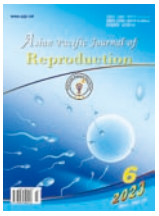

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Embryonic, genetic and clinical outcomes of fresh *versus* vitrified oocyte: A retrospective cohort study

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ABSTRACT

Objective: To compare embryonic development, ploidy status and clinical outcomes between fresh and frozen-thawed oocytes.

Methods: This retrospective cohort study evaluated 83 fertilization cycles including both fresh and frozen oocytes from 79 patients at the HP Fertility Center of Hai Phong International Hospital of Obstetrics and Pediatrics in Vietnam. The patient underwent several ovarian stimulation cycles to accumulate a certain number of oocytes that would be vitrified. In the last oocyte retrieval, all patient's oocytes including both frozen and fresh would be fertilized. The outcomes included the rates of oocyte survival, cleavage embryo, blastocyst, ploidy status, pregnancy, biochemical pregnancy and clinical pregnancy.

Results: The oocyte survival rate after thawing was 96.5%. No statistically significant difference was found when comparing fresh and frozen oocytes regarding fertilization rate (78.1% *vs.* 75.5%, *P*=0.461), usable cleavage embryo rate (86.9% *vs.* 87.2%, *P*=0.916) but usable blastocyst rate was found higher statistically in the frozen oocyte group (44.4% *vs.* 54.0%, *P*=0.049). The percentages of euploid, aneuploid and mosaic embryos between the fresh group and the vitrified group had no significant differences (33.8% *vs.* 31.6%, *P*=0.682; 51.0% *vs.* 54.2%, *P*=0.569; 15.2% *vs.* 12.4%, *P*=0.787; respectively). The rates of pregnancy, biochemical pregnancy and clinical pregnancy had no statistical difference (68.8% *vs.* 64.8%, *P*=0.764; 12.5% *vs.* 3.6%, *P*=0.258; 37.5% *vs.* 46.4%, *P*=0.565). 17 Mature oocytes are the minimum to have at least one euploid embryo.

Conclusions: Oocyte vitrification does not affect embryonic, genetic and clinical results. The number of mature oocytes should be considered for fertilization in some cases.

KEYWORDS: Clinical outcomes; Embryonic development; Genetic outcomes; Human oocyte; Oocyte accumulation; Oocyte cryopreservation; Ploidy status; Vitrified oocyte

1. Introduction

Many factors may affect gamete's health, such as environmental pollutant exposure, unhealthy lifestyle, medicine-taking habits and reproductive system dysfunction[1]. Nowadays, many research-based techniques are applied for patients to optimise infertility treatment results including oocyte freezing. Oocyte accumulation through cryopreservation can be an efficient approach for women with diminished ovarian reserve. Additionally, social oocyte freezing creates an opportunity for women to delay motherhood without concern about ovarian ageing.

The ovum is fertilized with sperm into an embryo and then transferred to the patient's uterus. In case of fertilization is not performed immediately after oocyte retrieval, these should be frozen

Significance

Oocyte cryopreservation with vitrification technique has been widely applied in recent decades. However, there is a gap in understanding the effectiveness of using frozen autologous oocytes. This study provides embryological and clinical evidence to bridge that gap. The study revealed that there is no difference when using autologous fresh and vitrified oocytes. The number of mature oocytes can predict at least one euploid embryo.

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to preserve their morphology and function. Among reproductive cells and tissues, oocytes are most vulnerable to damage during cryopreservation. This sensitivity is mainly due to the large cell size, low surface area relative to volume, and ice crystal formation resulting in only a 75% survival rate for the slow freezing method[2]. Oocytes damaged during cryopreservation may affect the structure of microfilaments and microtubules which are responsible for normal chromosomal segregation. Functional changes in mitochondria harden the zona pellucida that prevent natural sperm penetration[3]. However, it has been shown that the spindle could be reassembled again in most oocytes after the vitrification and thawing process[4,5]. Moreover, several studies stated that oocyte cryopreservation does not increase pregnancy complications and the frequency of developmental abnormalities[6,7]. Since the first baby was born from frozen oocytes[8], numerous studies conducted worldwide on the vitrification technique to demonstrate enhanced efficiency in oocyte cryopreservation. Outcomes have been improved for both embryonic development and clinical success rates[9–11]. Nevertheless, many studies which just give insights into ploidy status does not mention how many oocytes need to be obtained through accumulating cycles for euploidy result. This study aims for the following goals: (1) whether the potential development and pregnancy outcome of oocytes are affected by vitrification; (2) compare genetic results of embryos derived from fresh oocytes *vs.* frozen oocytes; and (3) give a cut-off number of mature oocytes to get at least one euploid embryo.

