Effects of Vitrification on Outcomes of In Vivo-Mature, In Vitro-Mature and Immature Human Oocytes

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Key Words
Vitrification • In vivo-mature human oocytes • In vitro-mature human oocytes • Immature human oocytes

Abstract
Background/Aims: To observe the effects of vitrification on spindle, zona pellucida, embryonic aneuploidy and DNA injury in in vivo-matured, in vitro-mature and immature human oocytes. Methods: Between January 2009 and February 2015, 223 immature oocytes from 450 infertile patients, and 31 in vivo-mature oocytes from 3 infertile couples were collected. Of the 223 immature oocytes, 113 were used for in vitro culture before vitrification. Some oocytes were randomly divided into in vivo-mature group (group A, n = 15), in vitro-mature group (group B, n = 88) and immature group (group C, n = 85), and then the oocytes with spindle in these three groups after freezing-thawing were selected to use for Polscope imaging, embryonic aneuploidy screening and embryo development evaluation. Other oocytes were randomly divided into group A (n = 16), group B (n = 25) and group C (n = 25) for detecting DNA injury. Results: After thawing, spindle occurrence rate, spindle Retardance value, and cleavage rate were significantly higher in groups A and B than in group C (all P < 0.05), but there were no statistical differences in fertility rate, high-quality embryo rate, blastulation rate and aneuploidy rate amongst the three groups (all P > 0.05). Zona pellucida density (ZPD) was significantly lower in group A than in groups B and C both before and after vitrification (all P < 0.05). ZPD was significantly higher after thawing than before vitrification (all P < 0.05), but zona pellucida thickness (ZPT) was not significantly changed in all the three groups (all P > 0.05). Rate of comet cells was significantly lower in group A than in groups B and C (all P < 0.01). Comet tail was significantly longer in group C than in groups B and A (all P < 0.05). Conclusion: In vivo- and in vitro-mature human oocytes are more suitable to vitrification than immature human oocytes. Spindle Retardance value has more predictive value for embryonic development potential than ZPD and ZPT.

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Introduction

Oocyte cryopreservation brings a hope to retain fecundity for some female patients [1-3]. With the increase in the number of babies born using frozen oocytes, effectiveness and safety of oocyte cryopreservation has become a sensitive topic. Vitrification is widely used in cryopreservation for human embryos, blastocysts and gametes because no ice crystal forms during vitrification. For oocyte cryopreservation, vitrification is better than traditional slow freezing because it can achieve more than 80% of anabiosis rate, 40-60% of pregnancy rate, but its live birth rate is lower [4-7].

Oocyte cryopreservation is associated with many factors, such as different human oocyte sources including in vivo-mature, in vitro-mature and immature oocytes. There are reports about babies born using vitrified oocytes of the three types [8-10], but it is yet unclear which kind of oocytes is more suitable to vitrification. Moreover, at present, most studies about human oocyte vitrification are clinically retrospective [11-13]. There is lack of comprehensive evaluation about which kind of oocytes is more suitable to vitrification.

Observe the cryodamage on oocytes, including oocytes’ spindle, zona pellucida, DNA injury and embryonic aneuploid, is the key to evaluate the quality of frozen-thawed oocytes. In recent years, Polscope system is used in fresh oocytes to observe oocytes’ spindle and zona pellucida, which may predict embryonic development potential [14, 15]. However, application of Polscope system in frozen-thawed human oocytes has not been reported. In this study, we observed and compared the effects of vitrification on spindle, zona pellucida, embryonic aneuploidy and DNA injury in in vivo-matured, in vitro-matured and immaturityd human oocytes to explore which kind of oocytes is most suitable to vitrification, and its possible mechanism.

Materials and Methods

All study methods were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Subjects

Between January 2009 and February 2015, 223 immature oocytes from 450 patients undergoing in vitro fertilization-embryo transfer (IVF-ET), and 31 in vivo-mature oocytes from 3 infertile couples were collected. The inclusion criteria for female patients included (1) age of 29.1 ± 1.7 years (range 21 - 34); (2) normal menses and endocrine; (3) normal chromosome; (4) no polycystic ovary syndrome; and (5) no histories of hereditary and familial diseases.

