Asian Journal of Andrology (2024) 26, 366–376
www.asiaandro.com; https://journals.lww.com/ajandrology

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ORIGINAL ARTICLE

Sperm Biology

Evaluation of microRNA expression profiles in human sperm frozen using permeable cryoprotectant-free droplet vitrification and conventional methods

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For sperm cryopreservation, the conventional method, which requires glycerol, has been used for a long time. In addition, the permeable cryoprotectant-free vitrification method has been continuously studied. Although the differences of cryopreservation effects between the two methods have being studied, differences in microRNA (miRNA) profiles between them remain unclear. In this study, we investigated the differences in miRNA expression profiles among conventional freezing sperm, droplet vitrification freezing sperm and fresh human sperm. We also analyzed the differences between these methods in terms of differentially expressed miRNAs (DEmiRs) related to early embryonic development and paternal epigenetics. Our results showed no significant differences between the cryopreservation methods in terms of sperm motility ratio, plasma membrane integrity, DNA integrity, mitochondrial membrane potential, acrosome integrity, and ultrastructural damage. However, sperm miRNA-sequencing showed differences between the two methods in terms of the numbers of DEmiRs (28 and 19 with vitrification using a nonpermeable cryoprotectant and the conventional method, respectively) in postthaw and fresh sperm specimens. DEmiRs related to early embryonic development and paternal epigenetics mainly included common DEmiRs between the groups. Our results showed that the differences between conventional freezing and droplet vitrification were minimal in terms of miRNA expression related to embryonic development and epigenetics. Changes in sperm miRNA expression due to freezing are not always detrimental to embryonic development. This study compared differences in miRNA expression profiles before and after cryopreservation between cryopreservation by conventional and vitrification methods. It offers a new perspective to evaluate various methods of sperm cryopreservation.

Asian Journal of Andrology (2024) **26**, 366–376; doi: 10.4103/aja202390; published online: 10 May 2024

Keywords: cryopreservation; cryoprotectant-free vitrification; early embryonic development; paternal epigenetic inheritance; sperm miRNAs; sucrose

INTRODUCTION

Cryopreservation of sperm is widely used in human assisted reproductive technology (ART) to preserve male fertility. The vitrification method in current use employs permeable-free cryoprotectants to rapidly freeze sperm. This has been recognized to be an effective approach to achieve good recovery of motility.[1–3] Sucrose is generally used as a permeable-free cryoprotectant in this method.[4,5]

Numerous comparative studies have evaluated the benefits and disadvantages of conventional cryopreservation and permeable cryoprotectant-free droplet vitrification methods. Most of them have focused on the effects on sperm motility, DNA integrity, mitochondria, acrosomes, and other parameters.[6] However, these studies have provided conflicting results.[2,5,7,8] This may be mainly attributed to inconsistencies in the freezing method and frozen sample. Sperm is responsible for embryonic development in addition to fertilization.[9,10] It carries microRNAs (miRNAs) that regulate maternal transcripts before activation of the zygotic genome, thereby regulating the first cell division of the embryo.[11]

MiRNAs are small noncoding RNAs that play a role in posttranscriptional regulation by binding to the 3′-untranslated regions (UTRs) of their target mRNA to suppress gene expression.[12,13] Numerous studies have evaluated the effect of changes in miRNA acquired by sperm *in vivo* on early embryonic development.[14,15] Additionally, emerging evidence suggests that several miRNAs affected by sperm cryopreservation are implicated in early embryonic development.[16] For example, expression of miR-34c is reduced following freezing and thawing of sperm, which may affect human embryonic development before transplantation.[16] The altered expression of miR-148b-3p after sperm cryopreservation may also influence fertilization and subsequent embryonic development through upregulation of phosphatase and tensin homolog deleted on chromosome ten (*PTEN*).[17]

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Received: 21 September 2023; Accepted: 18 January 2024

MiRNAs transmitted by sperm during fertilization are epigenetic factors that influence offspring. Animal studies have shown that obesity, metabolic diseases,[18] particulate matter in ambient air,[19] and mental stress[20] may change miRNA expression in sperm and mediate paternal genetic cross-generation.

Changes in osmotic pressure due to freezing and the development of ice crystals lead to cellular damage.[21] Permeable cryoprotectants, including glycerin, may affect the internal milieu of sperm, leading to changes in miRNA expression.[22] However, few studies have compared the overall differential expression of miRNAs between sperm frozen by permeable cryoprotectant-free vitrification or conventional cryopreservation. In particular, few studies have evaluated the differential effects on miRNA expression in the context of embryonic development or intergenerational epigenetics.

Our study focused on the effects of different cryopreservation treatments. Conventional cryopreservation and permeable cryoprotectant-free droplet vitrification methods were evaluated to compare their influence on miRNA expression profiles in mature human sperm. The effects of the two methods on embryonic development and intergenerational genetics were evaluated by RNA sequencing (RNA-seq). This study may provide a basis to select freezing methods for sperm preservation in the clinic.

PARTICIPANTS AND METHODS

Semen collection and processing

Ethical approval for this study was obtained from the Ethics Committee of The Affiliated Taizhou People's Hospital of Nanjing Medical University (Taizhou, China; Approval No. KY2020-025-01). Semen samples were obtained at the Department of Reproductive Medicine of The Affiliated Taizhou People's Hospital of Nanjing Medical University. The participants aged 24–41 years. They participated voluntarily and provided informed consent before their inclusion in the study. The World Health Organization (2010) standards[23] were used to evaluate routine seminal parameters. Normal semen was defined by the following parameters: (1) total sperm count $\geq 39 \times 10^6$; (2) sperm concentration $\geq 15 \times 10^6$ sperms per ml; (3) progressive motility (PR) + nonprogressive motility (Np) ≥40% and PR ≥32%; and (4) normal sperm morphology ≥4%. Semen samples were obtained from 43 men after 2–7 days of sexual abstinence. Sperm concentrations and motility were evaluated using a computer-assisted sperm analyzing system (Beion S3, Beionmed, Shanghai, China) after complete liquefaction (**Supplementary Table 1**). Among these, ten cases were involved in assessing sperm motility parameters, plasma membrane integrity, DNA fragmentation, and mitochondrial membrane potential. Eight cases were dedicated to evaluating sperm acrosome integrity, while six cases were allocated to scrutinizing sperm ultrastructure through electron microscope observation. Additionally, nineteen cases were devoted to conducting sperm miRNA-seq. During processing, semen samples were treated with a 40%/80% gradient centrifugation fluid (ART-2024; CooperSurgical Inc., Trumbull, CT, USA). After centrifugation at 300*g* for 15 min, deposited sperm was washed with washing medium (ART-1005; CooperSurgical Inc.) by centrifugation at 200*g* for 10 min (Universal 320; Andreas Hettich GmnH, Tuttlingen, Germany). Then, they were suspended in washing medium to assess sperm motility.