2. Materials and methods

2.1. Study design

In this retrospective study, outcomes including cleavage embryo, blastocyst, ploidy, and pregnancy were analyzed from women undergoing oocyte cryopreservation at HP Fertility Center of Hai Phong International Hospital of Obstetrics and Pediatrics, Vietnam from May 2021 and May 2023. A total of 83 fertilization cycles which met the inclusion and exclusion criteria (Figure 1). In the last fresh oocyte retrieval cycle, frozen oocytes were warmed and all oocytes including both fresh and frozen were fertilized at the same time and under the same culture condition. Most patients performed multiple oocyte cryopreservation cycles so patient characteristics were obtained from the first cycle to limit data missing.

2.2. Inclusion and exclusion criteria

Patients were included if they had a fertilization cycle with both fresh and frozen oocytes, blastocyst culture, and trophectoderm biopsy for preimplantation genetic testing for aneuploidy (PGT-A). Data collection was divided into fresh and frozen oocytes of each patient.

Cases excluded from this study included embryos frozen on day 3 and then the remaining embryos cultured into blastocysts. In those cases, a certain number of high-quality cleavage embryos will be frozen. The lower-quality embryos remaining may result in a lower usable blastocyst rate. Donation cycles were also excluded from the data to control maternal factors. Conventional *in vitro* fertilization (IVF) cycles were excluded. Another excluding criterion was a couple having infertility caused by male factor: moderate male factor (MMF), severe oligoasthenoteratozoospermia (OAT-S), obstructive azoospermia (OA), and nonobstructive azoospermia (NOA) that has been found impairing fertilization rate and developmental potential[12].

2.3. Variables

2.3.1. Primary outcomes

The primary objective of this study was comparing laboratory outcomes of fresh oocyte and frozen oocyte *via*, fertilization rate, usable cleavage embryo rate, usable blastocyst rate, euploidy rate, aneuploidy rate and mosaic rate. The fertilization rate was defined as the number of two pronuclei (2PN) zygotes per the number of MⅡ oocytes fertilized with sperm. The usable cleavage embryo rate and usable blastocyst rate were defined as the number of cleavage and blastocyst embryos per 2PN zygotes. Euploidy was the normal complete set of 46 chromosomes while aneuploidy was a genetic disorder where the total number of chromosomes does not equal to 46. Mosaicism was defined as the presence of two or more cell lines with different genotypes arising from a single embryo.

2.3.2. Secondary outcomes

The secondary objective was to evaluate the clinical outcomes of fresh oocyte compare to frozen oocyte and to estimate the number of MⅡ oocyte need to be obtained for at least one euploid blastocyst. Clinical outcomes included pregnancy rate, biochemical pregnancy rate and clinical pregnancy rate. Pregnancy was confirmed when a beta-hCG test on day 9 or 10 after embryo transfer showed a result greater than 25 IU/L. Biochemical pregnancy was defined as one where despite the beta hCG test being “positive”, the pregnancy fails to progress to the point of ultrasound confirmation and never reaches the stage where a gestational sac is seen on ultrasound examination. Clinical pregnancy was the actual pregnancy confirmed by transvaginal ultrasound with the presence of an amniotic sac, embryo, and fetal heart after 3-4 weeks of embryo transfer.

Our procedures were based on the revised guidelines for good practice in IVF laboratories[13] and we stated that the procedures were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000.

2.4. Oocyte vitrification

Oocyte freezing uses Vitrification Kit 101 (Reprolife, Japan) and

the Cryotec method[14]. After oocyte retrieval, oocyte cumulus complexes (OCC) were cultured in an incubator at 37℃, 5% O_2 and 6% CO_2 for 2 h before oocytes were denuded from granulosa and corona cells by hyaluronidase 80 IU/mL. Vitrification started with oocytes equilibrated in equilibration solution (ES) containing 7% ethylene glycol (EG), 7% dimethyl sulfoxide (DMSO) for up to 15 min before being transferred to vitrification medium (VS) containing 14.5% EG, 14.5% DMSO, 0.5 M trehalose for up to 90 s. The oocytes were loaded onto the Cryotec straw surface with minimal media volume and then directly embedded in the cooling rack containing liquid nitrogen. Close the Cryotec plastic cap and keep the oocyte in the storage container.

