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Research Article

Ultra-Rapid Freezing and Rapid Freezing Methods in Clinical ICSI Program: Effects on Sperm Biological Characteristics, DNA Methylation Stability, DNA Methyltransferase Activity, and Embryo Morphokinetics

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The purpose of this study was to evaluate the differences in sperm function and embryo morphokinetics following sperm cryopreservation by ultra-rapid freezing or rapid freezing methods compared to fresh spermatozoa. Thirty samples of normozoospermia were divided equally into fresh, ultra-rapid freezing, and rapid freezing groups. In the rapid freezing, sperm suspension was placed horizontally on nitrogen vapor. In the ultra-rapid freezing, sperm suspension with a straw-in-straw system was directly immersed in liquid nitrogen. Sperm function was assessed in terms of motility, morphology, viability, mitochondrial membrane potential, sperm DNA fragmentation, and acrosome reaction status. Also, the effects of two cryopreservation methods were assessed on global DNA methylation and DNA methyltransferase activity. Moreover, 730 embryos in three groups were cultured using time-lapse imaging until day 6 for embryo morphokinetics. Progressive motility (38.80 ± 4.21 vs. 34.86 ± 4.19; $p < 0.001$) and viability (64.30 ± 6.24 vs. 58.10 ± 8.69; $p < 0.01$) in ultra-rapid freezing were significantly higher than rapid group. DNA fragmentation and acrosome reaction were significantly increased in both cryopreserved groups ($p < 0.001$). However, DNA fragmentation (16.30 ± 1.14 vs. 14.33 ± 2.94; $p < 0.01$) was significantly higher in the rapid than the ultra-rapid freezing group. No significant differences were noted in global DNA methylation ($p > 0.05$) and DNA methyltransferases activity ($p > 0.05$) in fresh compared to cryopreservation groups. The kinetic times, including tPB2, tPNa, tPNf, t2, t3, t4, t5, t6, t7, t8, and tM, showed a significant delay in cell divisions in both cryopreservation groups. Furthermore, tPNa, tPNf, and t8 occurred with a significantly higher delay in embryos fertilized by sperm from the rapid freezing compared to the ultra-rapid freezing group. In addition, blastocysts formation was similar in both cryopreservation groups. Ultra-rapid freezing preserved the sperm biological integrity and lead to better embryo morphokinetics compared to the rapid freezing method. However, both methods of sperm cryopreservation were epigenetically safe.

1. Introduction

Sperm cryopreservation is routine in ART for male fertility preservation before cancer therapy, vasectomy, and cases with azoospermia. It is also useful for gamete donation programs and preventing the transmission of sexually transmitted diseases [1–3]. Therefore, it is necessary to choose the best method for sperm cryopreservation in assisted reproduction

programs. The main causes of cell damage during the freezing process are ice formation and osmotic shock, which are accompanied by changes in the cytoskeleton structure, acrosome membrane integrity, DNA structure, and motility [4–6]. Studies have reported that sperm cryopreservation is associated with increased DNA fragmentation and mitochondrial dysfunction, which has been linked to increased oxidative stress and reactive oxygen species (ROS) production [7, 8].

Increased ROS production, in addition to changes in DNA integrity, can induce site-specific hypermethylation by upregulating DNA methyltransferases (DNMTs) [9]. DNA methylation is one of the most important epigenetic mechanisms where a methyl group is covalently attached to 5-carbon of the cytosine ring. It mainly occurs in the cytosine phosphate guanine dinucleotides (CpGs) and plays an important role in the regulation of gene expression, embryonic genome activation, and embryo development [10]. Proper epigenetic modifications are necessary for cell differentiation and function, and it has been reported that genome imprinting is highly sensitive to environmental changes, [10]. DNA methylation is the most important epigenetic modification that controls genomic imprinting, and changes in this pathway in addition to affecting the sperm fertility potential, it can also lead to disruption of the embryo development [11, 12]. Only a few clinical studies have reported that sperm cryopreservation has no effect on DNA methylation mechanisms [8, 13, 14]. Considering limited information regarding sperm cryopreservation on DNA methylation and the concern of transferring methylation changes to children, it is necessary to investigate DNA methylation status and DNMTs activity.

Since sperm plays an important role in embryonic genome activation and subsequent development to the blastocyst stage, it is necessary to evaluate the role of sperm cryopreservation on the embryo morphokinetics [15, 16]. Time-lapse monitoring (TLM) is a noninvasive method that allows evaluating the kinetics of embryonic cell divisions without removing the embryo from the incubator [17, 18]. Without disturbing the temperature, humidity, pH, and osmolarity of the embryo culture medium, TLM provides us an accurate analysis of the embryo development [19, 20].

Currently, the most common methods suggested for sperm cryopreservation are rapid freezing and ultra-rapid freezing/vitrification. The rapid freezing is based on contacting the sample with liquid nitrogen vapor and using permeable cryoprotectants (CPAs) [21]. By contrast, the ultra-rapid freezing/vitrification method involves the sample directly contacting the liquid nitrogen, which is based on the cooling and warming rates and the use of high concentrations of CPAs [22]. Although this method is currently a successful method for oocyte and embryo freezing, it is still not recognized as a routine method for sperm cryopreservation due to the need for high concentrations of cytotoxic CPAs [23]. Lately, Schulz et al. [24] introduced a better method, in which they used a straw for sperm cryopreservation, without using CPA. This made it possible to freeze more sperm samples and prevented the transmission of contamination in a closed system. Compared to the rapid method, this method is simple and requires less time. Furthermore, due to high cooling rate and low water content of sperm, there is no need to use CPA [25]. Since, vitrification/ultra-rapid freezing is not suitable for high-volume samples, it is still not a popular method in ART compared to the rapid freezing method [5].

Considering the important role of sperm nuclear quality and epigenetic modifications in embryo development, we have compared the effects of rapid freezing and ultra-rapid freezing methods on sperm parameters, DNA fragmentation, acrosome status, mitochondrial function, global DNA methylation, DNMTs activity, as well as embryo morphokinetics up to the blastocyst stage in order to choose the best cryopreservation method with higher efficiency.

2. Materials and Methods

2.1. Selected Population. Semen samples were collected from 30 normozoospermic men referred to Yazd Research and Clinical Center for Infertility from March 2022 to April 2023. This study was approved by the Ethics Committee of our institution (IR.SSU.MEDICINE.REC.1400.306) and signed informed consent was obtained from all patients. Men with severe male factors, testicular samples, ejaculate samples with below 20×10^6/ml, and with history of varicocele, diabetes, alcohol consumption, and smoking were excluded. Also, women with BMI ≥ 30 and over 38 years old and history of endometriosis, polycystic ovary syndrome (PCOS), alcohol consumption, and smoking were excluded.

2.2. Semen Collection and Preparation. Semen samples were obtained by masturbation after 2–5 days of sexual abstinence and sperm parameters were assessed according to guideline of World Health Organization (WHO) 2021) [26]. After complete liquefaction, sperm preparation involves swim-up. The washed sperm were diluted with the sperm preparation basic medium, human tubular fluid (HTF) with 5 mg/ml human serum albumin (HSA) to reach a final concentration of 30×10^6/ml. Each diluted sample was divided into three groups equally. The groups were included: (a) fresh, (b) rapid freezing, and (c) ultra-rapid freezing (Figure 1).

