The Effect of Timing of Oocyte Denudation from Oocyte Retrieval in the Total Fertilization Failure Among in Vitro Fertilization – Intracytoplasmic Sperm Injection Cycles

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Objective: This study aimed to evaluate the effect of oocyte incubation after retrieval in TFF among IVF-ICSI and identify factors affecting total fertilization failure (TFF).

Methods: This is a retrospective cohort study, involving 995 IVF cycles using the antagonist protocol that were clustered into three timings of oocyte denudation from retrieval: Group 1: <1hour, Group 2: ≥1hour to <2hours and Group 3: ≥2hours. Other variables considered were etiology of infertility, female age, days of stimulation and total number of oocytes retrieved.

Results: Overall TFF was 4.5%. TFF among groups were 4.8%, 5.8% and 3.2%, respectively. Multiple logistic regression analysis showed that oocyte incubation prior to denudation for ≥ 2 hours tend to decrease TFF incidence. Among factors studied, male factor infertility and a low number of oocytes adversely affect TFF.

Conclusion: Timing of incubation of oocyte did not significantly affect the occurrence of TFF. Among factors studied, male factor infertility and a low number of oocytes adversely affect TFF.

Key words: oocyte denudation, total fertilization failure (TFF), in-vitro fertilization intracytoplasmic injection (IVF-ICSI)

Introduction

Infertility has been recognized as a global public health issue by the World Health Organization.[1] In a systematic analysis of 277 health surveys carried out by Mascarenhas, et al. in 2012,[2] infertility affects 48.5 million worldwide. Its prevalence was highest in South Asia, Sub-Saharan Africa, North Africa/ Middle East, and Central/Eastern Europe and Central Asia.

There is a growing population of infertile couples seeking advanced reproductive technology services in order to conceive. International Committee for Monitoring ART (ICMART) estimated that since the birth of Louise Brown in 1978, a total of 8 million babies worldwide were born as a result of in vitro fertilization.[3]

In vitro fertilization is the recommended treatment of choice for all couples with continued unresolved fertility problems.[4] The process involves ovarian stimulation, oocyte retrieval, assisted fertilization, and embryo transfer of cleavage stage embryos or day 5 blastocyst.

Total fertilization failure (TFF), defined as 'failure of all the available mature MII oocytes to fertilize'[5], affects 5-15% of IVF cases even with an apparently normal sperm quality.[6] It was suggested that by directly inseminating the oocyte via intracytoplasmic sperm injection (ICSI), the rate of fertilization would improve. ICSI achieves fertilization rate between 70% and 80% with ejaculated spermatozoa regardless of its functionality as long as the male gamete is viable. However, even with ICSI, TFF still occurs in 3 to

5% of cycles[5], with a 3.7% TFF rate documented in the Center for Advanced Reproductive Medicine and Infertility (CARMI), St. Luke's Medical Center, Global City.

Various studies looked into the factors contributing to TFF. Krog and colleagues[6] concluded in their study that female smoking, non-tubal factor infertility, decreased progressive motile spermatozoa after preparation, and decreased number of oocytes retrieved are the predictors of TFF following conventional IVF. In an extensive review by Neri, et al.[5], it was suggested that nucleus-cytoplasmic maturation asynchrony plays a role in the occurrence of TFF in ICSI cycles. Furthermore, several studies support delaying oocyte denudation after oocyte retrieval to improve fertilization outcomes.[7-10] However, literature is rather scarce in establishing the standard timing of oocyte denudation.[10,11]

Significance of the Project

This study will determine significance or trend in increasing fertilization rate in patients undergoing IVF-ICSI with proper timing of oocyte denudation. Establishing the relationship between the total fertilization failure and the number of fertilized embryos available for transfer may contribute to increased implantation rate and subsequently, increase in cumulative pregnancy and livebirth rates.