Ovarian stimulation

Ovarian stimulation was performed with a standard long protocol in all female patients [10]. After complete down-regulation using decapetyl (Germany), Gonal-F (FSH: follicle-stimulating hormone, Serono Company, Switzerland) was given on the third day of menstrual cycle. Follicular development was monitored by transvaginal ultrasound, and the levels of LH and E2 were determined. When the follicle diameter reached 18 mm at least in two follicles, 10000 IU of human chorionic gonadotrophin (hCG, Zhuhai Lizhu pharmaceutical company, Guangzhou, China) was given. Thirty six hours later, oocyte retrieval was performed under ultrasonic guidance.

Removal of granulosa cells

Oocytes were placed in hyaluronidase (60 IU/ml, Vitrolife Company, Sweden) for 30 s to remove mucous mass, and then were put in culture solution of G-MOPS (Vitrolife Company, Sweden) to remove granulosa cells followed by evaluating oocyte maturity under an invert microscope.

Specimen

In vivo-mature oocytes (MII-stage oocytes): Under invert microscope, the oocytes which had the first polar body and light cytoplasm with symmetrical granules, were regarded as mature oocytes. In this
study, 31 in vivo-mature oocytes all were from the infertile couples who refused sperm donation when their husbands had nonobstructive azoospermia and was able to obtain spermatozoa by testicular sperm extraction.

Immature oocytes included MI- and GV-stage oocytes. Under an invert microscope, MI-stage oocytes had light cytoplasm without both the first polar body and germinal vesicle (GV); and GV-stage oocytes possessed GV containing a nucleolus with nuclear membrane and refraction, and also had slightly-irregular cytoplasm with few black granules in its center. In this study, 223 immature oocytes all were from the infertile couples whose husbands had severe oligozoospermia or azoospermia.

In vitro-mature oocytes: MI- and GV-stage oocytes were incubated in IVM Media Kit (SAGE, USA) for 24 h. The removal of the first polar body from the oocytes indicated that the immature oocytes became mature oocytes.

Semen used in this study was from normal sperm liquid after gradient centrifugation and swim-up technique during IVF-ET.

**Grouping**

Some oocytes were randomly divided into in vivo-mature group (group A, n = 15), in vitro-mature group (group B, n = 88) and immature group (group C, n = 85). In the three groups, finally, only the oocytes with spindle were used for Polscope imaging, embryonic aneuploidy screening and embryo development evaluation. Other oocytes were randomly divided into group A (n = 16), group B (n = 25) and group C (n = 25) for detecting DNA injury 2 h after freezing-thawing.

**Vitrification and thawing for oocytes**

At room temperature, the oocytes were put in 7.5% Ethylene Glycol (EG, Sigma, USA) + 7.5% 1, 2 Propanediol (PROH, Sigma, USA) for 5 min (oocyte morphology: round→shrinkage→previous morphology), transferred into 15% EG +15% PROH +0.5 mol/L sucrose (Sigma, USA) for less than one minute, quickly placed on Cryoleaf (from Medicult, Canada), and directly thrown into liquid nitrogen.

Thawing was performed a month after vitrification. The Cryoleaf was placed in 1.0 mol/L sucrose at 37°C; then quickly transferred into 0.5 mol/L sucrose for one minute, 0.25 mol/L sucrose for one minute and basic solution for one minute, respectively, at room temperature; and then was put in basic solution at 37°C for 3 min followed by placement into overnight-balanced G-2 for 2 h.

**Observation on spindle density and zona pellucida using Polscope imaging system**

LC-Polscope imaging system (CRI, USA) and OosightTM software (VRI, USA) were used in this study. Observation and measurement for spindle density was performed according to the previous methods [16] (Fig. 1A, 1E and 1F). Under a polarimicroscope, we could see the thickness of zona pellucida containing three layers with different densities. The zona pellucida was divided into 8 equal parts with the center of oocyte as a point of intersection, and then the thickness (μm, ZPT) and density (nm, ZPD) of inner layer of the zona pellucida were measured [14] (Fig. 1B, 1C and 1D). Figures 1G and 1H show oocyte’s images taken using Polscope imaging system.