2.3. Rapid Freezing Method and Thawing. Rapid freezing was performed according to manufacturer's guidelines of sperm freeze solution (Kitazato, Japan). In short, 150 μl of the sperm freeze solution was added drop by drop to 150 μl of the sperm suspension (at a ratio of 1 : 1) and gently mixed. After 10 min of incubation at room temperature (RT) to reach equilibration, the mixed sample was transferred to 1.8 ml cryotube (Nunc, Denmark). The cryotube was placed horizontally at 3 cm above the surface of liquid nitrogen to freeze in the vapor phase and reduce the temperature difference between the two ends. After 30 min, the cryotube was immersed into liquid nitrogen and finally stored in the nitrogen tank. For thawing, the cryotube was removed from the nitrogen tank and placed in a water bath (37°C) for 10 min. HTF supplemented with 5 mg/ml HSA (Sigma Chemical Co.) was added drop by drop to the cryotube to reduce osmotic shock. For CPA removal, centrifugation (300 g, 5 min) was then performed. Finally, the postthawed sperm parameters were assessed [5].

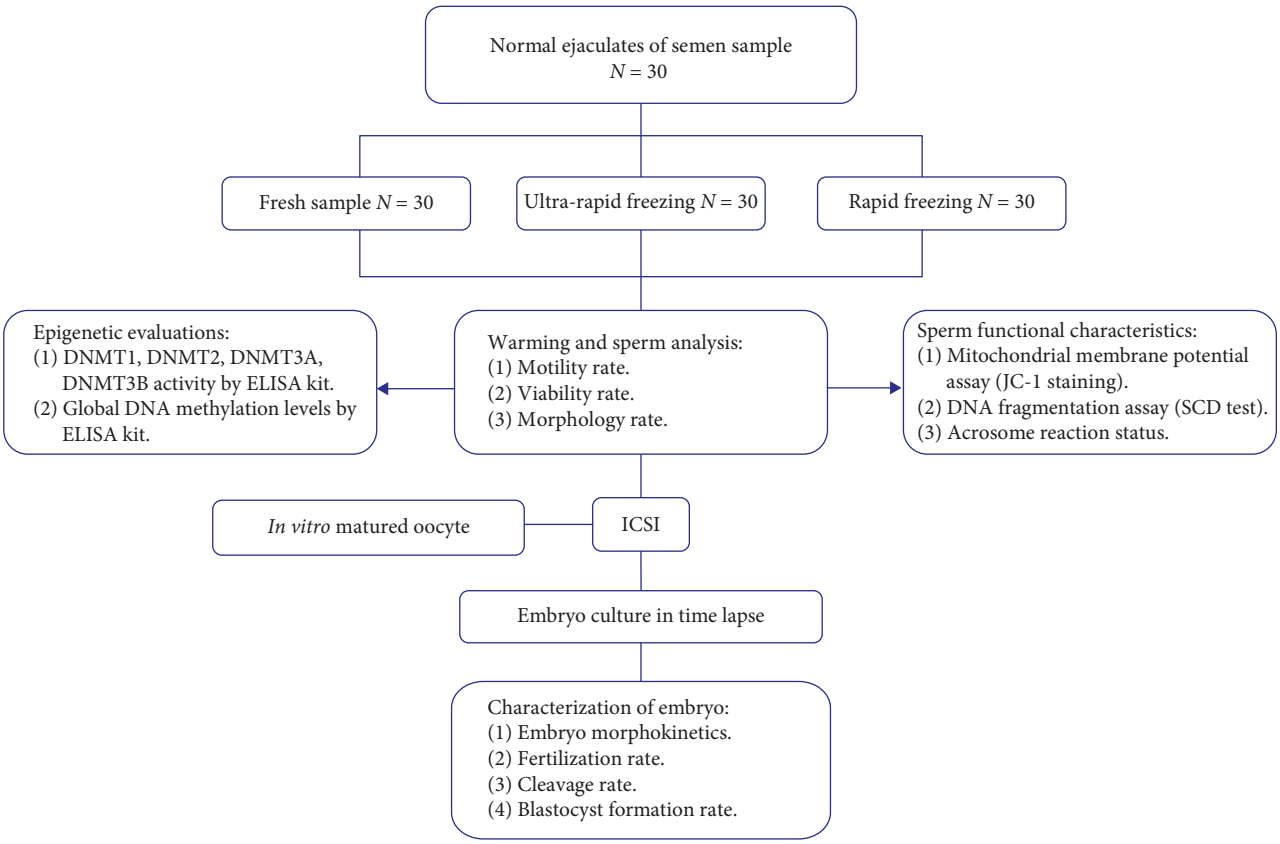

Figure 1: The schematic diagram of the study design.

2.4. Ultra-Rapid Freezing Method and Warming. Ultra-rapid freezing was performed according to Schulz et al. [24] using a closed system. The sperm suspension (processed with swim-up) was mixed with ultra-rapid freezing medium (HTF supplemented with 5 mg/ml HSA (Sigma Chemical Co.) and 0.5 mol/l sucrose (Sigma Chemical Co.) dissolved in distilled water with a final concentration of 0.25 M sucrose at a ratio of 1 : 1, at RT. Then, a 150 μl aliquot of this suspension (2.5×10^6–3.0×10^6 per straw) was aspirated into a 0.25 ml straw (inner straw) and this straw was placed inside a 0.5 ml straw (outer straw), and both ends of the outer straw was sealed (Figure 2). The sealed straw, in a horizontal position, was quickly immersed in liquid nitrogen for 1 min and stored in nitrogen tank. For warming, one side of the outer straw (0.5 ml) was cut and the inner straw (0.25 ml) was removed and immediately placed inside 15 ml of warming medium (HTF supplemented with 5% HSA) prewarmed at 37°C. The sample was washed and concentrated by centrifugation at 300 g for 5 min. The postwarmed sperm parameters were then assessed [24].

2.5. Sperm Parameters. At least 200 spermatozoa were assessed by light microscope (Olympus, Tokyo, Japan) in accordance with 2021 WHO guidelines. Sperm viability was evaluated using eosin–nigrosin staining. Unstained sperms in the head region were considered alive, and stained sperms with pink or red were considered dead. For determination of sperm morphology, 10 μl of sperm sample was smeared on a slide, then air-drying in RT. Finally, the slides were stained according to Diff-Quick kit procedure (Hooshman Fanavar, Tehran, Iran). The value of sperm morphology was evaluated based on the head shape, neck/midpiece status, tail, and presence of abnormal cytoplasmic droplets. The stained slides were evaluated under light microscope (Olympus, Tokyo, Japan) using 1,000x magnification with oil immersion. A total of 200 spermatozoa were evaluated for each sample.

Sperm motility and concentration were assessed using the Makler chamber (Sefi-Medical Instrument) by light microscope. Motility percentage was reported based on a four-category system of 2021 WHO included: rapidly progressive, slowly progressive, nonprogressive, and immotile [26].

