UNFERTILIZED OOCYTES AFTER INTRACYTOPLASMIC SPERM INJECTION AND WOMEN'S AGE

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This study assessed the characteristics of meiotic spindles in unfertilized oocytes following intracytoplasmic sperm injection (ICSI) and their relationship with the women's age. A total of 263 unfertilized oocytes were collected and were divided into 2 groups based on the age of the women. Meiotic spindles were stained and classified to identify causes of fertilization failure. Identified relationships between the spindle apparatus, causes of failed fertilization and the women's age. The number of unfertilized oocytes and the rate of unfertilized oocytes per cycle were not significantly different between women aged 35 years and younger and those older than 35 years (3.6 ± 2.4 versus 2.6 ± 2.2 unfertilized oocytes, p=0.083; 22.0 ± 12.1% vs 21.8 ± 12.9% unfertilized oocytes per cycle, p=0.947). The rate of disarranged spindles in oocytes collected from women older than 35 years was significantly higher than that of women 35 years and younger (73.5 vs. 52.5%; OR = 2.50, CI = 1.23 – 5.07, p = 0.011). The main factor associated with the failure of oocytes to fertilize after ICSI was failed oocyte activation, and there was no statistically significant difference between the two age groups. The women's age had a relationship with abnormal spindles.

Keywords: Failed oocyte fertilization, ICSI, meiotic spindle, women's age.

I. INTRODUCTION

Infertility is a global health issue, with an estimated prevalence of 12.5% of women and 10% of men facing fertility problems.[1] As a result, assisted reproductive technology (ART) is a growing area of interest. Conventional *in vitro* fertilization (IVF) has effectively helped cases of infertility due to maternal causes, and intracytoplasmic sperm injection (ICSI) is a revolutionary technique that helps cases of male factor infertility. However, despite these advances, failed fertilization following *IVF* attempts can happen and remains an important concern. Depending on the sperm source, the fertilization rate of oocytes during IVF ranges from 50% to 80%.[2]

In the fertilization process, many events occur. After penetrating of single sperm, a fully developed oocyte is capable of continuing meiosis, decondensing the sperm nucleus, then producing two pronuclei, creating a zygote, and having the ability to transform into a cleavage embryo. Understanding the causes of failed fertilization is essential to improving the ART success rate. Age has a great impact on fertility in women as there is decline in ovarian follicle reserve and decrease in oocyte quality in older women.[3] The aging of oocytes can lead to increased aneuploidy due to spindle apparatus disruption.[4] This is one of several abnormalities that significantly contributes to failure to fertilize. Due to ethical issues and limitations in oocyte collection during IVF procedures, studies on failed oocyte fertilization remains an issue of interest. Evaluation of spindle morphologies can

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help identify the causes of fertilization failure. Our study aimed to evaluate the relationship between the age of women and the meiotic spindle morphology in unfertilized oocytes after ICSI as well as describing some characteristics of ICSI cycles.

II. MATERIAL AND METHODS

1. Sources of oocytes with failed fertilization

Unfertilized oocytes were collected during IVF/ICSI procedures performed at the IVF and Tissue Engineering Center, Hanoi Medical University Hospital, from January 1st, 2020, to August 31st, 2022. Oocytes that failed to fertilize were defined as those without two pronuclei when observed 17–21 hours after ICSI and without embryogenesis by Day 2.

In this study, we collected 263 oocytes that failed to fertilize following ICSI from a total of 80 patients. Oocytes were divided into 2 groups according to the women's age: (1) oocytes taken from women aged 35 years or younger (201 unfertilized oocytes from 56 patients) and (2) oocytes taken from women aged older than 35 years (62 unfertilized oocytes from 24 patients).

2. Method

Study design

The study design is described in Figure 1.