**Intracytoplasmic sperm injection (ICSI), embryo culture and embryo grading**

ICSI was performed in the oocytes with spindle in groups A and B 24 h after freezing-thawing and in the oocytes with spindle which were in vitro incubated for 24 h after freezing-thawing in group C. Needling insertion should avoid the spindle during ICSI. Fertilization, embryo culture and embryo grading were observed 16-18 h after ICSI [16].

**Aneuploid screening for embryos**

Blastomere biopsy: D3 embryo was put in a liquid droplet of HEPES without Ca²⁺/Mg²⁺ (Sage, USA) for 10-15 min, then was placed in a biopsy dish, and then was fixed with a pin followed by laser drilling on the zona pellucida using OCTAX laser soft system. Blastomeres were sucked out using a biopsy needle through the laser hole (Fig. 2) and at the same time, we observed nuclei and took pictures. After biopsy, these embryos were again placed in G-2 medium (Vitrolife, Sweden) to D5 and D6 followed by observing and recording the number of blastocysts in each group.
Fig. 1. The measurement of spindle density and the measurement of thickness and density of inner layer of the zona pellucida using Polscope imaging system ×400. (A) Spindle and three-layer zona pellucida of an in vivo-mature oocyte before vitrification. (B) Spindle and zona pellucida of an in vivo-mature oocyte after thawing. (C and D) Measurements and results of inner layer-thickness and density of zona pellucida. ZPD: Zona pellucida density; ZPT: Zona pellucida thickness. (E and F) Measurements of spindle density and Retardance value on each point of spindle. (G) Polscope images of a MI-stage oocyte before vitrification. (H) Polscope images of a MI-stage in vitro-mature oocyte after thawing.

Blastomere fixation: Blastomeres were washed using PBS (Sigma, USA), and then were placed on a glass slide containing 1 - 2 µl of 0.01 Tween-20/0.01N of HCL (Sigma, USA) at 37°C to observe cellular
expansion and rupture, and nuclear liberation. The nucleus was free after membranolysis. After the free nucleus was dried, solution of methanol and acetic acid (3:1, Chemical Reagent Company, Shanghai, China) was added to remove residual impurities. The free nucleus was stored for future use after natural drying.

**FISH:** A glass slide was immersed in PBS for 20 min, and then was dehydrated in 70%, 85% and 95% of alcohol, respectively, each for 2 min at room temperature. Fluorescent probes, LSI 21 (21q22.13-q22.2) spectrum orange and LSI 13q14 spectrum green (Vysis, USA) were added in the dried hybridisation region followed by covering using coverslip. The glass slide underwent denaturation at 73°C for 5 min, was blocked

![Fig. 2. Blastomere biopsy ×200. (A) Blastomere biopsy; (B) A blastomere with nucleus.](image)

![Fig. 3. Fluorescent images of FISH ×1000. (A) Euploid signals: Two green signals on chromosome 13 and 2 red signals on chromosome 21 indicate normal embryos. (B) Aneuploid signals: Three green signals on chromosome 13 indicate a triploid embryo. Note: FISH: Fluorescence in situ hybridisation.](image)

![Fig. 4. Fluorescent images of oocytes after single-cell gel electrophoresis ×300. (A) Non-electrophoretic fluorescence images after DNA cleavage. (B) Comet tail along the direction of electrophoresis from left to right. Note: Scale bar = 20 μm.](image)
using sealing compound followed by overnight at 37°C. The next day, the sealing compound and coverslip were carefully removed in the dark, and then the glass slide was respectively immersed in 0.4SSC (bbott, USA) at 73°C for 2 min, in 2SSC/0.1%NP-40 (AppliChem, Germany) for one minute and in DAPI (Sigma, USA) for 20 min followed by observing signals under a fluorescence microscope (Olympus, Japan). Imstar fluorescence analysis software (Imstar, France) was used to take pictures (Fig. 3) and to analyze signals [17].

**DNA injury detected by single-cell gel electrophoresis (SCGE)**

The oocytes underwent SCGE 2h after freezing-thawing. SCGE was performed according to the method [18]. Fluorescent images were treated using software 3.01 (GP medical technology company, Beijing, China). Oocytes were divided into comet-like cells and non-comet-like cells (Fig. 4). The tail length (µm) of comet-like cell was the distance from the distal end to head end along the direction of electrophoresis. Incidence rate of comet-like cells and the tail length of comet-like cells are positively associated with DNA injury.