2.5. Oocyte thawing

Warming Solution Set 205 (Reprolife, Japan) was used for oocyte thawing. The thawing protocol was based on the Cryotec method[14]. Prepare the thawing medium (TS) containing 1.0 M trehalose and thawing plate in a heated incubator for at least 2 h before use. Remove the cap and place the straw in liquid nitrogen. Pour TS into a warming plate and insert straw immediately into TS (within 1 s) and stay still for 1 min. Transfer oocytes from TS to the diluent solution (DS) containing 0.5 M trehalose and keep in there within 3 min. After 3 min, transfer oocytes from DS to washing solution 1 (WS1) for 5 min and then transfer to WS2 for 1 min. In the end, oocytes were transferred into a culture dish and observed under a stereoscope.

2.6. Intracytoplasmic sperm injection and embryo culture

In the last oocyte retrieval cycle at 39–41 h post trigger for initiation of oocyte maturation, intracytoplasmic sperm injection (ICSI) was performed with fresh mature oocytes (MⅡ) and frozen MⅡ that had survived after thawing. Sperm was prepared by gradient or swim-up method before being used for injection. The oocytes after ICSI were cultured continuously in the single-step medium in a time-lapse incubator using mixed gas with a concentration of 6% CO_2, 5% O_2 and 37℃.

2.7. Embryo grading

Cleavage embryo was assessed on day 3 regarding the number of blastomere and fragmentation degree and blastocyst was based on 3 criteria including embryo expansion, inner cell mass (ICM) and trophectoderm (TE). Usable embryos are embryos with grades Ⅰ, Ⅱ, and Ⅲ according to the morphological classification criteria of David Gardner[15] and the Istanbul consensus workshop on embryo assessment[16].

2.8. Blastocyst biopsy

Cleavage embryos underwent laser-assisted hatching on day 3 or day 4 to create a tunnel on zona pellucida. On day 5 or day 6, several trophectoderm cells extruding from zona pellucida were removed without ICM disruption. These biopsied cells were tubed and transferred to a genetic laboratory for analysis.

2.9. Statistical analysis

Data were analyzed with IBM SPSS Statistics, Version 26.0. The independent sample *t*-test was used to find the significant difference between the bivariate samples in independent groups. Categorical variables including clinical outcomes and ploidy status between groups were shown as proportions and scrutinized using *Chi*-square test. To describe the data, descriptive statistics frequency analysis, percentage analysis was used for categorical variables and the mean and standard deviation (mean±SD) was used for continuous variables. The non-normally distributed data was expressed as median (IQR). The area under the receiver operating characteristics (ROC) curve (AUC) was used to determine the value of the predictor and the Youden index estimate the minimum number of MⅡ oocytes to obtain at least one euploid blastocyst for transfer. Statistical difference was defined when $P<0.05$.

2.10. Ethics statement

This research was approved by the Institutional Review Board in bio-medical research, Institute of Genome Research, Vietnam Academy of Science and Technology, with the clinical registration number of "04–2023/NCHG-HĐĐĐ". Informed and written consents were obtained from all participants.

3. Results

3.1. Demographic and clinical characteristics of patients

79 Patients underwent 83 ICSI cycles with a total of 410 fresh oocytes and 545 cryopreserved oocytes between May 2021 and May 2023. Figure 1 shows the cycles screening process. Patients accepted for oocyte freezing because of the following reasons: MⅡ accumulation (n=81), planned oocyte cryopreservation (n=2). Women's characteristics in this study were described by parameters in Table 1 including the mean age, infertility duration, body mass index (BMI), anti-Mullerian hormone (AMH), number of oocyte retrieval cycles and the average number of fresh and frozen oocytes per ICSI cycle.