Literature Review

Epidemiology of in vitro fertilization

A total of 225, 968 IVF cycles were reported by the Society for Assisted Reproductive Technology (SART)[12] among its member facilities across the United States in 2017. Locally, there are more than 2,000 IVF cycles since CARMI started in 2011. Initially used exclusively to alleviate tubal factor infertility, IVF has now evolved its utilization in infertile couples (Appendix 1), fertility preservation, and preimplantation genetic testing.[13]

Among couples undergoing IVF, top indications are male factor (34.8%), followed by diminished ovarian reserve (29.5%) and multiple male and female factors (17.8%). Interestingly, IVF successes were found to be determined by female age, duration of infertility, and previous pregnancy.[13]

Couples electing for IVF must undergo similar to that of basic infertility work-up: test for ovarian reserve and semen analysis. In addition, screening for infections is likewise routine in the evaluation.

The process of fertilization

Fertilization results from the fusion of oocyte and spermatozoon that induces a cascade of critical events leading to the development of the zygote.[5] Meiotic maturation of the oocyte and its subsequent activation by the spermatozoon are two distinct events that are absolute prerequisites for normal fertilization.[9]

In vivo, the capacitated sperm must penetrate the zona pellucida in order to bind and fuse with the oocyte plasma membrane. Soluble cytosolic factor carried by the sperm, in turn, activates a cascade of events leading to the oscillation of intracellular calcium via the phosphoinositide-specific phospholipase C (PLC), which activates the oocyte.[5]

Meiotic maturation of oocyte involves adequacy of both the nucleus and cytoplasm. Nuclear maturation involves extrusion of the first polar body, which is a characteristic of MII oocytes.[5,9,10] Cytoplasmic maturation, on the other hand, is poorly understood but is thought to be highly dependent on cumulus-oocyte complex.[5,10]

Fusion of the two parental gametes to form a zygote is followed by two important processes: the prevention of polyspermy via cortical and zona reactions; and the completion of meiosis. Three hours after insemination, the second polar body of the egg is released, leaving a haploid chromosome. This is complemented by the chromatin material derived from the sperm, which restores the diploid number of the fertilized egg. Each pronucleus derived from the male and female gamete migrates toward each other, forming a spindle, ready for the first cell division.[14]

Fertilization failure

Fertilization failure results from any defect in the abovementioned process – may it be an oocyte-related or a sperm-related deficiency. In conventional IVF, the most common cause of fertilization failure is failed sperm intrusion, thus, ICSI may provide

solution.[11] ICSI aims to assist male factor infertility by direct deposition of spermatozoon into the ooplasm, thus bypassing the zona pellucida.[5,11] The injected spermatozoon contributes to fertilization in two ways: 1) it provides paternal DNA that complements the haploid chromosome of the oocyte, and 2) it serves as a trigger for oocyte activation that leads to the completion of meiosis.[9]

Several studies have reported that the major cause of fertilization failure after ICSI is failure of the oocyte activation due to cytoplasmic immaturity of those gametes even after reaching nuclear maturity.[5,9] Furthermore, it has been shown that nuclear and cytoplasmic maturation in stimulated cycles are asynchronous.[7]

Cytoplasmic maturity is thought to involve maternal mRNA, proteins, substrates and nutrients that are required to achieve developmental competence. The oocyte starts to acquire large amounts of maternal mRNA during the folliculogenesis until germinal vesicle undergoes breakdown during meiosis.[15] Thus, allowing for cytoplasm to acquire its full potential via delaying oocyte denudation may facilitate fertilization success, and embryo development competence.

The precise timing of oocyte denudation from retrieval is widely varied among literatures, ranging from 1 hour to 12 hours.[7-10] This wide range in timing of oocyte incubation led to the formulation of the current study.

Objectives

The general objective of this study was to determine the effect of time of incubation of oocyte after retrieval on the total fertilization failure rate in IVF-ICSI cycles. Specifically, it aimed to determine which among the pre-incubation timings (< 1 hour, ≥ 1 hour to < 2 hours and ≥ 2 hours) significantly improved the incidence of the total fertilization failure rate, thereby, increasing the available embryos for transfer. Furthermore, this study likewise aimed to identify other clinical factors that affect total fertilization failure rate: female age, etiology of infertility (female, male, mixed, unexplained), length of ovarian stimulation (in days) and number of oocytes retrieved.

Methods

This retrospective cohort study was approved by Research and Biotechnology Division of St. Luke's Medical Center, Quezon City. A database generated from the patients undergoing in vitro fertilization with intracytoplasmic sperm injection (IVF-ICSI) at the Center for Advanced Reproductive Medicine and Infertility (CARMI), St. Luke's Medical Center-Global City from January 2012 to December 2018 was retrospectively reviewed.