**Calculation formulas for various rates**

- Survival rate\(=\)number of survival oocytes/number of retrieved oocyte ×100%
- Fertility rate\(=\)number of fertilized oocytes/number of ICSI oocytes ×100%
- Blastocyst rate\(=\)number of blastocysts/number of embryo biopsy ×100%
- Spindle occurrence rate\(=\)number of oocytes with spindle/number of survival oocytes ×100%
- Success rate of biopsy\(=\)number of blastomeres with successful biopsy/number of blastomere biopsy ×100%
- Rate of blastomere fixation\(=\)number of blastomeres with successful fixation/number of blastomeres with successful biopsy ×100%
- Signal rate\(=\)number of blastomeres with signals/number of blastomeres with successful fixation ×100%
- Aneuploid rate\(=\)number of aneuploid embryos/number of embryos with signals ×100%

**Statistical analysis**

Statistical treatment was performed with SPSS17.0 software. \(\chi^2\) test and Fisher exact test were used in the comparison of rates. Measurement data were expressed as (M ± SD). In comparisons between groups, if data were in line with normal distribution and homogeneity of variance; single factor analysis of variance was used; otherwise K independent sample test was adopted. Test criterion was set at \(\alpha = 0.05\) and statistical significance was established at \(P < 0.05\).

**Results**

**Comparisons of spindle density, ZPD and ZDT amongst the three groups**

Spindle occurrence rate and spindle Retardance values were significantly higher in groups A and B than in group C after thawing (all \(P < 0.05\)). ZPD was significantly lower in group A than in groups B and C both before and after vitrification (all \(P < 0.05\)). ZPD was significantly higher after thawing than before vitrification (all \(P < 0.05\)), but ZPT was not significantly changed in all the three groups (all \(P > 0.05\)) (Table 1).

**Comparisons of embryo development and aneuploid amongst the three groups in oocytes with spindle**

Cleavage rate in oocytes with spindle was significantly higher in groups A and B than in group C (all \(P < 0.05\)). There were no statistical differences in fertility rate, high-quality embryo rate, blastocyst rate and aneuploid rate amongst the three groups (all \(P > 0.05\)) (Table 2).

**Comparisons of DNA injury amongst the three groups**

Incidence rate of comet-like cells was significantly lower in group A than in groups B and C (all \(P<0.01\)). The comet tail was significantly longer in group C than in groups A and B (all \(P < 0.05\)) (Table 3).
Clinical significance of spindle density, ZPT and ZPD for human frozen-thawed oocytes

Spindle is a sensitive index to evaluate cryodamage because it is particularly sensitive to temperature. Vitrification readily leads to spindle injury in both in vivo-mature and in vitro-mature oocytes. Hence, we suggest that it is advisable to employ an alternative method to monitor spindle density, ZPT and ZPD for human frozen-thawed oocytes.