Droplet vitrification freezing and thawing

A sperm suspension was mixed with 0.5 mol l^{-1} sucrose (ST1672; Beyotime Biotechnology, Shanghai, China) containing 10% human serum albumin (100 mg ml^{-1}; ART-3001-5; CooperSurgical Inc.) at a 1:1 ratio.[5] Osmolarity of the vitrification medium was 708 mOsm l^{-1}. After mixing equal volumes of vitrification and sperm media, the osmolarity was reduced to 474 mOsm l^{-1}. Trace amounts of the sperm cryopreservation solution were added to the sperm suspension dropwise with rapid and thorough mixing between drops. The mixture was kept at room temperature (RT) for 3–4 min and then dropped into liquid nitrogen (LN) from a distance of 15 cm from the surface at approximately 30 μl per drop.[24] The collected pellets were then dropped into cryogenic vials (430659; Corning Incorporated, Corning, NY, USA) that were held by aluminum rods and transferred for storage into LN for at least 1 week.

A total of 5 ml sperm washing medium was prewarmed to 37°C in a water bath. A ball-shaped drop of sperm was retrieved from the cryogenic vial using metal tweezers. It was then dropped into the medium for thawing. A second drop was added after recovery of the temperature. No more than three drops were added per tube.

Conventional freezing and thawing

A sperm suspension was mixed with freezing medium (10670010F; ORIGIO A/S, Malov, Denmark) at a 1:1 ratio. The medium was added to the sperm suspension dropwise, ensuring thorough mixing after each drop. The mixture was then kept at RT for 10 min and subsequently transferred to 2.0 ml cryogenic vials fumigated by placing them in a holder at 1 cm from the LN surface for 30 min.[25] These vials were then transferred for storage into LN for at least 1 week. Frozen vials were placed in a water bath at 25°C and gently agitated for 3 min. After thawing and centrifugation at 200*g* for 10 min (Universal 320), the sperm precipitation was resuspended in 100 μl sperm washing medium and incubated at 37°C for 15 min. Sperm motility parameters were then assessed.

Determination of sperm motion characteristics

Sperm motility was evaluated using the computer-assisted sperm analyzing system. The recorded parameters included total motility (TM; %), progressive motility (PM; %), average path velocity (VAP; μm s^{-1}), curvilinear velocity (VCL; μm s^{-1}), linearity (LIN; %), straight line velocity (VSL; μm s^{-1}), and beat cross frequency (BCF; Hz).[5] Four microscopic areas containing at least 200 spermatozoa were randomly selected for analysis.

Determination of sperm plasma membrane integrity (PMI)

The integrity of the sperm plasma membrane was evaluated using the apoptosis detection kit (C1062L; Beyotime Biotechnology).[26] A total of 10 000 spermatozoa were counted using the FL2 channel of flow cytometer (BriCyte E6; Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China) to determine the propidium iodide (PI) positivity rate. The integrity rate of the plasma membrane was calculated as 1-PI%.

Determination of the DNA fragmentation index (DFI)

The DFI was determined using a sperm chromatin dispersion kit (BRED Life Science, Shenzhen, China),[27] following the manufacturer's protocol. Five hundred sperm were counted under a microscope. Sperm containing undamaged DNA, which was identified by large- or medium-sized halos of dispersed chromatin surrounding a compact and well-defined core, were then scored. No or small halos were indicative of DNA damage. The DFI (number of DNA-damaged sperm/total number of observed sperm ×100%) was then calculated.

Determination of the mitochondrial membrane potential (MMP)

The MMP was determined using a mitochondrial membrane potential assay kit with JC-1 (C2006; Beyotime Biotechnology),[28] following the manufacturer's protocol. Using flow cytometric evaluation, the MMP

(number of sperm emitting red fluorescence/number of total sperm ×100%) was then calculated.

Determination of acrosome integrity

Acrosome integrity was determined using the induced acrosome reaction detection kit (Pisum sativum agglutinin–fluorescein isothiocyanate [PSA-FITC]; BRED Life Science),[29] following the manufacturer's protocol. At least 200 spermatozoa were observed under a fluorescence microscope (Axio Vert. A1 FL-LED; Carl Zeiss, Oberkochen, Germany). Acrosome integrity represented by the acrosome-intact sperm (AIS) ratio was defined as demonstration of uniform bright green fluorescence by more than half of the sperm head.

Scanning electron microscopy (SEM)

Sperm samples were fixed using stationary liquid (G1102; Servicebio, Wuhan, China) at RT for 2 h. They were then rewashed, treated with osmium tetroxide for fixation, and rinsed again. Graded concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, 100%, and 100%) and isoamyl acetate were applied for dehydration. The samples were then dried in air at RT before gold coating under a high vacuum.[30] Images were obtained under a scanning electron microscope (HITACHI-SU8100; Hitachi, Tokyo, Japan). Fields of view were randomly selected for assessment. Structural damage to the head, middle, and tail of the sperm were evaluated.

Transmission electron microscopy (TEM)

Sperm samples were fixed using stationary liquid for 2 h at RT. After fixation in osmium tetroxide, the samples were exposed to graded concentrations (30%, 50%, 70%, 80%, 95%, 100%, and 100%) of ethanol and 100% acetone to prevent deterioration of the biological components. The samples were then treated with epoxypropane and incorporated into resin during the cutting process. They were then stained with a 2.6% lead citrate solution for 8 min.[30] Various ultrastructural changes in sperm morphology, including those in the head, mitochondria, midbody, and tail, were evaluated under a transmission electron microscope (HITACHI-HT7800; Hitachi).