2.6. Sperm Mitochondrial Membrane Potential (MMP). Mitochondrial activity was determined using a JC-1 mitochondrial membrane potential assay kit (Cayman Chemical Co., USA). According to the manufacturer's guideline, if the cells have healthy mitochondria ($\Delta\Psi m > 140$ mV) JC-1 form J-aggregates complex with intense red–orange fluorescence in the rhodamine isothiocyanate channel. If the mitochondria are damaged ($\Delta\Psi m < 100$ mV), JC-1 remains in a monomeric form and emits green fluorescence in the FITC channel. JC-1-staining solution was prepared by diluting the stock solution 1 : 10 (v/v) (v/v) in the culture medium. A droplet (1 μl) of JC-1 solution was incubated with 9 μl of sperm suspension (concentration of 100 times diluted) for 30 min in a humified incubator (37 C, 5% Co_2).

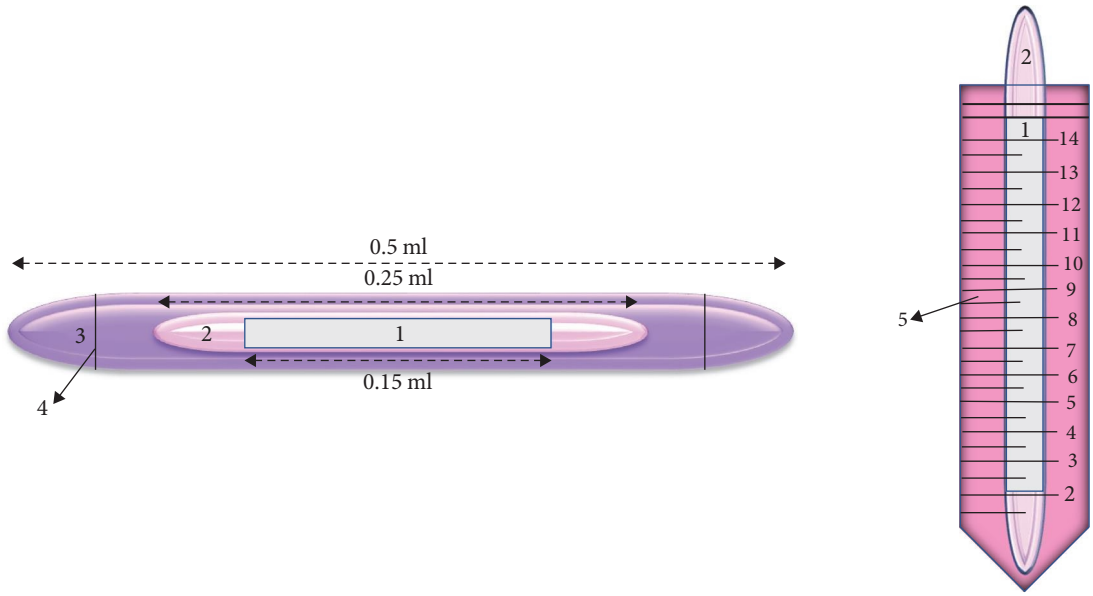

FIGURE 2: Ultra-rapid freezing method and warming: 1. sperm suspension (0.15); 2. inner straw (0.25 ml); 3. outer straw (0.5 ml); 4. sealed area of straw with heat; and 5. warming medium.

For evaluation of mitochondrial activity, fluorescence microscop (Olympus Co., Tokyo, Japan) was used under a coverslip with immersion oil at 1,000 magnification [27].

2.7. Sperm DNA Fragmentation Index (DFI). The level of sperm DNA fragmentation was evaluated using Halo sperm kit (SDFA kit, Tehran, Iran). Thirty microliters of the sperm suspension was mixed with 70 μl of 1% (w/v) low-melting-point agarose (Roche, Germany) at 37°C. Then, aliquots of 50 μl of the mixture were placed on glass slides precoated with 0.65% (w/v) standard agarose (Merck, Germany) and kept at 4°C/5 min for agarose solidification. The coverslip was removed and then denaturing solution (solution A) was added to slide for 7 min at RT in dark. The slide was covered with lysing solution (solution B) for 15 min at RT. Subsequently, the slide was washed with distilled water for 5 min, followed by placing in ethanol solutions of 70%, 90%, and 100%, each for 2 min and left to dry at RT. Finally, the slides were stained according to the manufacturer's guideline. The DNA fragmentation rate was evaluated according to the size of halo around the sperm head. Spermatozoa with large or medium halo were considered to have no DNA fragmentation, whist spermatozoa with small or no halo indicated fragmented DNA [28].

2.8. Acrosome Reaction (AR) Status. Acrosomal status after induction of the AR can be assessed by fluorescently labeled lectins, such as *Pisum sativum* agglutinin (PSA). According to the manufacturer's instruction (Sigma–Aldrich Company, USA), the sperm cells with acrosome-intact, more than half the head are brightly green, but those with only a fluorescing band at the equatorial segment or no fluorescing stain in the acrosome region are acrosome reacted. Briefly, the air-dried smear was prepared from 12 μl of sperm suspension on a glass slide and fixed in 95% (v/v) ethanol. For acrosome staining by PSA, 1 μl of prepared solution of the stock solution was diluted with 9 μL of PBS under dark conditions. Then, 12 μl from this solution was added to the prepared smears from the sperm sample. This staining was done in a dark room and a humid environment and then kept at 4°C for 60 min. The stained smears were washed indirectly with PBS and then allowed to dry completely. For evaluation of acrosomal status, fluorescence microscopy (Olympus Co, Tokyo, Japan) at 1,000 magnification and a U-MNIB3 filter was used with immersion oil [29].

2.9. DNA Extraction. DNA extraction was performed according to the manufacturer's guideline of Human Sperm DNA Extraction Kit (Favorgen, Biotch Corp., Taiwan). The input DNA quality and amount in each sample were measured using NanoDrop spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, USA) and adjusted to a concentration of 100 ng per reaction. Finally, total extracted DNA was stored in −20°C for further analysis.

2.10. Global DNA Methylation Analysis. The MethylFlash Global DNA Methylation (5-mC) ELISA Kit (Epigen Tek Group Inc., USA) was used for detection of global DNA methylation levels of sperm cells according to the manufacturer's instructions. In this assay, 100 ng of purified sperm DNA from each sample was added to binding solution and incubated at 37°C for 60 min. After removing binding solution and washing each well with 150 μl wash buffer for three times, 5-mC detection complex was added and incubated for 50 min. After washing the wells, developer solution was added and incubated for 10 min in order to develop the color. The absorbance was measured in a microplate spectrophotometer at 450 nm within 2–15 min. Finally, the percentage of methylated DNA (5-mc) in total DNA was calculated using the following formula:

$$5 - \text{mC}\% = \frac{\text{Sample OD} - \text{NC OD}}{\text{Slope} \times S} \times 100\%, \quad (1)$$

where S is the amount of input sample DNA in ng and NC is the negative control.

2.11. Nuclear Extraction. Nuclear proteins extraction of human sperm cells was done according to the manufacturer's guideline of the EpiQuik Nuclear Extaction Kit (Epigen Tek Group Inc., USA). A standard Bradford protein assay to measure the concentration of the nuclear extraction was done and adjusted to a concentration of 20 μg per reaction. Finally, total nuclear extractions were stored in −80C until further analysis.