Inclusion and Exclusion Criteria

All couples must satisfy the minimum prerequisites for IVF according to the Dutch Society of Obstetrics and Gynecology (see Appendix 1). Only the cycles using the gonadotropin-releasing hormone (GnRH) antagonist protocol were included. All cycles using surgically retrieved sperm samples were excluded.

In Vitro Fertilization-Intracytoplasmic Sperm Injection (IVF-ICSI) Protocol

Ovarian stimulation

Ovarian stimulation was performed using the GnRH antagonist protocol.[13] Urinary or recombinant follicle stimulating hormone (FSH), with or without human menopausal gonadotropin (hMG) were started on the second day of the menstrual cycle. Pituitary suppression was achieved by administration of GnRH antagonist (Cetrotide 0.25mg) once with at least 1 leading follicle, measuring 14mm in diameter. When at least 1 follicle reached 18mm, or 2 follicles reached 17mm, ovulation trigger with either urinary or recombinant human chorionic gonadotropin (uhCG 10 000 units or rhCG 250 ug, respectively) was administered.

Oocyte retrieval

Oocyte retrieval under general or spinal anesthesia was scheduled 36 hours after the ovulation trigger. Ultrasound-guided oocyte retrieval was carried out using a transvaginal probe, attached to a gauge 16 double-lumen needle, connected to a collecting tube. Each follicle was punctured by the

needle, and the follicular fluid was aspirated at 120 mmHg. The collecting tubes were immediately sent to the adjacent laboratory for evaluation for the presence of cumulus-oocyte complexes (COCs).

Oocyte denudation

Oocyte denudation was carried out by treating the COCs with 10% hyaluronidase followed by mechanical removal of cumulus cells using a Pasteur pipette. This was within 1 hour, 1 hour to 2 hours and greater than 2 hours after the oocyte retrieval. Oocytes that were pre-incubated and were not immediately denuded were washed and transferred to culture dishes containing GIVF medium and incubated under 6% CO_2 at 37°C. After denudation, the oocytes were examined for maturational stage.

Sperm preparation

The semen specimen were collected by masturbation into a sterile container. The ejaculate was allowed to liquefy and pre-wash semen analysis was performed. Semen was prepared by 100%–70%–40% gradient system using SpermGrad (Vitrolife AB, Göteborg, Sweden). Semen specimen were placed on the top of the discontinuous density gradient medium and then centrifuged at 250 RPM for 15 minutes. G-IVF+ medium was added to the 100% fraction of pellet and then re-centrifuged for 5 minutes at 300 RPM. The wash and centrifugation were then repeated. The final pellet was prepared via swim-up technique and the suspension was incubated under 6% CO_2 at 37°C, while preparing for ICSI.

Intracytoplasmic sperm injection (ICSI)

Intracytoplasmic sperm injection was performed on the heated stage of an inverted microscope under 400x magnification.[16] A single spermatozoon was selected and was immobilized by compressing the tail of the sperm with an injection pipette. The immotile sperm was aspirated into the tip of the injection pipette. Oocyte was stabilized by an injection pipette with the polar body at the 6 or 12 o'clock position and was pierced by the injection pipette containing the immotile sperm at the 3 o'clock position.[17] After the injection, oocytes were washed and incubated at 6% CO_2, 5% O_2 and 89% N_2.

Assessment of oocyte survival and fertilization

Injected oocytes were assessed for the presence of polar bodies and formation of two pronuclei (2PN) after 16 to 21 hours. Total fertilization failure was noted if none of the inseminated oocytes formed 2PN.