MiRNA-seq

To remove interference from nonsperm cells, samples were treated with 40% and 80% density gradient media.[31] After centrifugation at 300*g* for 15 min (Universal 320), the precipitate was centrifuged again with 40% density gradient medium. Sterile centrifuge tubes and pipettes were replaced at each step of the process.

Purified sperm samples used for RNA-seq were divided into three groups: fresh, conventional method, and permeable cryoprotectant-free droplet vitrification method.

Sperm samples that were frozen using conventional and droplet vitrification methods were resuscitated, washed, and centrifuged to obtain the precipitate as described previously. The samples were resuspended in 1 ml fertilization medium (10310060F; ORIGIO A/S) and incubated at 37°C with 5% CO_2 for 40 min. The cell suspension was then centrifuged, and the precipitate was transferred into 1.5 ml Eppendorf tubes (MCT-150-C, Axygen; Corning Incorporated) for storage in LN.

A HiPure Universal RNA Mini Kit (Magen Bio, Guangzhou, China)[32] was used to extract total RNA. RNA quality was examined by gel electrophoresis and a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was used to prepare a small RNA library for RNA-seq. The sequencing libraries were generated using a VAHTS™ Total RNA-seq (H/M/R) Library Prep Kit for Illumina®. The libraries were sequenced by Genergy Biotechnology Co., Ltd. (Shanghai, China) as 51-bp paired-end reads using the Illumina NovaSeq 6000 system in accordance with the manufacturer's instructions.

For data analysis, clean data were aligned to those in the miRBase database (http://www.mirbase.org/, last accessed on 05 May 2023), and expression reads of miRNAs were obtained using Bowtie read aligner (version 1.0).[33] Counts per million values were calculated using edgeR version 3.22.5. The DESeq software package was used to analyze differential expression between groups and screen differentially expressed miRNAs (DEmiRs). The expression level in each sample and the mean value in each group were calculated in addition to the fold change between groups. The $\log_2$ (fold change) was then calculated for subsequent screening of differentially expressed genes. Genes with $P < 0.05$ and $\log_2$ (fold change) > 1 were differentially expressed. Novel miRNAs or those with an expression level of 0 were excluded from DEmiRs. Target prediction of DEmiRs was performed using miRanda version 3.3a,[34] and functional analysis was performed on target genes of DEmiRs. Significantly enriched Gene Ontology (GO) entries and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were selected with a threshold of $P < 0.05$.

Total RNA in all samples was isolated, and first-strand cDNA was synthesized using a kit (B532453; Sangon, Shanghai, China) in accordance with the manufacturer's instructions.[35] The reaction mixture was incubated at 16°C for 30 min, 37°C for 30 min, and then 85°C for 5 min. MiRNA fluorescence quantitative real-time polymerase chain reaction (qRT-PCR) was performed using another kit (B532461; Sangon) in accordance with the manufacturer's instructions.[36] The qRT-PCR program consisted of 40 cycles of predenaturation at 95°C for 30 s, followed by denaturation at 95°C for 5 s and annealing and extension at 60°C for 30 s (LightCycler 480; Roche, Basel, Switzerland). *U6* was used as the reference gene. A melting curve was used to assess the specificity of PCR products. qRT-PCRs were repeated at least three times ($n = 3$). The primer sequences are shown in **Supplementary Table 2**.

Statistical analyses

Statistical analysis was performed using the IBM SPSS 21.0 software package (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was used to compare means in between-group comparisons. The Tukey's test was used for comparison between groups. The Dunnett's T3 test was used when the variance was not equal. The independent Samples *t*-test was used to compare the means of the two samples. The results were presented as mean ± standard error of the mean. $P < 0.05$ was considered statistically significant.

RESULTS

Assessment of sperm motility

To assess the effects of the cryopreservation methods on human sperm, we compared sperm motility parameters between samples processed by conventional freezing (CF) and droplet vitrification freezing (DF) methods before freezing and after thawing. Compared with samples in the fresh group, frozen-thawed samples in CF and DF groups demonstrated a decrease in sperm motility parameters, including TM, PM, VCL, VSL, and VAP (all $P < 0.05$, one-way ANOVA). Furthermore, a significant difference was observed in BCF between DF groups and fresh group ($P < 0.05$). Additionally, no significant difference was observed in TM, PM, LIN and BCF between CF and DF groups (all $P > 0.05$), meanwhile, VCL, VSL and VAP showed significant difference (all $P < 0.05$; **Figure 1a–1g**).

Assessment of the sperm structure

The cellular structure of sperm was evaluated before freezing and after thawing. Compared with the fresh group, CF and DF groups