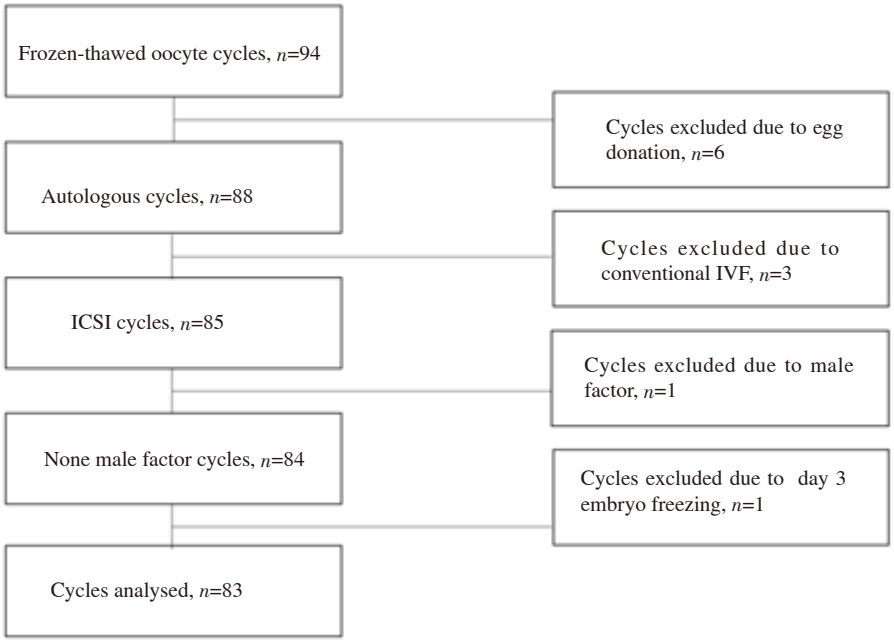

Figure 1. The cycles screening flowchart. ICSI: intracytoplasmic sperm injection, IVF: *in–vitro* fertilization.

Table 1. Demographic and clinical characteristics of patients.

Characteristics	Values
Maternal age, years	37.7±4.2
Infertility duration, years*	2.0 (2.0)
BMI, kg/m^2	21.8±2.1
Types of infertility#, *n*(%)	
Primary infertility	3 (3.6)
Secondary infertility	80 (96.4)
AMH*, ng/mL	1.3 (1.1)
FSH, ng/mL	9.88±0.86
LH, ng/mL	7.11±1.24
Number of oocyte retrieval cycles*	2.0 (1.0)
Mean MII oocyte per ICSI cycles*	4.7 (4.5)

Continuous data are expressed as mean±SD, *n*=83; non-normally distributed data (*) are expressed as median (IQR); categorical data (#) are expressed as *n*(%). BMI: body mass index, AMH: anti-Mullerian hormone, FSH: follicle-stimulating hormone, LH: luteinizing hormone, MⅡ: metaphase Ⅱ, ICSI: intracytoplasmic sperm injection.

3.2. Survival rate of the frozen–thawed oocyte group

A total of 565 oocytes obtained in previous cycles were vitrified and 545 of these were survival after thawing resulting in the oocyte survival rate standing at 96.5%.

3.3. Embryonic outcomes

The median MⅡ oocytes fertilized in the fresh group was lower significantly when compared to the frozen group (IQR: 5.00 *vs.* 66.00, *P*=0.005) but no differences were found in the rate of fertilization (78.1% *vs.* 75.5%, *P*=0.461) and the usable cleavage embryo rate (86.9% *vs.* 87.2%, *P*=0.916). The usable blastocyst rate was lower significantly in the fresh group compared with the vitrified group (44.4% *vs.* 54.0%, 95% *CI* 0.00-0.19; *P*=0.049) (Table 2, Figure 2).

In terms of morphology, there was no difference between fresh and thawed oocytes. Cleavage embryos and blastocysts from vitrified oocytes had similar morphology to embryos derived from fresh oocytes.

3.4. Genetic outcomes

When it comes to the ploidy status of embryos derived from fresh and frozen oocytes, the euploidy and mosaicism rates in the fresh oocyte group were lower than the frozen oocyte groups (33.8% *vs.* 31.6%, *P*=0.682; 15.2% *vs.* 14.1%, *P*=0.787) while the rate of

Table 2. Embryonic outcomes of the fresh and frozen oocyte groups.

Embryonic outcomes	Fresh (*n*=410)	Frozen (*n*=545)	*P* value
Median MⅡ ICSI, IQR	5.00 (5.00)	6.00 (3.00)	0.005
Fertilization rate, *n*(%)	320 (78.1)	411 (75.5)	0.461
Usable cleavage embryo rate, *n*(%)	356 (86.9)	475 (87.2)	0.916
Usable blastocyst rate, *n*(%)	182 (44.4)	294 (54.0)	0.049

Table 3. Genetic outcomes of the fresh and frozen oocyte groups [*n* (%)].