Data Collection and Analysis

The demographics that were collected included female age, etiology of infertility (female, male, mixed, unexplained), length of ovarian stimulation (in days) and number of oocytes retrieved to assure homogeneity across study populations. Timing of oocyte denudation from ovum pickup was classified into three groups: Group 1, < 1hour; Group 2, ≥ 1 hour to < 2 hours; and Group 3, ≥ 2 hours. The corresponding fertilized eggs per group were recorded. Percent fertilized was computed as shown below. Fertilization failure was defined as percent fertilized = 0%.

$$\frac{\text{Number of fertilized oocytes (2PN)}}{\text{Number of mature oocytes inseminated}}$$

Sample size

Sample size was calculated based on the comparison of total fertilization failure (TFF) rate among IVF-ICSI cycles at three different periods of timing of oocyte denudation from oocyte retrieval. Assuming that the TFF rate in general IVF-ICSI population is 5%[18] and if timing is beyond 2 hours, TFF is hypothesized to be reduced to 1%, with an α error of 5%, power of 80% and a 1- tailed alternative hypothesis, sample size required is 223 per group or 669 for 3 groups. Controlling for 4 more variables in the analysis, with an additional 10% for each control variable, final sample size required was 937.

Statistical analysis

TFF rates across different timing of oocyte denudation were analyzed using chi square test. Odds ratio and the 95% confidence interval were calculated using the immediate denudation as the reference. Control of confounders was analyzed using multiple logistic regression. Level of significance was set at α=0.05.

Ethical Considerations

The study abided by the Principles of the Declaration of Helsinki (2013) and conducted along the Guidelines of the International Conference on Harmonization-Good Clinical Practice (ICH-GCP), E6 (R2) and other ICH-HCP 6 (as amended); National Ethical Guidelines For Health and Health-Related Research (NEG HHRR), 2017. The Clinical Protocol and all relevant documents were reviewed and approved by the SLMC Ethics Review Committee. Patient confidentiality was respected by ensuring anonymity of patient records. Each patient document was CODED and did not contain any identifying information in order to ensure confidentiality. All study data were recorded and investigators were responsible for the integrity of the data i.e accuracy, completeness, legibility, originality, timeliness and consistency. The manner of disseminating and communicating the study results guaranteed the protection of the confidentiality of patient's data. All study-related documents such as the all versions of the protocol, ethical clearance, data collection forms, hard copies of source documents, signed informed consent forms shall be kept and stored by the Principal Investigator in strict confidentiality for at least 5 years' after which they will be shredded.

Results

Out of 1,373 cycles performed in CARMI, SLMC GC between January 2012 and December 2018, a total of 995 IVF-ICSI cycles were included and analyzed. The parameters and distribution of variables across different timing of oocyte denudation (Group 1 < 1hour; Group 2, ≥ 1 hour to < 2 hours; and Group 3, ≥ 2 hours) in this study are shown in Table 1.

Among the 995 IVF-ICSI cycles, 393 patients had denuded oocytes within 1 hour from retrieval, 260 patients had an interval of 1 to 2 hours from oocyte retrieval to denudation, and 342 patients had an interval of at least 2 hours prior to denudation. Mean age of female partner and days of stimulation were similar across the groups (Group 1: 36.3 ± 4.83 years and 10.9 ± 1.73 days, Group 2: 36.8 ± 4.92 years and 11 ± 1.89 days, Group 3: 36.4 ± 4.92 years and 11 ± 1.42 days). Female infertility was the most common etiology of infertility in all groups (52.5%, 46.9% and 59.1%), followed by mixed factors (29%, 39.6% and 30.7%) and male factor (10.9%, 9.2% and 5.6%), while unexplained infertility was the least common cause (7.9%, 4.2%, 4.7%). Although the etiologies of infertility across groups were similar, the differences in the proportions across groups were found to be statistically significant (p-value = 0.002).

Furthermore, mean total oocytes retrieved were highest in Group 3 with 11.3 ± 7.5 oocytes, followed by Group 1 with 9.5 ± 6.31oocytes. Group 2 had a significantly least number of oocytes retrieved with 8.7 ± 5.69 (p <0.001).

A univariate logistic regression analysis (Table 2) was performed to investigate the existence of any correlation between the variables and the occurrence of TFF.

Table 1. Baseline parameters and distribution of variables.