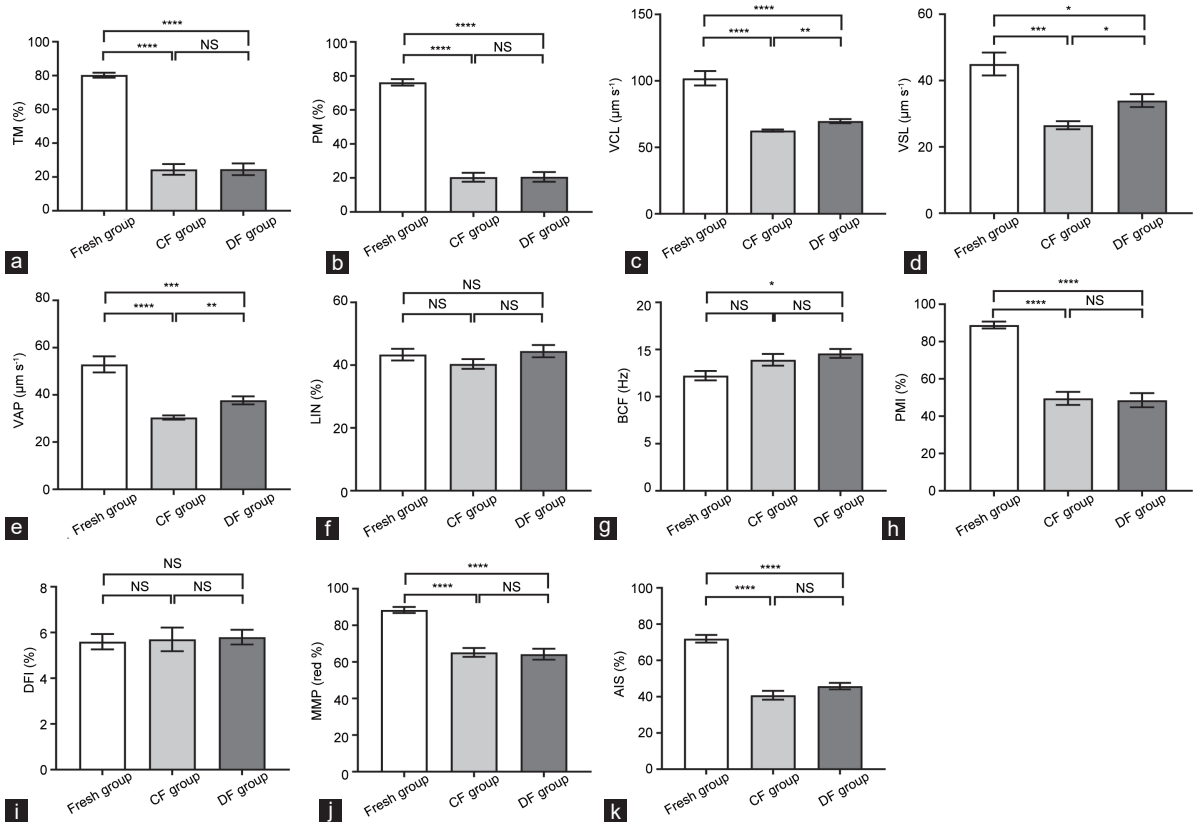

Figure 1: Comparison of parameters of sperm motility and cryoinjuries, including (**a**) TM, (**b**) PM, (**c**) VCL, (**d**) VSL, (**e**) VAP, (**f**) LIN, (**g**) BCF, (**h**) PMI, (**i**) DFI, (**j**) MMP, and (**k**) AIS, before and after the freeze–thaw cycle by CF and DF methods. Data are presented as mean ± standard error of the mean and one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. NS: no significance; CF: conventional freezing; DF: droplet vitrification freezing; TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; LIN: linearity; BCF: beat cross frequency; PMI: plasma membrane integrity; DFI: DNA fragmentation index; MMP: mitochondrial membrane potential; AIS: acrosome-intact sperm; ANOVA: analysis of variance.

demonstrated decreases in PMI, MMP, and AIS (all $P < 0.05$, one-way ANOVA). However, no significant difference was observed in DFI (both $P > 0.05$). CF and DF groups also showed no significant differences in PMI, DFI, MMP, or AIS (all $P > 0.05$; **Figure 1h**–**1k**).

Assessment of the sperm ultrastructure by SEM

Common injuries of sperm include head injury, neck fracture, tail breakage, and increased membrane space. SEM was employed to observe injuries in fresh and frozen–thawed samples. In particular, the extent of injury in the head, midsection, and tail of frozen–thawed sperm obtained by CF and DF methods was approximated (**Figure 2a**–**2i**).

Assessment of the sperm ultrastructure by TEM

Samples of fresh sperm showed slight detachment of the plasma membrane in some cases. Besides, fresh sperm usually displayed an orderly arrangement and homogeneous and electron-dense mitochondrial ultrastructure. The uniformity of nuclear chromatin was slightly lower in conventional frozen–thawed sperm than in fresh counterparts. They also showed obvious detachment of plasma membranes and conspicuous mitochondrial swelling and vacuolization. Frozen–thawed sperm that underwent DF showed slightly lower chromatin uniformity in the nucleus. Plasma membrane detachment was also evident. Additionally, mitochondria demonstrated a low electron density and disorganized arrangement. No specific abnormalities or damage was found using either freezing method (**Figure 2j**–**2r**).

MiRNA profiles under cryopreservation conditions

Sperm frozen using the CF method had 19 DEmiRs compared with fresh sperm samples. Among them, 11 were upregulated and eight were downregulated (**Figure 3a**). GO enrichment and KEGG pathway analyses of the target genes of these DEmiRs are shown in **Figure 3b** and **3c**. Expression of miR-132-3p, miR-223-3p, miR-375-3p, and miR-4516 was verified by qRT-PCR (all $P < 0.05$; **Figure 3d**–**3g**).

Sperm frozen by DF had 28 DEmiRs compared with fresh sperm samples, of which 25 were upregulated and three were downregulated (**Figure 4a**). On GO enrichment analysis, the target genes of these DEmiRs were mainly found to be related to DNA-binding transcription activity and elements (**Figure 4b**). KEGG pathway analysis suggested that the DEmiR targets were involved in the following pathways: PI3k-Akt signaling, cellular senescence, and so on (**Figure 4c**). Expression of miR-132-3p, miR-223-3p, miR-363-3p, miR-375-3p, miR-516b-5p, and miR-4516 was verified by qRT-PCR (all $P < 0.05$; **Figure 4d**–**4i**).

Frozen–thawed sperm using DF had nine DEmiRs compared with samples subjected to the CF method, of which seven were upregulated and two were downregulated (**Figure 5a**). GO enrichment analysis indicated that the target genes of these DEmiRs predominantly pertained to DNA-binding transcription activities and elements (**Figure 5b**). Moreover, KEGG pathway analysis indicated the involvement of DEmiR targets in various pathways such as PI3K-Akt signaling and cell cycle regulation (**Figure 5c**). Expression of let-7b-3p, miR-34b-5p, and miR-516b-5p was verified by qRT-PCR (all $P < 0.05$; **Figure 5d**–**5f**).