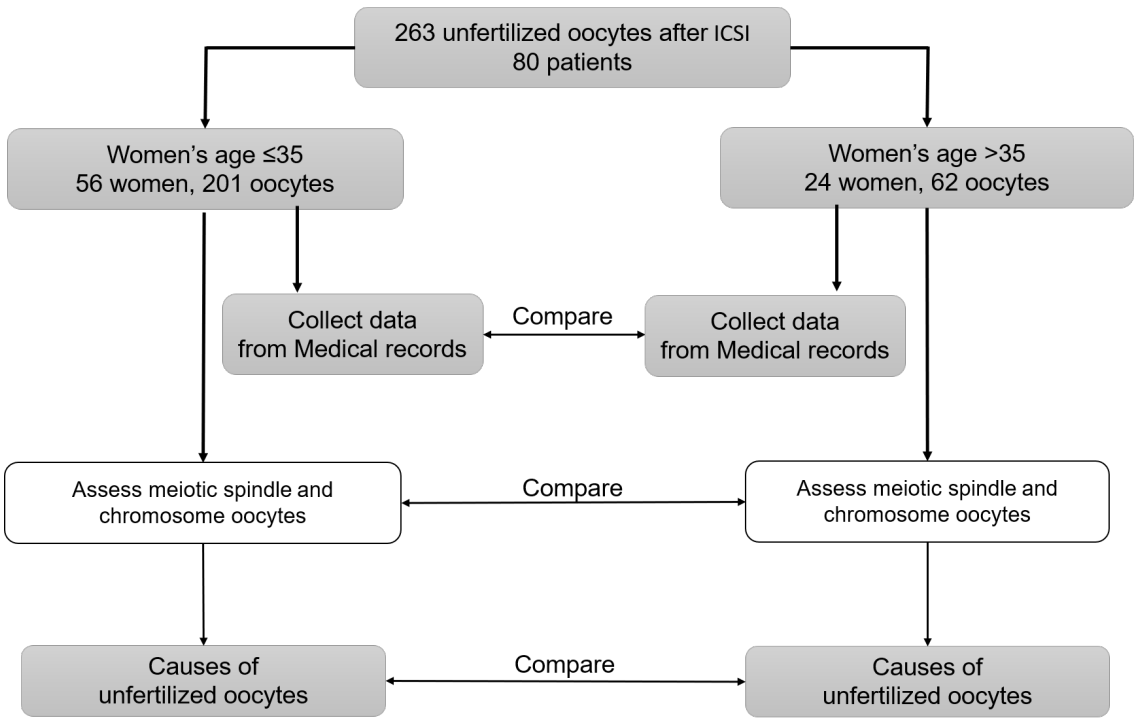

Figure 1. Research model

Fluorescent staining technique

Oocytes that failed to fertilize were incubated in PHEM spindle stabilization buffer (60mM PIPES, pH 6.9; 25mM HEPES; 2mM $MgCl_2$; and 10mM EGTA) containing 0.5% TritonX-100 for 30 second, followed by fixation in 2% paraformaldehyde (#P6148, Merck) in phosphate-buffered saline (PBS) for 30 minutes at room temperature. Antigen retrieval was performed in 0.5% Triton X-100 at

room temperature for 30 minutes. Nonspecific antibody binding sites were blocked using 5% normal goat serum in 1× PBS for 1 hour. After, oocytes were incubated with anti–alpha-tubulin antibody (alpha-tubulin polyclonal antibody, #PA5-16891, ThermoFisher Scientific; 1:200) at 4°C overnight or 2 hours at room temperature. Oocytes were then washed three times for 15 minutes each with 1× PBS containing 0.1% Tween 20 and 0.01% Triton X-100, followed by incubation with a fluorescent secondary antibody [goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 546, #A-113035, ThermoFisher Scientific; 1:200] for 1 hour at room temperature. After washing three times for 15 minutes each in 1× PBS, chromosomes were stained using Sytox Green (S7020, ThermoFisher Scientific) for 5 minutes. The oocytes were washed three times for 15 minutes each with 1× PBS then mounted on concave microscope slides using Fluoromount-G™ mounting medium.

Evaluation of meiotic spindles and chromosomes

We used fluorescent microscopy to evaluate the meiotic spindle and chromosome morphology. The meiotic spindles in oocytes were assessed as follows. The presence of a meiotic spindle was categorized as "Yes" or "No". The spindle shapes were categorized as bipolar or disarranged. The chromosomes in the spindles were categorized to be aligned or unaligned. The sperm chromosomes were evaluated for decondensation and categorized as condensed, partially decondensed, prematurely condensed, and in metaphase. The number of polar bodies was also assessed (1 or 2).

We classified the causes of failed fertilized oocytes into 4 types based on spindle morphology and chromosome alignment according to Luo et al.[5] Type I (**no sperm in oocyte**) is characterized by the presence of female chromosomes and the absence of sperm chromatin in the cytoplasm. Type II (**oocyte activation failure**) is characterized by the presence of female chromosomes associated with varying degrees of sperm decondensation or premature chromosome condensation. Type III (**defects in pronucleus formation or migration**) is characterized by the presence pronucleus formation or migration defect associated with groups of condensed chromosomes or disorganized chromatin. And type IV (**other**) includes the issues other than cytoskeletal organization, such as polyspermia.