Variables	Group 1 <1 hour	Group 2 ≥1 hour to < 2hours	Group 3 ≥ 2 hours	p-value
Number of samples	393	260	342	
Etiology (n, %)				0.002
Female factor	205 (52.2)	122 (46.9)	202 (59.1)	
Male factor	43 (10.9)	24 (9.2)	19 (5.6)	
Mixed factors	114 (29.0)	103 (39.6)	105 (30.7)	
Unexplained	31 (7.9)	11 (4.2)	16 (4.7)	
Female age in years (mean ± SD)	36.3 ± 4.83	36.8 ± 4.92	36.4 ± 4.92	0.375
Mean days of stimulation (mean ± SD)	10.9 ± 1.73	11 ± 1.89	11 ± 1.42	0.939
Mean total oocytes retrieved (mean ± SD)	9.5 ± 6.31	8.7 ± 5.69	11.3 ± 7.5	<0.001

Table 2. Univariate analysis of the predictors of total fertilization failure.

Variables	Status		p-value[a]
	TFF	Successful	
Timing of denudation [n (%)]			0.305
Group 1	19 (4.8)	374 (95.2)	
Group 2	15 (5.8)	245 (94.2)	
Group 3	11 (3.2)	331 (96.8)	
Etiology [n (%)]			0.862
Female	22 (4.2)	507 (95.8)	
Male	4 (4.7)	82 (95.3)	
Mixed	17 (5.3)	305 (94.7)	
Unexplained	2 (3.4)	56 (96.6)	
Mean female age (mean ± SD)	38.02 ± 5.15	36.40 ± 4.9	0.632
Days of stimulation (mean ± SD)	11.02 ± 2.07	10.95 ± 1.65	0.025
Total oocytes retrieved (mean ± SD)	3.4 ± 3.09	10.2 ± 6.64	<0.001

[a]statistical test – logistic regression

Overall, the total fertilization failure rate reported in this study was 4.5%. Among the timings of oocyte denudation studied, Group 3 has the lowest TFF rate at 3.2%, followed by Group 1 at 4.8%, while TFF rate was highest in Group 2 at 5.8%. Among the etiologies of infertility, unexplained infertility yielded the lowest TFF rate (3.4%), followed by female (4.2%) and male (4.7%) infertility, with highest TFF rate in mixed infertility (5.3%). Women who had TFF were older by 2 years compared to those with successful fertilization (38 years vs 36 years). Timing of denudation, etiology of infertility and mean female age however, all did not reach statistical significance in the univariate logistic regression analysis. Days of stimulation were longer in TFF group (11.022 vs 10.946, p-value=0.025). Finally, the total number of oocytes retrieved were significantly higher in those with successful fertilization, with an average of 10 oocytes, compared to those with TFF, averaging 3 oocytes retrieved per cycle (p-value<0.001).

After adjusting for all potentially confounding variables in the multivariate analysis using multiple logistic regression (Table 3), only etiology of infertility and total oocytes retrieved were significantly related to TFF. Still, timing of denudation was not found to be significantly related with TFF.

The etiology of infertility significantly influenced TFF in the study population (p-value=0.027). Using female factor infertility as the reference, the odds of having TFF were increased by sevenfold in couples with male factor infertility (OR 7.13 95% CI 1.96 – 25.98). Other etiologies of infertility such as mixed and unexplained, female age and days of stimulation all did not significantly affect TFF.

The total number of oocytes retrieved showed a statistically significant effect on the occurrence of total fertilization failure (p<0.001). That is, for every single increase in total oocytes retrieved, the odds of TFF decrease by 36% (OR 0.64, 95% CI 0.56-0.74).

Discussion

Total fertilization failure (TFF) affects 5-15% of IVF cases despite an apparently normal sperm quality.[6] With the advent of ICSI, the occurrence of TFF decreased to 3- 5% of cycles. The total fertilization failure in ICSI cycles in this study was low (4.5%), thus, comparable with international standards.

A spectrum of factors that influence fertilization rate in conventional IVF and ICSI cycles were found in studies. These include delaying oocyte denudation from retrieval[7-10], etiology of infertility[6,21], female age[6,21,24], length of ovarian stimulation phase[23], number of oocytes retrieved[21,24], BMI and duration of infertility 6, to name a few. For the purpose of

Table 3. Multiple logistic regression analysis of the predictors of total fertilization failure.