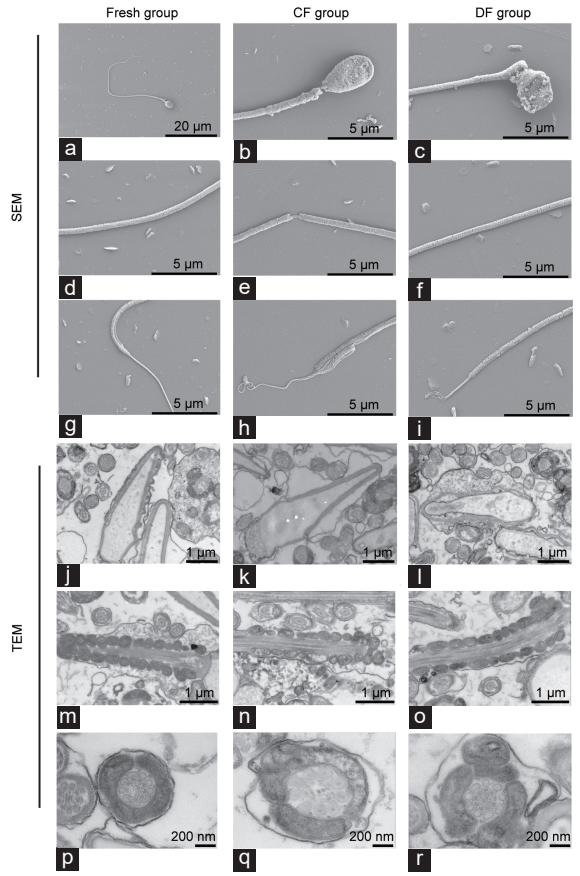

Figure 2: SEM and TEM images showing the most relevant sperm cryodamage after freezing by CF and DF methods compared with fresh samples. (**a**–**g**) SEM images. (**a**) Fresh sample showing a normal smooth surface and neck, midpiece, and tail. (**b**) CF sperm showing a broken neck. (**c**) DF sperm showing a broken neck and damaged membrane on the head with a rough surface. (**d**) Mid-piece from a fresh sample showing a smooth surface. (**e**) Broken midpiece of CF sperm with the axoneme exposed. (**f**) Midpiece of DF sperm showing a slight increase in the gap on the membrane surface. (**g**) Tail from a fresh sample showing normal morphology. (**h**) Broken tail of CF sperm with the axoneme exposed and an increased gap on the membrane surface. (**i**) Tail of DF sperm showing an increase in the gap on the membrane surface, which was damaged, and the axoneme scattered. (**j**–**r**) TEM images. (**j**) Fresh sperm sample showing a sperm with a slight detachment of the plasma membrane. (**k**) CF sperm samples showing considerable detachment of the plasma membrane. (**l**) DF sperm showing slight deterioration of nuclear chromatin uniformity and obvious detachment of the plasma membrane. Fresh sperm showing an orderly arrangement, and a homogeneous and electron-dense mitochondrial ultrastructure in (**m**) longitudinal section and (**p**) cross-section. CF sperm with vacuolization and swelling of mitochondria, which showed reduced electron density in (**n**) longitudinal section and (**q**) cross section. DF sperm showing a low mitochondrial electron density and disordered arrangement in (**o**) longitudinal section and (**r**) cross section. SEM: scanning electron microscopy; TEM: transmission electron microscopy; CF: conventional freezing; DF: droplet vitrification freezing.

DEmiRs in the three groups were compared in pairs (**Figure 6a** and **6b**). Compared with fresh sperm, the two frozen groups showed an intersection of the same eight DEmiRs (common DEmiRs; co-DEmiR). The expression of these eight identical DEmiRs was changed in the same direction relative to the fresh sperm, and none of them overlapped with DEmiRs between CF and DF groups. The upregulated genes included hsa-miR-1273g-3p, hsa-miR-132-3p, hsa-miR-181a-5p, hsa-miR-212-5p, hsa-miR-375, hsa-miR-4516, and hsa-miR-92b-3p; and hsa-miR-223-3p was downregulated. GO enrichment and KEGG pathway analyses of the target genes of these DEmiRs are shown in **Figure 6c** and **6d**.

Association of DEmiRs with early embryonic development and paternal epigenetics

A search of the relevant literature suggested that DEmiR expression in sperm was related to early embryonic development and paternal epigenetics, particularly in the case of co-DEmiRs (**Supplementary Table 3**).

DISCUSSION

Sperm samples that have been cryopreserved using vitrification by placing plastic tubes containing the samples directly into LN without an osmotic protective agent show poor viability after thawing and resuscitation.[37,38] Microcapillary tubes (inner diameter: 200 μm; volume: 2 μl) with a high thermal conductivity that are directly placed in LN during the vitrification of sperm do not confer any benefits.[39] To improve the effect of vitrification, we adopted the DF method. Sperm showed good resilience using this method. It employs rapid cooling of spermatozoa by directly dropping small volumes into LN using a nonpermeable cryoprotectant.[40] Cryopreservation of semen by vitrification has been increasingly studied in recent years.[1,37,41–43] The main factors in vitrification are the rates of cooling and heating, because crystallization and recrystallization may occur during the process.[44] The advantages of DF may be related to the fact that it requires one layer of medium less compared with other carrier systems. This results in more rapid cooling and heating during freezing and recovery, respectively. Additionally, the dilution of the cryoprotectant after heating is significantly accelerated using this method. In this context, previous studies[45] on sperm vitrification have shown that a higher heating rate (higher temperature and lower exposure time) yields superior sperm motility and plasma membrane integrity than a lower heating rate. However, the short duration of high-temperature rewarming (as low as 5 s) may harm the sperm. Accurate timing of the procedure is also a challenge.[2] Because the normal body temperature in humans is 37°C, thawing and vitrifying frozen sperm at this temperature has shown favorable outcomes in numerous studies.[1,5,46] A rewarming temperature of 37°C was therefore used in this study and yielded favorable outcomes.

Sperm motility, membrane integrity, acrosome integrity, and MMP are commonly used indicators to assess the effects of sperm cryopreservation methods on fertilization ability and embryo quality.[5,47–50] Our findings demonstrated that major sperm motility-related parameters (TM, PM, VCL, VSL and VAP), PMI, MMP and AIS in frozen-thawed samples exhibited reductions compared with fresh sperm. However, no significant difference was observed in sperm motility-related parameters (TM, PM, LIN and BCF), PMI, DFI, MMP and AIS between DF and CF methods.