2.12. DNA Methyltransferases (DNMTs) Activity Analysis. The EpiQuik™ DNA Methyltransferase Activity/Inhibition Assay Kit (Epigen Tek Group Inc., USA) was used for measuring total DNMTs (DNMT1, DNMT2, DNMT3A, DNMT3B) activity in human sperm cells according to manufacturer's instructions. DNA methyltransferase (DNMT) activity was measured by adding 20 μg nuclear extracts to the sample wells in accordance with the user manual. Colorimetric analysis was read on a microplate reader at 450 nm. Finally, the activity of DNMTs was calculated using the following formula:

$$\text{DNMT activity}(\text{OD/hr/mg}) = \frac{\text{Sample OD} - \text{blank OD}}{\text{Protein amount } (\mu\text{g}) \times \text{hour}} \times 1{,}000. \quad (2)$$

2.13. Controlled Ovarian Hyperstimulation (COH) and Oocyte Preparation. The classical COH cycles were performed using the standard GnRH antagonist protocols. When at least two follicles had grown to a diameter of 18–19 mm, 10,000 IU human chorionic gonadotrophin (Organon, Netherlands) was given to induce oocyte maturation [30]. Follicle aspiration was performed after 35–36 hr of hCG administration. Cumulus oocyte complexes were washed in SynVitro Flush media (Origio, Denmark) and incubated in Universal IVF Medium (Origio, Denmark) under the conditions of 37°C, 5% CO_2, 6% O_2. After 2 hr, cumulus cells were removed with exposure to 80 IU/ml prewarmed hyaluronidase (Irvine Scientific®, CA, USA) and mechanical pipetting [31]. Then, denuded oocytes were assessed and immature oocytes (GV) were matured under the IVM protocol.

2.14. GV Oocytes Collection and In Vitro Maturation. Immature oocytes were cultured in 25 μl droplets of SAGE 1-Step medium (Origio, Cooper Surgical, Denmark) at 37°C in 6% CO_2, 6% O_2 with high humidity under paraffin oil (Life Global, USA) for 24 hr. Oocyte maturation was confirmed by extrusion of a visible PB1 [32].

2.15. Intracytoplasmic Sperm Injection. For ICSI, mature oocytes were placed in 5 μL droplet of buffered medium (SynVitro Flush media, Origio, Denmark) in a culture dish covered with paraffin oil (Life Global, USA). Prepared sperm samples were placed in a central droplet of polyvinylpyrrolidone (PVP) solution (Irvine Scientific, CA), and injection was performed with an inverted microscope (Olympus IX71, Tokyo, Japan). After ICSI, all injected oocytes were loaded in a pre-equilibrated Primo Vision Embryo Culture Dish™ (Vitrolife Company, Sweden) in a standard incubator at 37°C, 5% O_2, 6% CO_2. The primo Vision dish containing nine straight-sided circular wells filled with 40 μl of 1-Step (SAGE, Origio) medium and covered by 3 ml of mineral oil [33].

Table 1: The evaluation of spermatozoa parameters before semen processing.

Parameters	Mean ± SD
Concentration ($\times 10^6$ cell/ml)	89.75 ± 5.08
Rapidly progressive (%)	44.01 ± 4.20
Slowly progressive (%)	18.08 ± 2.01
Nonprogressive (%)	14.03 ± 3.01
Immotile (%)	23.88 ± 2.91
Normal morphology (%)	6.75 ± 1.08
Viability (%)	79.11 ± 5.02
MMP (%)	72.11 ± 4.66
AR (%)	24.44 ± 2.11

Note. MMP, mitochondrial membrane potential; AR, acrosome reaction.

2.16. Embryo Development with TLM Culture. The TLM system took images of the embryos every 10 min, for 6 days through 11 focal planes. The optimal time ranges for evaluating the embryos kinetics were set according to previous studies [34–38]. The strict time points of embryonic development that were evaluated include t2 (24–28 hr), t3 (30–38 hr), t4 (35–41 hr), t5 (48–57 hr), t8 (50–59 hr), time of morula (tM: 81.28–96 hr), and expanded blastocyst (tEB ≤ 116 hr). Cell cycles intervals cc2: t3-t2 (8–12 hr), s2: t4-t3 (≤1 hr), cc3: t5-t3 (9.7–21 hr), and s3: t8-t5 (≤8.78) were calculated. The evaluated abnormalities included fragmentation, vacuole, direct cleavage (DC), reverse cleavage (RC), uneven blastomere size at two cell stage, multinucleated blastomere at four cell stage. Also, the blastocyst score was determined based on the expansion of the blastocyst cavity and the integrity of inner cell mass and trophectoderm cells. Moreover, the grading system proposed by Motato et al. [37] was used to determine the percentage of embryos that have passed the optimal time points for blastocyst formation. Based on Motato's algorithm, the optimal timings include s3 and tM.

2.17. Statistical Analysis. Statistical analysis was performed using SPSS version 26. The Shapiro–Wilk test was used to estimate the variables' distribution characteristics. One-way ANOVA test with Tukey's post-hoc test was used to evaluate the significant differences between the groups. Chi-square tests were used to compare categorical data and significance level for multiple comparisons were done using the pairwise comparisons with Bonferroni method. Correlations between DNA methylation and DNMTs activity with sperm parameters were analyzed with the Spearman–Rho correlation test. The results for numeric variables were expressed as mean ± SD and percentage for categorical variables. Differences at the level of $p < 0.05$ were considered significant. GraphPad Prism software version 9.5.1 (GraphPad Software, Inc., San Diego, CA) was used to generate graphs.

Table 2: Evaluation of spermatozoa parameters after semen processing in fresh, ultra-rapid freezing, and rapid groups.

Sperm parameters	Fresh group mean ± SD	Ultra-rapid freezing group mean ± SD	Rapid group mean ± SD
Progressive motility (rapidly + slowly) (%)	76.66 ± 3.08	38.80 ± 4.21***	34.86 ± 4.19***†††
Immotile (%)	11.04 ± 2.48	47.66 ± 2.65***	50.76 ± 4.34***††
Total motility (%)	88.66 ± 2.44	51.96 ± 4.61***	49. 56 ± 4.62***
Normal morphology (%)	8.36 ± 1.47	3.46 ± 0.68***	3.96 ± 0.76***
Viability (%)	88.93 ± 4.50	64.30 ± 6.24***	58.10 ± 8.69***††

***$p < 0.001$; the difference between fresh with ultra-rapid freezing and rapid groups. ††p-value < 0.01, †††$p < 0.001$; the difference between ultra-rapid freezing with rapid groups.

3. Results

3.1. Sperm Parameters. Evaluation of sperm parameters before semen processing is summarized in Table 1. After semen processing (Table 2), progressive motility, total motility, morphology, and viability were significantly decreased in both cryopreserved groups compared to fresh group ($p < 0.001$). Both, progressive motility and viability in ultra-rapid freezing group were significantly higher compared to rapid group ($p < 0.01$).

3.2. DNA Fragmentation Index, Mitochondrial Membrane Potential, and AR Status. DFI was significantly increased in both cryopreserved groups compared to fresh group ($p < 0.001$). Moreover, DNA fragmentation was significantly higher in the rapid compared to the ultra-rapid freezing group ($p < 0.01$) (Figure 3). MMP was decreased after cryopreservation ($p < 0.001$), but this parameter was significantly lower in the rapid group than the ultra-rapid freezing ($p < 0.01$) (Figure 3). AR increased after cryopreservation ($p < 0.001$), but this parameter was similar in both cryopreserved groups ($p > 0.05$) (Figure 3).