Table 1. Comparisons of spindle density, ZPD and ZPT amongst the three groups. Notes: * indicates $P < 0.05$ as compared with other two groups. $^*$ indicates $P < 0.05$ as compared with that after thawing within the same group. ZPT: Zona pellucida thickness; ZPD: Zona pellucida density.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
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</thead>
<tbody>
<tr>
<td>No. of obtained oocytes</td>
<td>15</td>
<td>88</td>
<td>85</td>
</tr>
<tr>
<td>No. of in vitro-mature oocytes before vitrification [n (%)]</td>
<td>74 (84.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of oocytes with spindle before vitrification [n (%)]</td>
<td>13 (86.6)</td>
<td>50 (67.6)</td>
<td></td>
</tr>
<tr>
<td>No. of frozen oocytes [n]</td>
<td>13</td>
<td>50</td>
<td>85</td>
</tr>
<tr>
<td>No. of obtained oocytes after thawing [n]</td>
<td>13</td>
<td>50</td>
<td>85</td>
</tr>
<tr>
<td>No. of survival oocytes after thawing [n (%)]</td>
<td>12 (92.3)</td>
<td>41 (80.0)</td>
<td>60 (70.6)</td>
</tr>
<tr>
<td>No. of in vitro-mature oocytes after thawing [n (%)]</td>
<td></td>
<td>41 (68.3)</td>
<td></td>
</tr>
<tr>
<td>No. of oocytes with spindle after thawing [n (%)]</td>
<td>11 (91.6)</td>
<td>38 (87.5)</td>
<td>28 (56.0)*</td>
</tr>
<tr>
<td>Spindle density before vitrification (nm) (M±s)</td>
<td>2.26±0.71</td>
<td>1.53±0.12</td>
<td></td>
</tr>
<tr>
<td>Spindle density after thawing (nm) (M±s)</td>
<td>2.66±0.67</td>
<td>2.17±0.05</td>
<td>0.81±0.17*</td>
</tr>
<tr>
<td>ZPT before vitrification (µm) (M±s)</td>
<td>8.53±1.04</td>
<td>8.21±0.15</td>
<td>8.26±1.01</td>
</tr>
<tr>
<td>ZPT after thawing (µm) (M±s)</td>
<td>8.74±0.04</td>
<td>8.53±0.22</td>
<td>8.23±0.32</td>
</tr>
<tr>
<td>ZPD before vitrification (nm) (M±s)</td>
<td>1.23±1.14*</td>
<td>2.34±1.14*</td>
<td>2.72±1.22*</td>
</tr>
<tr>
<td>ZPD after thawing (nm) (M±s)</td>
<td>2.15±0.53*</td>
<td>3.04±0.92</td>
<td>3.47±1.25</td>
</tr>
</tbody>
</table>

Table 2. Comparisons of embryo development and aneuploidy amongst the three groups. Notes: * indicates $P < 0.05$ as compared with other two groups. ICSI: Intracytoplasmic sperm injection.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of oocytes undergoing ICSI [n]</td>
<td>11</td>
<td>38</td>
<td>28</td>
</tr>
<tr>
<td>Fertility rate [n (%)]</td>
<td>10 (90.9)</td>
<td>32 (84.2)</td>
<td>21 (75.0)</td>
</tr>
<tr>
<td>Cleavage rate [n (%)]</td>
<td>9 (90.0)</td>
<td>28 (87.5)</td>
<td>12 (57.1)*</td>
</tr>
<tr>
<td>High-quality embryo rate [n (%)]</td>
<td>6 (66.7)</td>
<td>12 (42.8)</td>
<td>5 (41.7)</td>
</tr>
<tr>
<td>Success rate of biopsy [n (%)]</td>
<td>18 (100)</td>
<td>54 (96.4)</td>
<td>24 (100)</td>
</tr>
<tr>
<td>Rate of blastomere fixation [n (%)]</td>
<td>17 (94.4)</td>
<td>50 (92.6)</td>
<td>22 (91.7)</td>
</tr>
<tr>
<td>Signal rate [n (%)]</td>
<td>15 (88.2)</td>
<td>45 (90.0)</td>
<td>19 (86.7)</td>
</tr>
<tr>
<td>Aneuploid rate [n (%)]</td>
<td>3 (33.3)</td>
<td>9 (32.1)</td>
<td>5 (41.7)</td>
</tr>
<tr>
<td>Blastocyst rate [n (%)]</td>
<td>4 (66.7)</td>
<td>6 (21.4)</td>
<td>1 (8.3)</td>
</tr>
</tbody>
</table>

Table 3. Comparisons of DNA injury amongst the three groups. Notes: * indicates $P < 0.01$ as compared with other two groups. ** indicates $P<0.05$ as compared with other two groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of oocytes</td>
<td>16</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>No. of comet-like cells [n (%)]</td>
<td>4 (25)*</td>
<td>24 (80.0)</td>
<td>23 (92.0)</td>
</tr>
<tr>
<td>Length of comet tail (µm) (M±s)</td>
<td>14.5±4.2</td>
<td>20.3±10.3</td>
<td>29.2±11.4**</td>
</tr>
</tbody>
</table>

Discussion

Clinical significance of spindle density, ZPT and ZPD for human frozen-thawed oocytes
mature oocytes [19-21], but for immature oocytes, there are different reports [22, 23]. Zona pellucida, as a channel of material exchange between oocytes and internal environment, plays an important role in sperm-egg fusion and embryo development. It has been reported that vitrification enhances the hardness of zona pellucida, which increases polyspermic fertilization and decreases fertilization in IVF [24]. Most studies above used animal oocytes as research subjects, and required fixation and staining. These factors affect the observation on embryo development potential.