SEM is more sensitive to detect surface changes of sperm, including head loosening, acrosome alterations, and breakage, bending, or coiling of tails.[49] We found no differences between CF and DF methods in terms of damage to the sperm head, membrane, or tail. TEM revealed that both methods increased dissociation of the plasma membrane, mitochondrial vacuolization, and conspicuous mitochondrial swelling.

The DF method demonstrated similar effects on sperm motility and injury to the CF method. In this regard, there appears to be little difference in their application to *in vitro* fertilization (IVF). Because both methods caused similar damage to sperm, we investigated differences in embryonic development and epigenetics-related miRNA expression by miRNA seq.

Sperm miRNA-seq requires highly purified RNA. In addition to sperm, semen contains somatic cells, including leukocytes and

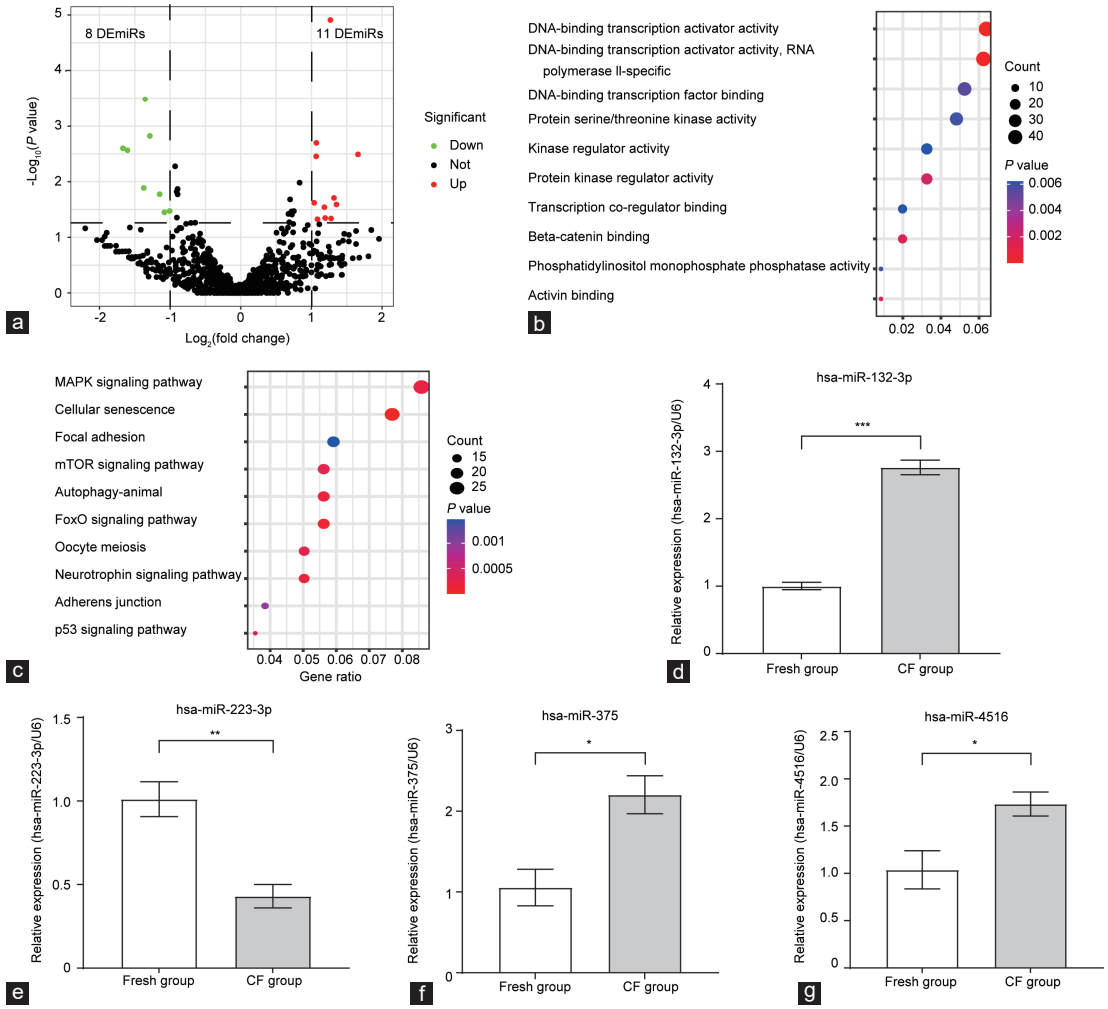

Figure 3: Analysis of differentially expressed miRNAs (DEmiRs) in human sperm. (**a**) Volcano plot of DEmiRs between fresh and frozen–thawed sperm obtained by the CF method. (**b**) Gene Ontology (GO) enrichment analysis of DEmiRs. (**c**) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEmiRs. Expression levels of miRNAs in human sperm were measured by qRT-PCR of (**d**) hsa-miR-132-3p, (**e**) hsa-miR-223-3p, (**f**) hsa-miR-375, and (**g**) hsa-miR-4516. Data are presented as mean ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CF: conventional freezing; qRT-PCR: quantitative real-time polymerase chain reaction.

epithelial cells. Because each sperm contains minimal amounts of RNA (femtograms), and somatic cells have considerably larger quantities (picograms),[51] it is essential that somatic cells are removed to prevent contamination of sperm transcripts.[52,53] Studies have used various methods to purify sperm and remove somatic or other cells. These methods include density gradient centrifugation, hypo-osmotic solution treatment,[54,55] sodium dodecyl sulfate, and Triton X-100.[56,57] However, these agents increase membrane permeability, which may affect transcriptome comparison results and introduce experimental uncertainty. To improve the accuracy of transcriptome alignment and reduce experimental uncertainty, we employed a density gradient centrifugation method (40% and 80%), followed by an additional purification step using a 40% solution for centrifugation. Both the centrifuge tube and pipette were replaced at each step to minimize contamination from somatic cells. We found that the proportion of somatic cells in the sperm suspension was <0.05%. cDNA-PCR analysis of various somatic cell-associated RNAs yielded negative results (**Supplementary Figure 1** and **Supplementary Table 4**).