3. Statistical analysis

All statical analyses were performed using SPSS software (version 20.0, SPSS Inc.). Data are presented as the mean ± standard deviation ($\overline{X}$ ± SD) or frequency and percent. Student's T-test was used to compare the means of two groups. Relationship between categorical variables were assessed using the Chi-Square test or Fisher's exact test when a cell value in a contingency table was < 5. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to evaluate associations. A p value < 0.05 was considered statistically significant.

4. Research ethics

Patients' personal information remained confidential throughout the study. Oocytes that fail to fertilize and/or display no embryogenesis were routinely removed during the IVF/ICSI process, and the collection of these oocytes had no effect on IVF treatment outcomes. The oocytes used in the study were obtained with the patient's consent. The study was approved by the Ethics Committee of Hanoi Medical University (IRB-VN01.001/IRB00003121/FWA00004148) on March 17th, 2020.

III. RESULTS

1. Characteristics of study group

In this study, 263 oocytes that failed to fertilize were collected from 80 patients undergoing IVF using the ICSI technique at the IVF and Tissue Engineering Center, Hanoi Medical University Hospital. The oocytes were divided into two groups according to the women's age. The characteristics of two groups are described in Table 1.

Table 1. Characteristics of the study population

Characteristic	$\bar{X}$ ± SD / n (%)		p
	Ages ≤35 (n = 56)	Ages >35 (n = 24)	
BMI	22.2 ± 2.8	22.3 ± 2.2	0.766 [a]
Types of infertility			
Primary	26 (46.4%)	4 (16.7%)	0.012 [b]/*
Secondary	30 (53.6%)	20 (83.3%)	
Semen analysis			
Density (10^6/ml)	62.8 ± 39.5	45.3 ± 35.5	0.065 [a]
≥ 15	55 (98.2%)	21 (87.5%)	0.078 [c]
< 15	1 (1.8%)	3 (12.5%)	
Motility (%)	43.4 ± 16.8	36.9 ± 16.4	0.113 [a]
Normal	42 (75.0%)	13 (54.2%)	0.065 [b]
Abnormal	14 (25.0%)	11 (45.8%)	
Normal morphology (%)	3.3 ± 3.6	2.3 ± 1.4	0.187 [a]
≥ 4	24 (42.9%)	7 (29.2%)	0.249 [b]
< 4	32 (57.1%)	17 (70.8%)	

BMI: Body mass index; : Mean; SD: Standard Deviation;

[a]Student's T-test; [b] Chi-Square test; [c] Fisher exact test

The average BMI values of the two groups were not significantly different from each other (22.2 ± 2.8 versus 22.3 ± 2.2, p > 0.05). The mean values of sperm quality, such as density, motility, and normal morphology, were also not significantly different between the two groups (p > 0.05) (Table 1). The rate of primary infertility in the group 35 years and younger was significantly higher than that in the group older than 35 years (46.4% versus 16.7%, p < 0.05).

Data on IVF cycles were collected from medical records. The characteristics of oocytes from IVF cycles are described in Table 2.

Table 2. Characteristics of IVF cycles

	$\bar{X}$ ± SD / n (%)		p
	Ages ≤ 35 (n=56)	Ages > 35 (n=24)	
Retrieved oocytes N	1170	348	
Per cycle	20.9 ± 9.6	14.5 ± 7.1	0.004 a /*
Mature oocytes	949	293	
%	81.1	84.2	0.190 b
Per cycle	16.9 ± 7.8	12.2 ± 6.5	0.011 a /*
% per cycle	64.5 ± 13.8	65.9 ± 16.9	0.703 b
ICSI fertilization oocytes	748	231	
%	78.8	78.8	0.994 b
Per cycle	13.4 ± 6.7	9.6 ± 5.5	0.019 a /*
% per cycle	78.0 ± 12.1	78.2 ± 12.9	0.947 b
Unfertilized oocytes	201	62	
%	21.2	21.2	0.994 b
Per cycle	3.6 ± 2.4	2.6 ± 2.2	0.083 a
% per cycle	22.0 ± 12.1	21.8 ± 12.9	0.947 b

X : Mean; SD: Standard Deviation; [a] Student's T-test; [b] Chi-Square test

**p<0.05*

The rate of ICSI oocytes, the rate of ICSI fertilization, and unfertilized oocytes per cycle were not significantly different between the two groups. On average, women aged 35 years and younger had significantly higher number of retrieved oocytes, number of mature oocytes, and number of fertilized oocytes per cycle than women aged older than 35 years (20.9 ± 9.6; 16.9 ± 7.8; and 13.4 ± 6.7, respectively, with 14.5 ± 7.1; 12.2 ± 6.5 and 9.6 ± 5.5; p <0.05).

