0.514*	***<0.001****
Peak E2 (pg ml^{-1})	*0.370*	*NS*
Duration of stimulation (days)	0.103	NS
No. of oocytes	*0.665*	***<0.001****
No. of MII oocytes	*0.787*	***<0.001****

NS, not significant.
*$P < 0.001$: statistically significant independent variables.

and the outcome mainly depends on AOA, higher number of oocytes retrieved and spermatozoon with normal morphology. In men with severe OAT, Ca++ ionophore for AOA preceding HOST or pentoxifilline/Sperm Mobile for the selection of spermatozoa for ICSI seems to resolve the problems associated with sperm motility and oocyte activation.

Intracytoplasmic sperm injection is the method of choice to achieve fertilisation even in cases where sperm motility and ability to penetrate the zona pellucida is vicious. In the absence of motile spermatozoon, viability assessment before ICSI procedure is a key step to procure successful fertilisation. As performed in this study, HOST is the most common practice to identify a viable spermatozoon for ICSI with acceptable and comparable pregnancy rates. Other than HOST, Polscope for the selection of birefringent spermatozoa or stimulation of motility with pentoxifilline can be employed in these cases with motility issues. (Sallam *et al.*, 2005; Hattori *et al.*, 2011; Mangoli *et al.*, 2011). Following accurate viability assessment, total immotility cannot be the sole factor for TFF like in the current analysis. Supporting this, morphologic features rather than motility were positively correlated with successful fertilisation in the latter cycles of cases with prior TFF. As previously reported, normal sperm ultrastructure correlates well with ART success (Malgorzata *et al.*, 2007). Aggregated data for 5% normal sperm morphology threshold (strict criteria) reported 59.3% overall fertilisation rates per oocyte for ≤4% normal sperm morphology and 77.6% per oocyte for the >4% normal sperm morphology group (Coetzee *et al.*, 1998). The abnormal morphology has been linked to premature chromosomal condensation and protamine deficiency presenting as enlarged or slightly amorphous headed spermatozoa that significantly affect fertilisation rate when selected for ICSI (Malgorzata *et al.*, 2007; Nasr-Esfahani *et al.*, 2008). Therefore, teratozoospermia seems to be a significant contributing factor for TFF.

Normal morphology of the spermatozoa is regarded as a surrogate marker for the quality of spermatogenesis as

poor morphology is associated with increased DNA fragmentation, chromosomal abnormalities, poor chromatin packaging and high sperm aneuploidy rates (World Health Organization, 2010). A novel method for detailed morphological evaluation of spermatozoa called 'motile sperm organelle morphology examination' (MSOME) relies on the assessment of spermatozoa morphology under a magnification. This modified technique is called intracytoplasmic morphologically selected sperm injection (IMSI). By this method, spermatozoa with a normal nucleus and nuclear content can be identified (Balaban *et al.*, 2011). If the sperm head contains one or more vacuoles occupying more than 4% of the normal nuclear area, then the nuclear chromatin content is considered as abnormal (Ebner *et al.*, 2014). Another sperm selection technique is mainly based on cell surface hyaluronic acid (HA) binding glycoprotein in the human spermatozoa called PICSI technique (ICSI with HA-bound spermatozoa; physiologic ICSI-PICSI). This method allows selection of spermatozoon with minimal DNA fragmentation and low frequency of chromosomal aneuploidies (Woodward *et al.*, 2008). Albeit the beneficial effects of IMSI and PICSI are debatable, couples with prior TFF history might benefit from these methods in their subsequent cycles (Setti *et al.*, 2013; Mokánszki *et al.*, 2014). Another option for such cases could be the application of testicular spermatozoon for ICSI in the subsequent cycle as testicular samples reveal significantly lower DNA damage compared with ejaculated spermatozoa (14.9% $\pm$ 5.0 versus 40.6% $\pm$ 14.8, $P < 0.05$) (Moskovtsev *et al.*, 2012). However, the origin of the spermatozoa used in ICSI does not have a major influence on fertilisation if the selected spermatozoon is motile and morphologically normal (Wennerholm *et al.*, 2000; Bukulmez *et al.*, 2001).

Defective oocyte activation plays a major role in the etiology of TFF, as more than 80% of these oocytes contain a spermatozoon (Flaherty *et al.*, 1998). Oocyte activation process is a result of complex interactions that are triggered by spermatozoa. Intracellular calcium rise starting shortly after spermatozoon–oocyte fusion is the triggering mechanism of oocyte activation (Miyazaki & Ito, 2006; Ramadan *et al.*, 2012). Following ICSI, the inability of a spermatozoon to initiate calcium oscillations or cytoplasmic immaturity of the oocyte is one of the main causes of TFF. The immobilization of the spermatozoon and rupture of the oolemma are two major steps required for calcium oscillations following ICSI (Vanderzwalmen *et al.*, 1996). Even though spermatozoon- or oocyte-related activation deficiency could be managed by artificial means, beneficial effects are not justified in all cases with previous TFF history. To distinguish oocyte-related activation deficiency from other technical/biological factors, using sibling oocytes has been suggested previously (Vanden Meerschaut *et al.*, 2012). However, in our study, such an approach has not been applied due to legal issues. Assisted oocyte activation with calcium ionophores A23187, GM 508, ionomycin, puromycin or strontium chloride help to activate the oocyte by increasing the calcium permeability of the cell membrane, resulting with successful fertilisation of injected oocytes. Other than chemical agents, mechanical and electrical methods are also used to stimulate the calcium oscillations necessary to activate the oocyte after ICSI (Vanden Meerschaut *et al.*, 2014). Electrical stimulation is another type of AOA that induces calcium influx through the pores that are generated by direct current voltage in the plasma membrane and has been related with successful outcomes (Yanagida *et al.*, 1999). In our study, chemical method of AOA was used in subsequent cycles of TFF cases, which yielded a 50% fertilisation rate. In case of severe male factor infertility with a history of TFF, AOA can be useful especially in severe teratozoospermia as previously suggested by Nasr-Esfahani *et al.* (2010).

Diminished ovarian reserve is not only associated with reduced oocyte yield, but it also contributes to TFF and cycle cancellations. When the retrieved number of oocytes is <5, then the risk of TFF and cycle cancellation is presumed to be higher (Melie *et al.*, 2003). Therefore, some authors suggest that the success rate of ICSI is independent of typical semen analysis (Mansour *et al.*, 1995; Oehninger *et al.*, 1995) and mainly depends on the number of available oocytes (Esfandiari *et al.*, 2005). Correspondingly, enhanced mature oocyte yield was found to be a significant contributor of successful fertilisation in our dataset. Subtle improvements in oocyte yield can increase the chance of fertilisation in subsequent cycles of these patients.

Sporadic TFF event in the first and only cycle is usually a technically modifiable condition but repeated TFF might be indicative of gamete defects, which might not be overcomed in the next ICSI cycle. Previous trials reported that the majority of couples, who underwent further ICSI procedure after a single episode of TFF, experienced subsequent successful fertilisation up to 87% (Flaherty *et al.*, 1998; Kinzer *et al.*, 2008; Shinar *et al.*, 2014). In the present study, successful fertilisation was achieved in 85% of the cases with previous TFF. In conclusion, improving oocyte quantity and then AOA in ICSI cycles are crucial steps to overcome TFF. More studies are needed to be conclusive on TFF in ART. Future development in the field of AOA has enormous potential for clinical benefit, particularly for those with TFF history.

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