Because of the low RNA content in sperm (approximately 0.015 pg RNA per sperm), the volume of ejaculate obtained from a single individual was inadequate for RNA-seq.[53,58] Therefore, we mixed samples of human sperm for analysis.

GO enrichment and KEGG pathway analyses of the target genes of DEmiRs in the three groups compared in pairs showed no functions or pathways related directly to embryonic development.

We found eight common DEmiRs (co-DEmiRs) between the two freezing methods and fresh sperm. These common DEmiRs showed no significant difference in expression. This may be attributed to the similarities in structural and microenvironmental damage caused by the freezing methods and partly reflects the common mechanisms of the different freezing methods.

Among these co-DEmiRs, a target gene of miR-1273g-3p, E-cadherin (*CDH1*), plays an important role in preimplantation embryo densification[59], and kirsten rat sarcoma viral oncogene (*K-ras*)[60], insulin like growth factor 1 (*IGF1*)[61], cyclin-dependent kinase 19 (*CDK19*)[62], and cAMP-regulated phosphoprotein 19 (*Arpp19*)[63] genes are related to early embryonic development. These processes are associated with the proliferation and differentiation of embryonic cells. Among the target genes of miR-4516, sex determining region Y box 10 (*SOX10*) may affect blastomere differentiation during cleavage,[64] and

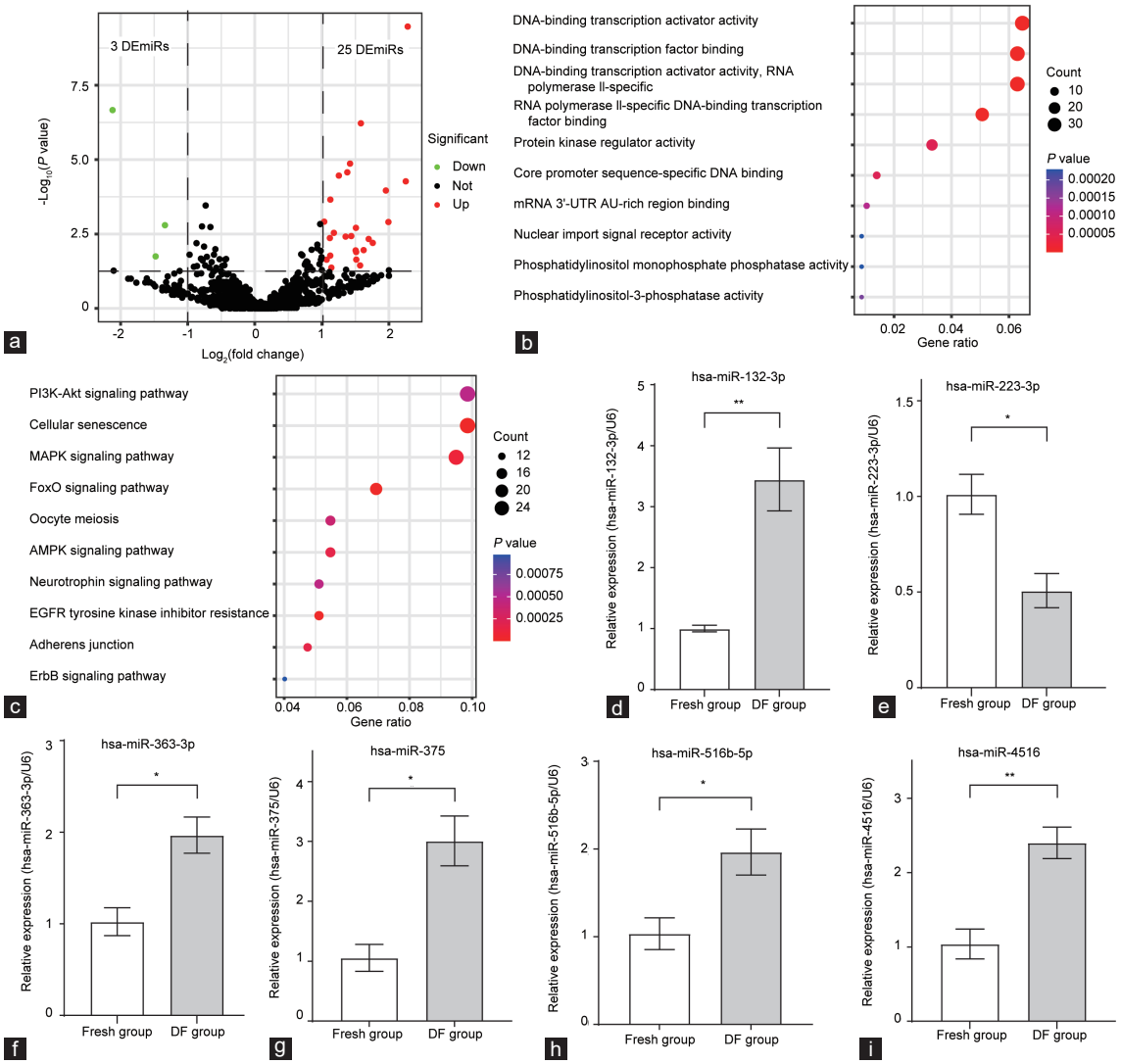

Figure 4: Analysis of differentially expressed miRNAs (DEmiRs) in human sperm. (**a**) Volcano plot of DEmiRs between human fresh and frozen–thawed sperm obtained by the DF method. (**b**) Gene Ontology (GO) enrichment analysis of DEmiRs. (**c**) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEmiRs. Expression levels of miRNAs in human sperm measured by qRT-PCR of (**d**) hsa-miR-132-3p, (**e**) hsa-miR-223-3p, (**f**) hsa-miR-363-3p, (**g**) hsa-miR-375, (**h**) hsa-miR-516b-5p, and (**i**) hsa-miR-4516. Data are presented as mean ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$. DF: droplet vitrification freezing; qRT-PCR: quantitative real-time polymerase chain reaction.

cyclin-dependent kinase 2-associated protein 2 (*CDK2AP2*) acts as a novel regulator of embryonic stem cell self-renewal.[65] Freezing induces high expression of miR-1273g-3p and miR-4516 in sperm, which may inhibit the expression of the above-mentioned genes and affect the early development of embryos on fertilization.