3.3. Global DNA Methylation and DNMTs Activity. Our study showed that there was no significant difference in the level of global DNA methylation ($p > 0.05$) and the activity of DNMTs ($p > 0.05$) in fresh group compared to cryopreservation groups (Figure 4). The results of our study showed that DNA methylation and DNMTs activity have a positive relationship in fresh ($r = 0.675$, $p = 0.011$), ultra-rapid freezing ($r = 0.732$, $p = 0.003$), and rapid group ($r = 0.617$, $p = 0.019$). Also, a positive relationship between DNA methylation level and DNMTs activity with progressive motility was observed in fresh, ultra-rapid freezing, and rapid groups, while no significant relationship was observed with DFI, viability, and morphology (Table 3).

3.4. Embryo Development. A total of 730 embryos were evaluated using time-lapse until day 6: fresh ($n = 249$), ultra-rapid freezing ($n = 243$), and rapid freezing groups ($n = 238$) (Table 4). According to Table 5, the kinetic times, including tPB2, tPNa, tPNf, t2, t3, t4, t5, t6, t7, t8, tM, showed a significant delay in cell divisions in both sperm cryopreservation groups compared to the fresh group until the blastocyst stage. Furthermore, tPNa, tPNf, and t8 occurred with a significantly delay in the rapid compared to the ultra-rapid freezing group. Blastocyst formation and cleavage rate showed a significant decrease in rapid freezing compared to the fresh group, but these parameters were not different in the cryopreservation groups (Table 4).

The optimal time ranges of embryo kinetics (cc2, s2, t5, cc3, s3, and tM) are shown in Figure 5. This graph describes percentage of embryos falling within proposed optimal time ranges in fresh, ultra-rapid freezing, and rapid groups. In the assessment of cleavage abnormalities, cryopreservation groups showed a significant increase in the percentage of fragmentation and uneven blastomeres compared to the fresh group, while no significant difference was observed in other abnormalities between fresh and cryopreservation groups (Table 6).

4. Discussion

Our results showed that progressive motility and viability decreased significantly after sperm cryopreservation, but these two parameters were significantly better in the ultra-rapid freezing group compared to the rapid group. Our data confirm the previous studies with the similar vitrification protocol (straw in straw system) [24, 39]. In using the drop-wise technique of sperm vitrification method, progressive motility and viability in rapid freezing group were significantly higher than vitrification [40].

Moreover, a meta-analysis study has indicated that the vitrification/ultra-rapid freezing method is a more efficient technique compared to the rapid method in terms of total and progressive motility. They also stated that these results are dependent on the vitrification/ultra-rapid freezing protocol and sperm quality [41]. One of the most important differences between vitrification/ultra-rapid freezing and rapid freezing is the use of permeable CPAs in the rapid freezing, which can induce oxidative stress to membrane phospholipids [42, 43].

In our study, a significant increase in DFI and AR was observed in cryopreservation groups compared to the fresh. But no significant difference was observed in the acrosome reaction in both cryopreservation groups. However, Schulz et al. [24] reported minor damage in the acrosome integrity in vitrified sperm cells compared to the slow-freezing method. They also stated that by increasing the warming temperature to 42°C and modifying the composition of the vitrification medium (adding dextran and antioxidants), the integrity of the plasma membrane was better maintained in the acrosome region. They increased the warming temperature for optimization of the vitrification technique. Their

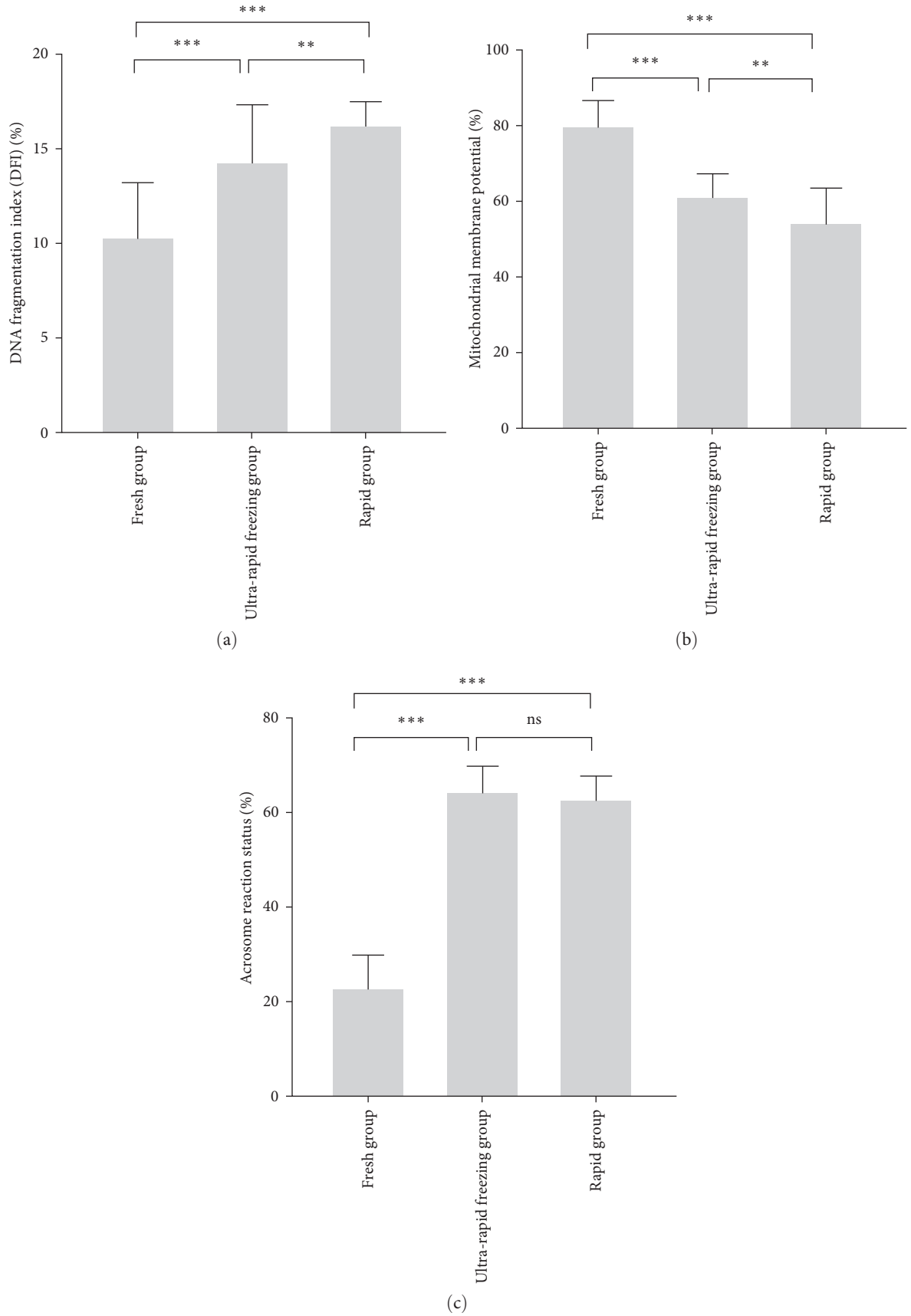

Figure 3: The effects of ultra-rapid freezing and rapid freezing methods on DNA and acrosome integrity and mitochondrial activity of spermatozoa: (a) DNA fragmentation index, (b) mitochondrial membrane potential, and (c) acrosome reaction status. $^{**}p < 0.01$; $^{***}p < 0.001$. ns shows $p > 0.05$.