Variables	Odds Ratio	95% Confidence Interval		p-value
		Lower	Upper	
Timing of denudation				0.848
Group 1*				
Group 2	1.13	0.53	2.40	
Group 3	0.88	0.39	1.98	
Etiology				0.027
Female*				
Male	7.13	1.96	25.98	0.003
Mixed	1.58	0.79	3.14	0.192
Unexplained	1.58	0.33	7.58	0.570
Female age	0.94	0.87	1.02	
Days of stimulation	1.07	0.92	1.23	
Total oocytes retrieved	0.64	0.56	0.74	<0.001

this study, the authors analyzed the effect of timing of oocyte denudation from retrieval, etiology of infertility, female age, days of ovarian stimulation and number of oocytes retrieved on TFF.

Nuclear and cytoplasmic maturation in stimulated cycles are asynchronous. Delaying the denudation of oocyte upon retrieval is hypothesized to promote cytoplasmic maturity[9,10], thereby, improving the fertilization rate.[8] However, the recommended timing is still debatable.

Rienzi, et al.[9] concluded from 95 ICSI treatment cycles included in their study that the fertilization and embryo development rates improve with pre-incubation of 3-12 hours after oocyte retrieval. In another study by Yanagida and colleagues[8] involving 544 treatment cycles, it was evident that oocyte maintains sufficient fertilization ability, with good pregnancy rate and embryo quality when cultured between 1 and 9 hours. Similarly, in a retrospective study by Isiklar, et al.[7] including 1,260 patients, fertilization rate was highest in 2-4 hours of pre-incubation, whereas Patrat, et al.[10] suggested 3 hours between oocyte retrieval and denudation to achieve the maximum chance of fertilization.

Although the lowest TFF was seen when oocytes were incubated by at least 2 hours (Group 3), the authors found that the timing of oocyte denudation did not show a statistical significance when ran in univariate and multivariate analyses. This could be explained that apart from nuclear-cytoplasmic asynchrony, other factors are as equally vital in the occurrence of TFF. Such factors include: 1) oocyte yield and quality; 2) male gamete aberrations; and 3) activation deficiency due to decreased sperm activity or impaired oocyte response.[5]

In the multivariate analysis, it has been shown that the etiology of infertility was significantly related to the occurrence of TFF (p=0.027). Taking into account each variable separately, with female infertility being the reference variable, mixed and unexplained infertility both did not significantly affect TFF. It has been shown, however, that the odds of TFF increased by seven-fold in patients having male infertility (OR 7.13 95% CI 1.96-25.98).

Decreasing quantitative value of conventional semen analysis parameters such as motile spermatozoa[6,21], mean percentage of morphologically normal sperm and sperm concentration[20,21] all significantly affect the occurrence of TFF. This suggests that sperm defects likewise contribute to the fertilization rates in IVF cycles. This was evident in a retrospective study conducted by Zhu, et al.[20] among 2,429 conventional IVF cycles – that when the morphologically normal sperm drops below 5%, the risk of TFF is evidently increased due to decreased sperm-zona binding rate.

Introduction of ICSI has dramatically increased fertilization and pregnancy outcomes in conventional IVF cycles by bypassing the acrosome reaction and zona penetration. In ICSI cycles, however, the fertilization rate is still flawed. Lee and colleagues[21] suggested that the quality of sperm could influence fertilization, implantation and subsequent outcome in ICSI cycles. In their retrospective study involving 206 ICSI cycles, there was a significantly lower fertilization rate in cycles with poor sperm quality than the normal. They theorized that the paternal effect that is responsible for failed ART cycles, is possibly mediated by centrosome dysfunction or by deficiency of oocyte activating factor.

Such theory is supported by Neri and colleagues.[5] In their extensive review, oocyte activating factor is found to be carried by the sperm head via the molecule, PLC, which triggers Ca^{2+} oscillations eliciting oocyte activation, and early embryonic development up to the blastocyst stage. Thus, it is highly plausible that defective sperm may cause certain types of male factor infertility through oocyte activation failure. In summary, not only sperm quantity but more importantly, the sperm quality plays a role in occurrence of TFF, especially in ICSI cycles. Thus, inactive form of oocyte activating factor in the sperm cell can probably account to more cases of TFF than what most used to believe.