2. Meiotic spindle and chromosome morphology in oocytes that failed to fertilize after ICSI

The characteristics of the study sample, including the meiotic spindle shapes and chromosomal alignment in oocytes and the decondensation of chromosomes in sperm, are described in Table 3.

Table 3. Characteristics of meiotic spindles and chromosomes in unfertilized oocytes after ICSI

	n	Percentage (%)
Presence of spindle (n =263)		
No	56	21.3
Yes	207	78.7
Shape of spindles (n = 207)		
Bipolar	92	44.4
Disarranged	115	55.6
Chromosomes in the spindles (n=207)		
Aligned	61	29.5
Unaligned	146	70.5
Decondensation of sperm chromosomes (n = 263)		
Lack of sperm nuclei	15	5.7
Condensed	58	22.1
Decondensed	140	53.2
PCC	39	14.8
Metaphase	11	4.2

ICSI, intracytoplasmic sperm injection; PCC, premature chromosome condensation

Among the 263 oocytes that failed to fertilize, 56 (21.3%) oocytes contained no meiotic spindle and 207 (78.7%) oocytes contained a meiotic spindle. There were 92 (44.4%) oocytes whose meiotic spindles were bipolar. In the majority of oocytes (146/263, 70.5%), the chromosomes in the spindle system are unaligned. The absence of sperm nuclei was observed in 15 (5.7%) oocytes. In 140 (53.2%) oocytes, varying degrees of sperm chromatin decondensation were observed. Premature chromosome condensation (PCC) was observed in 39 (14.8%) oocytes.

The 207 unfertilized oocytes were divided into four groups based on meiotic spindles and chromosome characteristics per De Santis et al. as follows:

(i) bipolar spindle with aligned chromosomes;

(ii) bipolar spindle with unaligned chromosomes;

(iii) disarranged spindle with aligned chromosomes;

(iv) disarranged spindle with unaligned chromosomes.[6] (Figure 2)

The proportions of oocytes classified into each of these four groups were compared between patients 35 years or younger and those older than 35 years. Oocytes from patients older than 35 years had a significantly higher rate of abnormal spindle shape morphology and unaligned chromosomes than those from patients younger than 35 years (63.3% versus 43.0%; OR = 2.28; CI = 1.17 – 4.41; $p < 0.05$) (Table 4).

Table 4. Correlation between spindle morphology and patient age

	n	Ages ≤ 35 years 56 women, 158 oocytes	Age > 35 years 24 women, 49 oocytes	OR (95% CI)	p
i	40	33 (20.9%)	7 (14.3%)	1.58 (0.65–3.85)	0.307
ii	48	42 (26.6%)	6 (12.2%)	2.60 (1.03–6.54)	0.038*
iii	20	15 (9.5 %)	5 (10.2%)	0.92 (0.31–2.68)	1.000
iv	99	68 (43.0%)	31 (63.3%)	0.44 (0.22–0.85)	0.013*

OR: odds ratio; CI: confidence interval; All variables compared using the Chi-square test;

**p < 0.05*

Based on the characteristics of the spindle system, the presence of sperm nuclei, and the degree of sperm chromatin decondensation, the 263 oocytes were classified into four types, according to Luo et al. as described in section 2.2.3.[5] The frequencies and percentages of oocytes in each group are presented in table 5.

Table 5. Classification of oocytes that failed to fertilize after ICSI

Type	n	Ages ≤ 35 years 56 women, 201 oocytes	Age > 35 years 24 women, 62 oocytes	p
I	15	11 (5.5%)	4 (6.4%)	0.758 [a]
II	199	154 (76.6%)	45 (72.6%)	0.517 [b]
III	49	36 (17.9%)	13 (21.0%)	0.589 [b]
IV	0	0 (0.0%)	0 (0.0%)	

[a] Fisher exact test; [b] Chi-Square test

The most common cause of fertilization failure in both age groups was oocyte activation failure (154/201 [76.6%] in the group 35 years and younger; 45/62 [72.6%] in the group older than 35 years; and in total 199/263 [75.7%] in both groups). Defects in pronucleus formation or migration were the second most common cause in our study with 49/263 (18.6%) oocytes. Unfertilized oocytes due to pronucleation or migration defects was more likely in the group older than 35 years than in the group 35 years or younger, but the difference was not statistically significant (21.0% versus 17.9%, p>0.05). This study was conducted in the absence of unfertilized oocytes due to other causes, such as polyspermia.