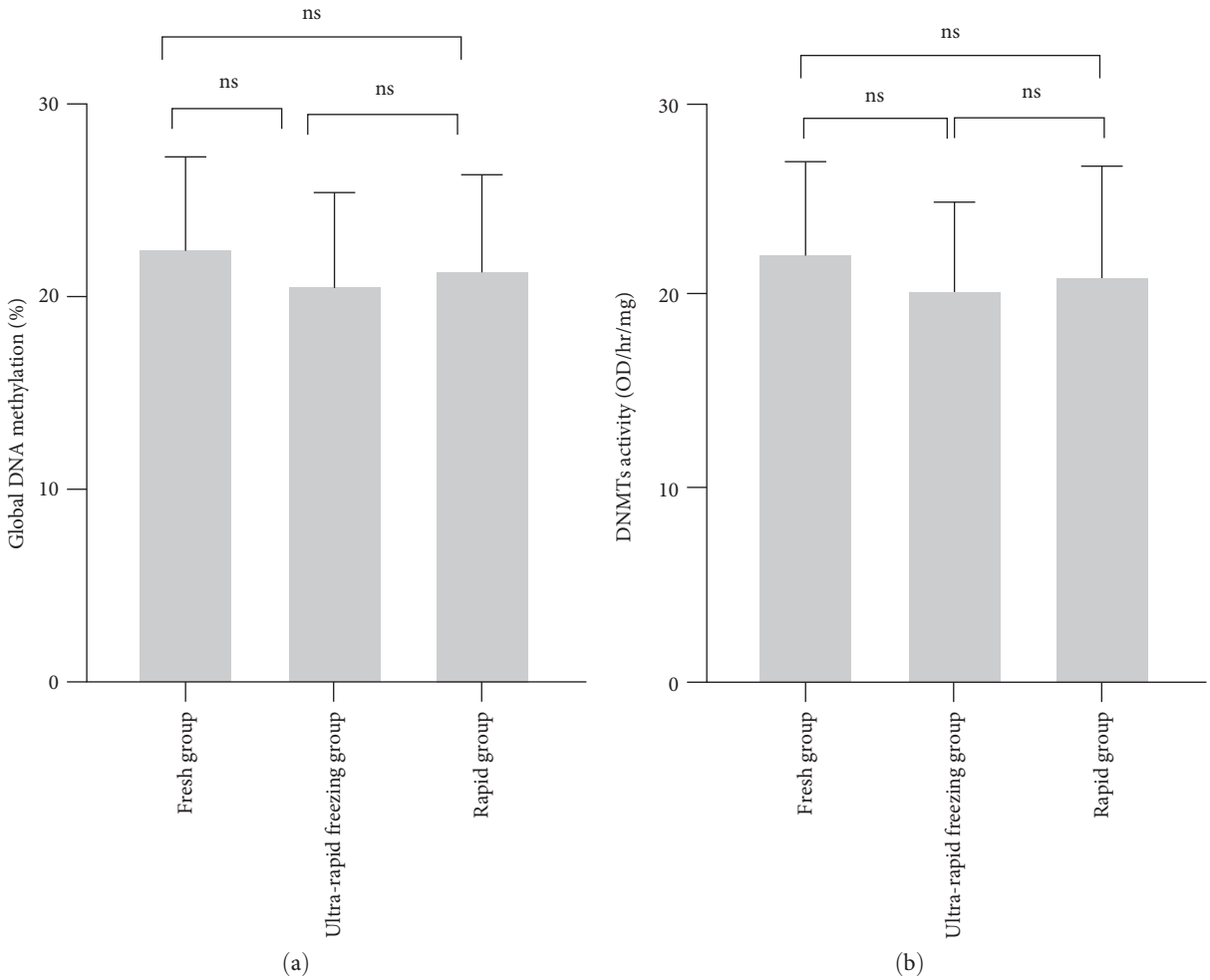

Figure 4: The effects of ultra-rapid freezing and rapid freezing methods on DNA methylation stability and DNMT activity: (a) global DNA methylation and (b) DNMTs activity analysis in fresh, ultra-rapid freezing, and rapid groups. ns shows $p > 0.05$.

Table 3: Spearman's correlation analysis of DNA methylation and DNMTs activity with DFI and sperm parameters in fresh, ultra-rapid freezing, and rapid groups.

		DNA methylation	DNMTs	DFI	Progressive motility	Viability	Morphology
Fresh group							
DNA methylation	Correlation	1	0.657[b]	0.029	0.561[b]	−0.155	0.401
	p-Value	—	0.011	0.921	0.037	0.596	0.156
DNMTs	Correlation	0.657[b]	1	−0.054	0.604[b]	0.063	0.096
	p-Value	0.011	—	0.855	0.022	0.832	0.744
Ultra-rapid freezing group							
DNA methylation	Correlation	1	0.732[a]	−0.247	0.672[a]	0.014	−0.082
	p-Value	—	0.003	0.395	0.009	0.962	0.781
DNMTs	Correlation	0.732[a]	1	−0.081	0.637[b]	−0.261	0.009
	p-Value	0.003	—	0.782	0.014	0.367	0.976
Rapid group							
DNA methylation	Correlation	1	0.617[b]	0.354	0.629[b]	−0.324	0.199
	p-Value	—	0.019	0.214	0.016	0.258	0.495
DNMTs	Correlation	0.617[b]	1	0.215	0.724[a]	−0.326	−0.077
	p-Value	0.019	—	0.46	0.003	0.255	0.793

[a]Correlation is significant at $p < 0.01$ (two-tailed); [b]correlation is significant at $p < 0.05$ (two-tailed).

Table 4: Comparison of laboratory characteristics between different groups.

Characteristics	Fresh	Ultra-rapid freezing	Rapid	p-Value
Total number of MII oocytes (n)	330	330	330	—
Fertilization rate (%), (n/n)	75.5%, (249/330)[a]	73.6%, (243/330)[a]	72.1%, (238/330)[a]	0.62
Cleavage rate (%), (n/n)	90.4%, (225/249)[a]	86.8%, (211/243)[a,b]	81.5%, (194/238)[b]	0.01
Blastocyst rate (%), (n/n)	42.2%, (105/249)[a]	34.2%, (83/243)[a,b]	30.3%, (72/238)[b]	0.02

Note. The used superscript letters that are similar do not show a significant difference ($p > 0.05$), but the dissimilar letters show a significant difference ($p < 0.05$).

Table 5: Comparison of morphokinetics individual and calculated variables between groups.