It has long been documented that there is an inverse relationship between advanced female partner age and fecundity. Aging results to reduced ovarian reserve and decreased oocyte and embryo competence. Furthermore, mitochondrial dysfunction, telomere-mediated oocyte aging, loss of cohesion between sister chromatids and meiotic spindle instability all are postulated to contribute to age-related infertility. Mitochondria, the cell powerhouse, is crucial for efficient oocyte cytoplasmic and nuclear maturation during folliculogenesis. It also participates in Ca^{2+} signaling pathway particularly important for fertilization and early development. With aging, mitochondria within the oocyte undergo structural changes and decrease metabolic activity, ultimately leading to impairments in embryo development and implantation.[22]

In this study, the authors found that the female partner age did not affect the occurrence of TFF. This is in congruence with the study of Krog, et al.[6] TFF may not be related to chronological age, rather, biological age – that is, the ovarian reserve. The number of collected oocytes as a proxy of the ovarian reserve appears to be more appropriate predictor of TFF than the female chronological age.

Conflicting data were found regarding the effect of length of ovarian stimulation on ART outcomes. In a retrospective study by Maxwell, et al.[23], which investigated 295 fresh, autologous IVF cycles, shortened cycle length, defined by <8 days, was associated with lower pregnancy rates and oocyte yield from mature oocytes when compared to normal length (9-12 days). On the other hand, Alport and colleagues[19] reported that SPL did not increase the probability of fertilization. In our study, the ovarian stimulation phase length was similar for both groups, thus, we cannot draw conclusions as to its effect on TFF.

As in other studies[6,18,24,25], the authors observed that the number of oocytes retrieved inversely relate to the occurrence of TFF. In a retrospective study by Esfandiari, et al.[18], the retrieval of three or less MII oocytes is known to be a major contributing factor to complete failed fertilization. On the other hand, when patients were used as their own controls, Kahyaoglu, et al.[25] observed that the number of total and mature oocytes retrieved were found to be three times more compared to TFF cycles. These studies were in congruence to present findings: the mean number of oocytes retrieved in TFF group was 3.4 ± 3.09, threefold lower compared to that of successful fertilization (10.2 ± 6.64). Moreover, the lowest number of oocytes retrieved among groups were in Group 2. Hence, if the authors are to follow the notion that the higher the oocytes retrieved, the less likely the occurrence of TFF, the low number of oocytes retrieved in Group 2 probably explains the highest TFF rate among groups.

Among the variables studied, only the number of oocytes retrieved has consistently showed a significant impact in the occurrence of TFF when ran on univariate and multivariate analyses. That is, for every single oocyte retrieved increase, the odds of having TFF decrease by 36%.

Conclusion

Timing of incubation of oocyte did not significantly affect the occurrence of TFF. Among

factors studied, male factor infertility and a low number of oocytes adversely affect TFF.

Recommendation

Male factor infertility may be further analyzed as to which semen parameter significantly affects TFF. Not only does the TFF can be studied, also, the embryo quality and its development.

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Appendix

Appendix 1. Indications for in vitro fertilization
(as recommended by the Dutch Society of Obstetrics and Gynecology)

1	Tubal pathology	If tubal surgery is not a realistic option. In cases of impaired tubal function but no occlusion is present, or following tubal surgery after an infertility duration of ≥ 2 years. Depending on female age, IVF can be done after a shortened duration of infertility.	
2	Unexplained infertility	IVF is indicated if the duration is ≥ 3 years. If woman is ≥ 36 years, IVF may be considered earlier.	
3	Male infertility	TMC < 1M	First treatment of choice is ICSI
		TMC > 1M and < 10M	IVF if infertility duration is ≥ 2 years.
		TMC > 10M	Treat as unexplained infertility
4	Endometriosis	In case of mild or moderate, treat as unexplained infertility. In case of severe, treat as tubal pathology.	
5	Cervical factor/ Immunological infertility	After an infertility duration of ≥ 2 years, IVF is indicated. If woman is ≥ 36 years, IVF may be considered earlier.	
6	Hormonal disturbances	Anovulatory cycle abnormalities are indications for IVF if 12 cycles of treatment with ovulation induction have been unsuccessful.	

**IVF: in vitro fertilization, TMC: total motile count, M: million*
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