IV. DISCUSSION

During in vivo and conventional IVF, sperms must pass through the culumus cell layers, generate an acrosome reaction to pass through the zona pellucida, and fuse with the oocyte membrane. Following the ICSI procedure, the culumus cells are removed by mechanical and chemical treaments. Then the embryologist uses a microneedle to insert a single sperm through the natural barriers of zona pellucida and oocyte membrane into the oocyte cytoplasm to

induce fertilization. The ICSI technique can be particularly effective in cases of sperm defects such as abnormal density, abnormal motility, or abnormal morphology, but ICSI cannot completely avoid failed fertilization. Even with ICSI, fertilization failure can occur depending on the number and quality of oocytes and the degree of sperm DNA damage.[7]

The results of our study showed that the numbers of retrieved oocytes, mature oocytes, and fertilized oocytes per cycle were significantly higher in the group of young patients (≤35 years) than in the older group (>35 years). This is consistent with published studies that showed women's fertility potential decreases significantly with age due to two main reasons: oocyte quantity and quality, especially after the age of 35.[3] The number of oocytes in the ovarian gradually decreases throughout life until reaching menopause (about 10^6 at birth to about 10^3 at menopause).[8] However, there were not significant differences in the mature oocytes rate and fertilization rate between the two age groups ($p>0.05$) (Table 2). The fertilization rate was not affected by the increasing age of women in IVF and ICSI that demonstrated by previous studies.[9,10]

When comparing the rate of unfertilized oocytes of two age groups, we did not find a significantly difference (21.2 vs 21.2%, p=0.994). The number of unfertilized oocytes per cycle was higher in the group 35 years and younger but the difference was not statistically significant. This could be explained by the observation that the total number of mature oocytes was significantly higher in women aged 35 years and younger than women older than 35 years. However, the rate of unfertilized oocytes per cycle did not significantly differ between the two age groups (Table 2). Thus, the percentage of unfertilized oocytes was relatively stable across the two age groups. Many studies have shown that oocyte quality declines with increasing maternal age, but this decline seems to affect embryogenesis, causing aneuploid embryos rather than fertilization. [11,12]

Additionally, many potential mechanisms have been proposed to explain the age related decline in oocyte quality, such as mitochondrial dysfunction, epigenetic changes, DNA damages, and chronic exposure to oxidative stress. An important factor related to oocyte quality is the aging of the meiotic spindle apparatus.[13] In our analysis, abnormal meiotic spindle morphology and/or chromosome misalignment in unfertilized oocytes accounted for 167/207 (80.7%) oocytes. Although we studied meiotic spindle morphology in unfertilized oocytes, we found that the percentage of oocytes with disarranged spindles was significantly higher in the group of women >35 years than in the group of women ≤35 years (36/49 oocytes [73.5%] and 83/158 oocytes [52.5%]; OR = 2.50; CI = 1.23 – 5.07; p = 0.011]. Long incubation periods in the laboratory may increase the proportion of oocytes with meiotic spindle and chromosome abnormalities, but they would not cause the significantly higher incidence of abnormalities observed in women older than 35 years. This is because maternal age has an effect on the expression of genes involved in cell cycle regulation (such as assembly of the meiotic spindle apparatus and chromosomes during the M phase of meiosis) in metaphase II oocytes in women undergoing IVF or ICSI treatment.[14] With long-term exposure to harmful agents, the rate of abnormal spindles in the oocytes of older women has been observed to increase.

In this study, most oocytes were not fertilized due to failed oocyte activation with 199/263 (75.7%) oocytes and there was no difference between the two age groups. The percentage of

unfertilized oocytes due to the absence a sperm in the ooplasm accounted for 5.7%. Failure to fertilize due to lack of spermatozoa in the oocyte also occurs in IVF. But in conventional IVF, the absence of sperm in the cytoplasm was due to poor sperm quality (e.g., poor motility, inability to cause an acrosome reaction, penetration of the transparent oocyte membrane, and fusion of the oolemma). Following ICSI, the absence of spermatozoa in the cytoplasm by technical failure led to the failure of the ICSI needle tip to perforate the oocyte membrane, preventing sperm from reaching the ooplasm. Oocyte membranes have large elasticity, which increases the difficulty of penetration and does not guarantee membrane rupture during ICSI. In such case, the sperm is expelled into the perivitelline space. Otherwise, the ICSI technique ensures that only a single sperm enters the oocyte's cytoplasm. Therefore, the case of an unfertilized oocyte due to polysperm did not appear in this study.

V. CONCLUSIONS

Women's age was not related to the rate of unfertilized oocytes during ICSI cycles. Maternal age older than 35 years increased the risk of unfertilized oocytes due to meiotic spindle morphology and chromosome abnormalities. The main cause of unfertilized oocytes after ICSI procedures was failed oocyte activation, which was not related to maternal age.

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