In recent years, Polscope imaging system has become a major topic in clinical reproduction because it can evaluate embryo development-related spindle and zona pellucida in living oocytes without the requirement of cell fixation and staining. Most studies are about fresh human oocytes. It has been confirmed that the oocytes with spindle have higher development potential, and spindle density is directly correlated with embryo development [25-27]. Shen et al. [26] have reported that ZPT and ZPD may be used as important indexes to select embryo transfer in fresh human oocytes because they can reflect embryo development potential. GA Rama Raju et al. [15] have believed that the oocytes with spindle Retardance value >3 nm and ZPT of 10-12 μm have higher development potential in fresh human oocytes; and comprehensive analysis of spindle, ZPT and ZPD can predict embryo development potential. Besides our previous study about animal oocytes [16], there has not been a report about effects of vitrification on spindle, ZPT and ZPD in frozen-thawed human oocytes.

Our study indicated that spindle occurrence rate, spindle Retardance values and cleavage rate all were significantly higher in groups A and B than in group C (all \(P < 0.05\)); ZPD was significantly lower in group A than in groups B and C (all \(P < 0.05\)), but there was no statistical difference in ZPT amongst the three groups both before and after vitrification (all \(P > 0.05\)). Our results suggest that spindle density is positively related to embryo development because both Retardance values and cleavage rate all were significantly higher in groups A and B than in group C, which is similar to the results of our previous study [16]. Our results also suggest that ZPD and ZPT are not related to cleavage rate. This is different from the results reported by GA Rama Raju et al. [15] probably because of different research subjects. Our results suggest that Retardance value has more predictive value than ZPD and ZPT for vitrified oocytes. Our study also indicated that ZPD was significantly higher after thawing than before vitrification (all \(P < 0.05\)) in all the three groups, and ZPD was significantly lower in group A than in groups B and C both before and after vitrification (all \(P < 0.05\)), suggesting that vitrification may increase ZPD. Therefore, we propose that assisted hatching should be performed on frozen-thawed oocytes before ICSI and embryo transfer, which may be conducive to improvement of fertility rate and pregnancy rate.

**Quality of vitrified-thawed oocytes in the three groups**

Although the babies born using vitrified oocytes of the three types have all been reported, there still has been considerable debate about which kind of oocytes is more suitable to vitrification. Early study indicated that immature oocytes were more suitable to vitrification because its chromosomes were protected within GV and micro-tubular structure had not yet formed until the time of initial meiosis [28]. However, there were also converse views which believed that there still were abnormal chromosomes and spindle, zona pellucida hardening and cytoskeleton injury in vitrified immature oocyte [29, 30]. Since the first baby was successfully born using vitrified in vitro-mature oocytes, the number of babies born using vitrified in vitro-mature oocytes has been increasing. However, there is lack of comprehensive evaluation information about which kind of oocytes is more suitable to vitrification.

Intact DNA of oocytes has important significance for normal fertilization and embryo development. Vitrification-induced free radicals lead to DNA injury [31], so DNA injury may be used as an index of evaluating cryodamage. For animal oocytes, DNA injury is more severe in vitrified in vitro-mature oocytes than in vitrified in vivo-mature oocytes [18]. There has not yet been a report on DNA injury for vitrified human oocytes. Our results indicated that spindle occurrence rate after thawing, spindle Retardance value and cleavage rate were
significantly lower, but comet tail was significantly longer in immature oocytes than in both in vivo- and in vitro-mature oocytes (all $P < 0.05$); and there were no statistical differences in aneuploid rate amongst the three groups (all $P > 0.05$). Our results suggest that vitrified in vivo- and in vitro-mature oocytes are better than vitrified immature oocytes, and lower spindle occurrence rate and Retardance value may be associated with DNA injury in vitrified immature oocytes.

In summary, it may obtain better clinical outcomes in IVF-ET to vitrify in vitro- and in vitro-mature oocytes and to transfer oocytes with higher spindle Retardance value. However, large-sample studies are necessary to confirm our conclusion because the sample size of oocytes was small in our study.

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**Disclosure Statement**

There are no conflicts of interest in this study.

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