Genetic outcomes	Fresh oocyte (*n*=145)	Frozen oocyte (*n*=177)	*P* value
Euploidy rate	49 (33.8)	56 (31.6)	0.682
Aneuploidy rate	74 (51.0)	96 (54.2)	0.569
Mosacism rate	22 (15.2)	25 (12.4)	0.787

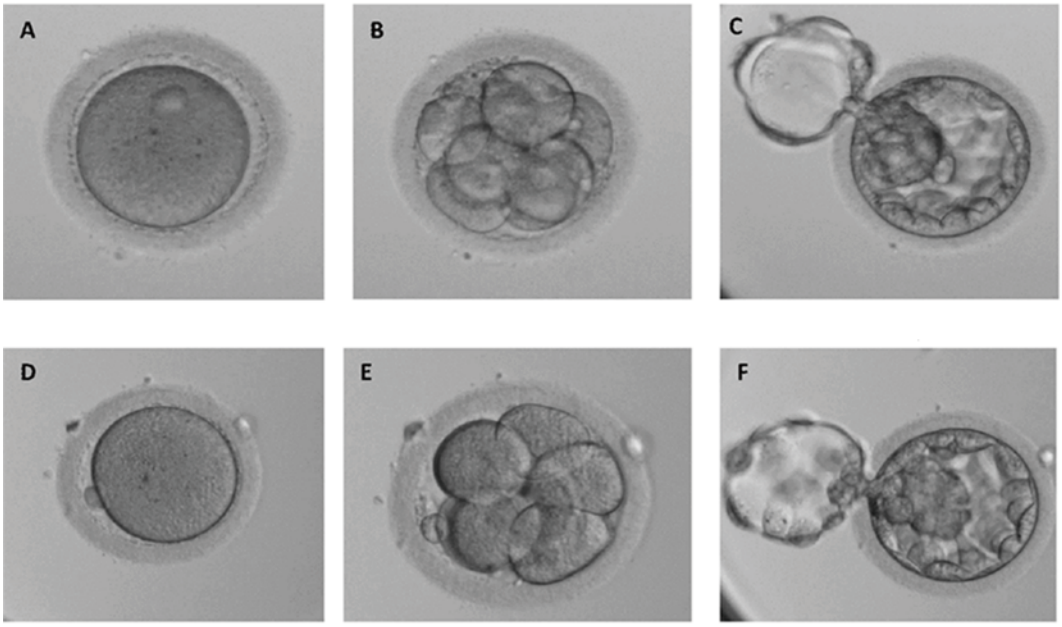

Figure 2. Morphology of fresh and frozen-thawed oocytes, cleavage embryos and blastocysts. A: Fresh oocyte; B: Cleavage embryo from fresh oocyte; C: Blastocyst from fresh oocyte; D: Oocyte thawing after vitrified; E: Cleavage embryo from vitrified oocytes; F: Blastocyst from vitrified oocyte.

Table 4. Clinical outcomes [*n* (%)].

Pregnancy outcomes	FET from fresh oocyte (*n*=16)	FET from frozen oocyte (*n*=28)	*P* value
Pregnancy rate	11 (68.8)	18 (64.3)	0.764
Biochemical pregnancy rate	2 (12.5)	1 (3.6)	0.258
Clinical pregnancy rate	6 (37.5)	13 (46.4)	0.565

FET: frozen embryo transfer. Data are expressed as *n* (%).

aneuploid embryos tends to go on the opposite side (51.0% *vs*. 54.2%, *P*=0.569). However, these numbers did not reach statistical significance (Table 3).

3.5. Clinical outcomes

A total of 44 frozen embryo transfer (FET) cycles were performed, of which 16 were derived from fresh oocytes and 28 developed from frozen oocytes. Outcomes between the two groups including the number of beta-hCG (+) cases, the number of biochemical pregnancy cases, the number of clinical pregnancy cases, pregnancy rate, biochemical pregnancy rate and clinical pregnancy rate were described in Table 4. The pregnancy rate was higher in the fresh oocyte group (68.8%) compare to the frozen oocyte group (64.3%) but the biochemical pregnancy rate and clinical pregnancy rate were better in the frozen oocyte group (3.6% *vs*. 12.5% and 46.6% *vs*. 37.5%, respectively). However, these data did not reach the significance.

3.6. Predictor for euploid blastocyst

To account for the value of the MⅡ quantity predictor detecting euploid blastocyst, the ROC curve was used depending on two variances including the number of euploid embryos and the number of MⅡ being fertilized. The result showed that AUC was 60.3% (*P*=0.124, 95% *CI* 47.8–72.8) which was valid in detecting euploid blastocyst (Figure 3). The Youden index identified 17 mature oocytes per ICSI cycle as the threshold for at least 1 euploid blastocyst with optimal sensitivity and specificity standing at 19% and 100%, respectively.