Time lapse parameters	Fresh group ($n = 249$) mean ± SD	Ultra-rapid freezing group ($n = 243$) mean ± SD	Rapid group ($n = 238$) mean ± SD
tPB2	3.97 ± 0.91	$4.67 \pm 1.16^{***}$	$4.61 \pm 1.12^{***}$
tPNa	7.97 ± 0.90	$8.40 \pm 0.81^{***}$	$8.63 \pm 0.99^{***\dagger}$
tPNf	23.55 ± 1.76	$24.14 \pm 2.47^{*}$	$24.79 \pm 3.03^{***\dagger}$
t2	26.9 ± 1.85	$28.22 \pm 3.50^{***}$	$28.15 \pm 3.60^{***}$
t3	37.04 ± 2.54	$37.78 \pm 3.48^{*}$	$37.83 \pm 3.05^{*}$
t4	39.69 ± 2.61	$40.48 \pm 3.74^{*}$	$40.54 \pm 3.01^{**}$
t5	49.04 ± 3.48	$50.19 \pm 4.34^{**}$	$49.92 \pm 3.23^{*}$
t6	51.57 ± 2.71	$52.53 \pm 3.51^{**}$	$52.22 \pm 2.74^{*}$
t7	53.00 ± 2.61	$53.77 \pm 3.31^{*}$	$54.06 \pm 2.69^{***}$
t8	57.30 ± 3.42	$58.31 \pm 4.06^{*}$	$59.39 \pm 5.11^{***\dagger}$
tM	95.20 ± 2.75	$96.02 \pm 1.74^{*}$	$96.44 \pm 3.40^{**}$
tB	107.39 ± 4.11	108.00 ± 4.89	108.45 ± 5.00
tEB	115.95 ± 2.45	116.54 ± 2.83	116.77 ± 2.55
cc2	10.13 ± 3.23	9.56 ± 4.63	9.68 ± 4.60
s2	2.65 ± 1.69	2.70 ± 1.82	2.68 ± 1.75
cc3	12.00 ± 4.14	12.41 ± 5.46	12.10 ± 4.43
s3	8.28 ± 5.08	8.06 ± 4.30	9.32 ± 5.91

Note. tPB2, time to detached the second PB from oolema; tPNa, time to PN appearance; tPNf, time to PN disappearance; t2, first cleavage (2-cell stage); t3, second cleavage (3-cell stage); t4, 4-cell stage; t5, 5-cell stage; t6, 6-cell stage; t7, 7-cell stage; t8, 8-cell stage; tM, time to morula; tB, time to blastocyst; tEB, time to expanded blastocyst; cc2 = t3–t2; s2 = t4–t3; cc3 = t5–t3; s3 = t8–t5. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; the different between fresh with ultra-rapid freezing and rapid groups. $^{\dagger}p < 0.05$; the different between ultra-rapid freezing with rapid groups.

findings showed that sperm progressive motility at 42°C (65%) was higher than that at 38°C (26%) [24]. We considered 37°C as the optimal temperature of warming, similar to recent studies [5, 39, 44, 45].

DNA fragmentation was significantly higher in the rapid group compared to the ultra-rapid freezing. This finding supports that of Slabbert et al. [46] in closed straw systems. However, in the study of Schulz et al. [24] and Isachenko et al. [47], no significant difference in DNA fragmentation was reported in any of the cryopreservation methods. Nevertheless, there is no agreement about the effect of sperm cryopreservation on sperm DNA integrity, which can be related to different sperm cryopreservation protocols and different methods used to evaluate sperm DNA fragmentation [41]. Studies have reported that one of the important reasons for sperm DNA fragmentation is the increase in oxidative stress and ROS production during the freeze–thaw process [7].

In addition, our results showed a significant decrease in MMP in the rapid group compared to the ultra-rapid freezing group (Figure 3). Previous studies have reported that CPAs used during sperm cryopreservation caused disruption of mitochondrial function by increasing ROS production [43, 48]. In the rapid method, due to the low cooling rate in the vapor phase, CPA is needed to avoid the intracellular and extracellular ice crystal formation and reduce osmotic changes. However, in the vitrification method/ultra-rapid freezing, there is no need to use CPA due to the high cooling and warming rates [25]. No significant differences were observed in the investigation of two cryopreservation methods on the sperm DNA methylation status (Figure 4). DNMTs activity, which is mainly responsible for DNA methylation, was also evaluated. The results of our study did not show a significant difference in DNMTs activity in cryopreservation groups compared to fresh group (Figure 4). Moreover, in the correlation analysis between global DNA methylation and DNMTs activity, a significant positive correlation was observed in all three studied groups (Table 3).

This study confirmed that DNA methylation is not affected by cryopreservation methods. In agreement with our results, Lu et al. [49] reported that no significant

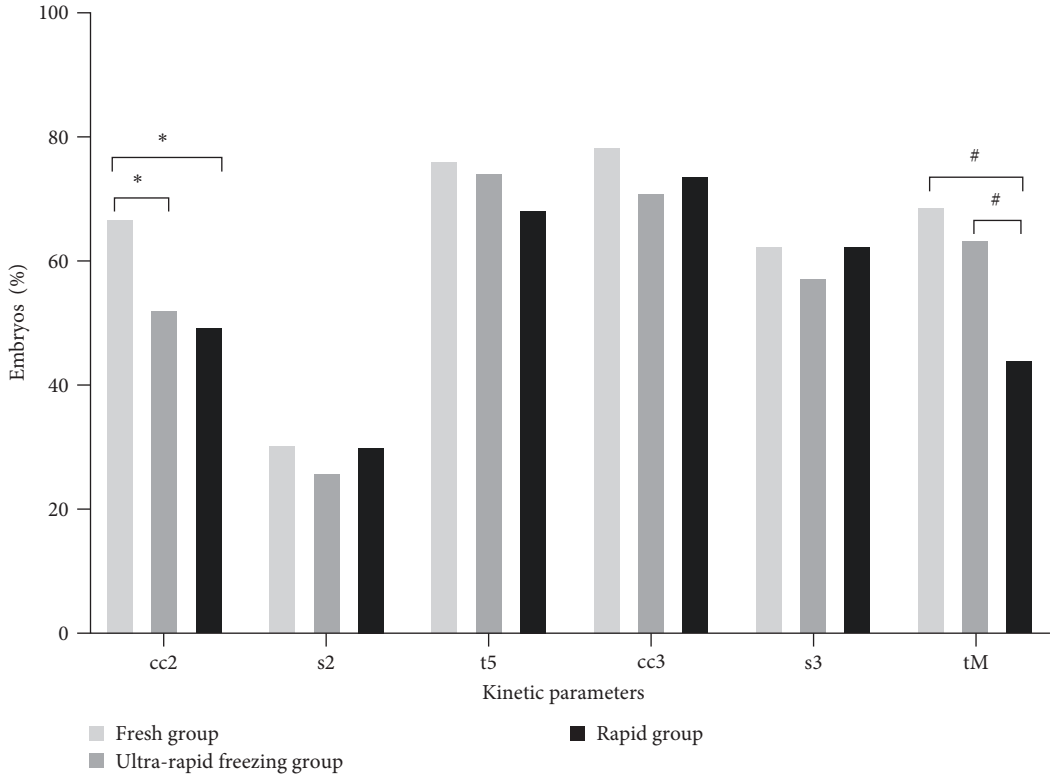

Figure 5: Embryo development kinetics in fresh, ultra-rapid freezing, and rapid groups. Percentage of embryos falling within optimal ranges. Proposed optimal time ranges are included: cc2 (>5 and ≤11.9 hr), s2 (≤1 hr), t5 (48–57 hr), cc3 (9.7–21 hr), and tM (81.28–96 hr). $p < 0.05$ is considered as statistically significant. *Shows the significantly different between rapid and ultra-rapid freezing with fresh groups. #Shows the significantly different between rapid with fresh and ultra-rapid freezing groups.