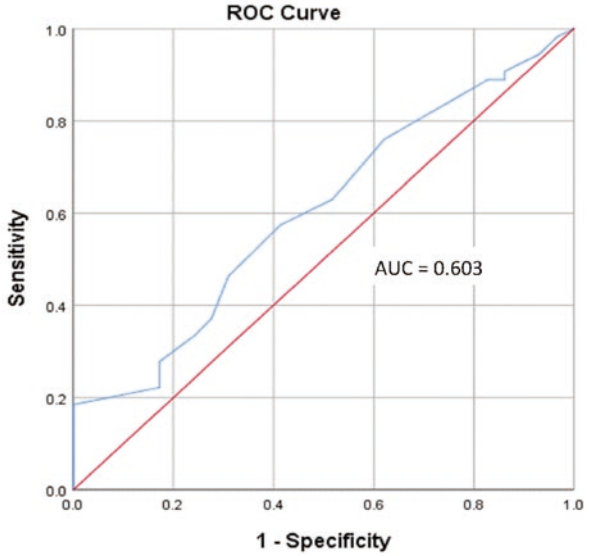

Figure 3. ROC curve showing true positive (horizontal axis) and false positive (vertical axis) of the factor of MⅡ oocyte quantity in detecting euploid embryos in ICSI cases using frozen oocytes.

4. Discussion

In this study, the patient baseline characteristics did not belong to the good prognosis group with the average age of the study population being (37.7±4.2) years old; Infertility duration lasts a median of 2 years; Diminished ovarian reserve with a median of AMH 1.3 ng/mL. The median number of oocyte retrieval cycles was 2 and low corresponding to the median number of oocytes per ICSI cycle was 4.7. Oocyte quantity and quality gradually decrease with age. According to the American Society For Reproductivse Medicine, women beyond 35 years old should be evaluated for diminished ovarian reserve status through the AMH test[17]. The mean age of women in this study was quite high compared to previous research in relation to ploidy status[18,19] because most cases included had signs of diminished ovarian reserve with a mean AMH serum level of 1.5 being considered low. Because diminished ovarian reserve is associated with poor ovarian response[20], only 5.2 oocytes per cycle of ovarian stimulation are obtained in our study. This was the reason why there was a difference in the average number of MⅡ oocytes per ICSI cycle between the fresh and frozen groups. Many studies have shown that the oocyte survival rate is significantly improved with the vitrification method ranging from 92% to 97%[21,22] and so similar to our result of 96.5%.

Our results demonstrated no difference between fresh and cryopreserved oocytes in fertilization rate and usable cleavage embryo rate but the usable blastocyst rate was found to lower significantly in fresh group and this could be explained by the significant difference in the median MⅡ oocyte per ICSI cycle between two groups. Thus, it might be a confounder factor in our study. Despite the usable blastocyte rate difference, embryos that survive culture to the blastocyst stage display equivalent rates of euploidy, aneuploidy, and mosaicism compared with blastocysts derived from fresh oocytes. Our data suggested that oocyte vitrification has no detrimental effect on chromosomal competence, further supporting the efficacy and safety of this technology. Clinical outcomes reflected better results in vitrified oocytes in comparison with the fresh group but differences were not statistically significant. Results of this retrospective study indicated that, overall, the number of mature oocytes can be the predictor of at least one euploid blastocyst. Nevertheless, the AUC did not reach statistical significance because out of the 83 cycles, only 10 with at least 17 mature oocytes were fertilized, representing 12% of the dataset. In our study, an MⅡ cut-off value of 17 had a sensitivity of 19% and a specificity of 100%. When it comes to the need to have euploid blastocyst for transfer, a predictor with 100% specificity may be considered acceptable for aged women. However, we suggested that subsequent studies may further evaluate other maternal-related predictors such as age, AMH, and BMI to find a better predictor of both sensitivity and specificity. The main limitations of this study are that the sample distribution was non-random and that the study population included oocytes with abnormal morphology.

In conclusion, the embryonic and clinical results of the oocytes are less affected by vitrification. Oocyte vitrification does not affect ploidy status. The number of MⅡ oocytes fertilized per ICSI cycle can be used to predict ploidy in the group using frozen oocytes.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

Phuong Dao Thi, Van Hanh Nguyen, and Thuan Nguyen Duc performed the concept and design of the study. Collection and processing of material were carried out by Anh Pham Van, Son Dang Truong, and Anh Do Tuan. Phuong Dao Thi was responsible for writing the manuscript. Phuong Dao Thi and Van Hanh Nguyen edited the manuscript. All authors proofread and approved the final manuscript.

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