Table 6: Percentage and frequency of cleavage abnormalities between groups.

Cleavage abnormalities	Fresh group ($n = 249$)	Ultra-rapid freezing group ($n = 243$)	Rapid group ($n = 238$)	p-Value
Reverse cleavage	10.4% (26)[a]	14.8% (36)[a]	14.3% (34)[a]	0.29
Direct cleavage	4.8% (12)[a]	7.4% (18)[a]	5.0% (12)[a]	0.39
Multinucleation	2% (5)[a]	3.3% (8)[a]	3.8% (9)[a]	0.31
Uneven blastomeres	35.5% (88)[a]	48.6% (118)[b]	52.1% (124)[b]	0.001
Vacuoles	26.1% (65)[a]	31.3% (76)[a]	35.7% (85)[a]	0.07
Fragmentation	60.6% (151)[a]	73.3% (178)[b]	80.7% (192)[b]	<0.001

Note. The used superscript letters that are similar do not show a significant difference ($p > 0.05$), but the dissimilar letters show a significant difference ($p < 0.05$).

difference was observed in the methylation status of genome imprinting (H19 and MEST ICR) under the influence of cryopreservation techniques. Also, Kläver et al. [13] stated in the analysis of nine imprinted genes that sperm cryopreservation and cryoprotectant medium had no effect on sperm DNA methylation status. A recent study reported that in transcriptome analysis of human sperm, vitrification method induces very minor epigenetic changes compared to slow freezing, accepting that this study reported that sperm cryopreservation was an epigenetically safe method to preserve fertility [14].

Results of studies regarding the effect of cryopreservation on sperm DNA methylation are contradictory. In some studies, on nonhuman species, significant alterations in DNA methylation have been reported after sperm cryopreservation [50–53]. In the present study, a significant relationship was observed between DNA methylation and DNMTs with progressive motility, but no significant relationship was observed with DFI. In agreement with our study, Khosravizadeh et al. [8], noticed no significant relationship between DNA methylation and DFI. Montjean et al. [54] reported a significant correlation between DNA methylation and motility as well as with DFI. DNA methylation is essential for normal sperm chromatin compaction and prevention of DNA damage. In some studies, a negative correlation between DFI and DNA methylation has been reported [54, 55]. However, other studies have shown that DNA methylation alterations can be caused by disruption in spermatogenesis and defects in DNMTs function [56, 57]. The inconsistency in the results can be related to the difference in DNA methylation evaluation methods, the type of genes studied, semen samples, the CPAs used, and the type of species studied

[58, 59]. However, few studies have investigated the effect of cryopreservation on DNA methylation in human spermatozoa. To clarify the effect of cryopreservation on DNA methylation status, more studies with a larger sample size are needed.

Regarding embryo morphokinetics following fertilization with cryopreserved spermatozoa, we observed a significant delay in kinetic times (tPB2, tPNa, tPNf, t2, t3, t4, t5, t6, t7, t8, tM) in both sperm cryopreservation groups compared to the fresh group. According to previous studies, one of the important reasons for this delay in embryo kinetics is the sperm nuclear quality. Esbert et al. [60] stated that high sperm DNA fragmentation can lead to a delay in early embryo kinetics, which is related to the increase in the time required before the first cell division to activate DNA repair mechanisms in oocyte. Also, Ribas-Maynou et al. [61] observed that the overall DNA damage in sperm was associated with a delay in tPNa, tPNf, and t2 which led to an increase in the time of transition from eight cells to the morula stage.

Furthermore, it has been reported that the chromatin integrity of mouse sperm is related to the kinetics of the first cell division, and sperm chromosomal abnormalities can lead to delays in tPNf and t2 [18]. In our study, there was a significant delay in the kinetic times of tPB2, tPNa, tPNf, and t2 in both cryopreservation groups, which may be related to the significant increase in sperm DNA fragmentation in cryopreservation groups. However, this parameter was significantly higher in the rapid group compared to the ultra-rapid freezing group. This is most likely the reason for the significant delay of kinetic times tPNa and tPNf in the embryos of the rapid group compared to the embryos of the ultra-rapid freezing group.

Based on previous literatures, one of the important cellular events of embryo development is embryonic genome activation that is initiated from paternal genome at the 8-cell stage [16]. Our results showed a delay in t8 in both cryopreservation groups compared to the fresh group. This morphokinetic time showed a longer delay in the rapid compared to the ultra-rapid freezing group. Previous studies have shown that paternal factors can affect the early stages of embryo development through embryonic genome activation [16]. In addition, it was reported that sperm quality, especially sperm DNA integrity, can affect the early embryo development [62]. Studies have shown that the increase in sperm DNA fragmentation is associated with the decrease in blastocyst development rate after IVF or ICSI [61, 63].

Braga et al. [64] stated that sperm cryopreservation has no effect on embryo quality on days 2 and 3 and blastocyst formation in the presence of normal oocytes and this effect was related to the oocyte quality. Previous studies have reported that viable oocytes can repair some of paternal DNA defects, but the oocyte capacity to repair sperm DNA defects is limited and depends on the degree of sperm DNA damage and oocyte quality [60, 65]. Moreover, it was stated that equine embryos derived from cryopreserved spermatozoa showed significant downregulation in genes involved in DNA binding, DNA replication and oxidative phosphorylation. This group suggested that the oxidative stress occurring during cryopreservation and thawing is as an important reason for this event [66]. Thus, the reason for the delay in t8 in the rapid group compared to the ultra-rapid freezing may be due to the higher DNA fragmentation in rapid group.

Based on optimal kinetic times (tM = 81.28–96 hr and s3 $\leq$ 8.78 hr) proposed by Motato's algorithm [37] for potential of blastocyst formation and quality, our tM showed significant delays in both cryopreservation groups compared to the fresh group. This was accompanied by a decrease in blastocyst formation in both cryopreservation groups. Whilst, blastocyst formation was not significant in the cryopreservation groups, the ultra-rapid freezing group showed a significantly higher percentage of embryos with optimal time ranges, based on Motato's algorithm, compared to rapid embryos (63.6% vs. 44.2%) (Figure 5).

5. Conclusion

The superiority of sperm progressive motility, viability, mitochondrial activity, DNA integrity, and embryo kinetics was recognized in the ultra-rapid freezing compared to the rapid method. Also, due to the high sensitivity of sperm cells to the toxic effects of permeable CPAs used in the rapid freezing and the lower cooling rate in vapor phase, the ultra-rapid freezing appears to be less harmful for sperm quality. In addition, the ultra-rapid freezing results in better embryo development compared to rapid freezing method. Both sperm cryopreservation methods were epigenetically safe. Further studies are needed to confirm the efficacy of ultra-rapid freezing compared to rapid freezing.

Data Availability

Data are available upon request.

Ethical Approval

This study was approved by the Ethics Committee of our institution (IR.SSU.MEDICINE.REC.1400.306).

Consent

Signed informed consent was obtained from all patients.

Disclosure

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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