A study on sperm miR-375 has shown that its upregulation might play a positive regulatory role during the embryonic cleavage stage and, therefore, affect embryo quality in patients who undergo IVF.[66] A study that assessed the expression of small noncoding RNAs in sperm compared groups with high- and low-quality embryo rates during IVF.[67] Evaluation of the DEmiRs showed that the low-quality embryo rate group demonstrated downregulation of miR-132-3p. This may serve as a distinguishing factor between the two groups. These findings suggest that sperm small RNA expression is potentially associated with embryo quality, even in cases where conventional semen parameters appear normal. In this study, the freeze–thaw cycle upregulated sperm miR-375 and miR-132-3p expression. Notably, these alterations in miRNA expression might play a positive regulatory role during embryonic development. This suggests that, although sperm are damaged by freezing, early embryonic development may be affected by a combination of various factors.

Sperm miRNAs are also associated with patriarchal epigenetic differences in expression. MiRNAs in sperm reduce target mRNA levels in the zygote and therefore indirectly alter the epigenetic status of a developing embryo.[68] In addition to their association with early embryo quality, sperm miR-375 and miR-132-3p expression is also involved in paternal epigenetics. Gapp *et al.*[69] established a mouse model of early traumatic stress. They found upregulated expression of miR-375-3p in the sperm of traumatized mice and traumatized offspring mice. Injecting the zygote with sperm RNA from these traumatized mice led to the recurrence of related behaviors and metabolic phenotypes. Additionally, miR-132-3p, miR-132-5p, miR-212-3p, and miR-212-5p expression was upregulated in the sperm and hippocampi of the mice after 10 weeks of physical exercise and cognitive training. This also significantly enhanced long-term memory and cognitive function in the hippocampi of their

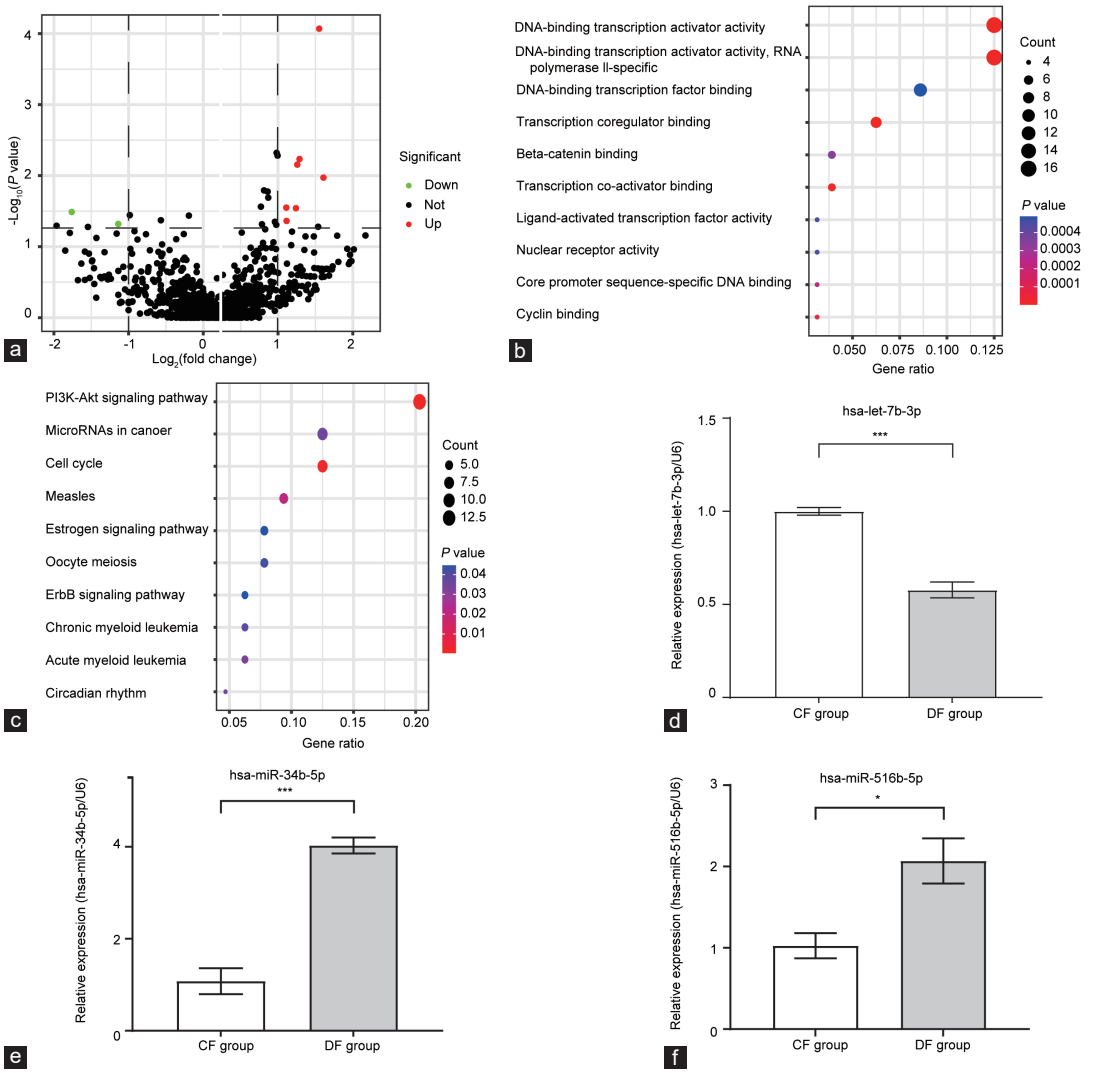

Figure 5: Analysis of differentially expressed miRNAs (DEmiRs) in human frozen–thawed sperm. (**a**) Volcano plot of DEmiRs in frozen–thawed sperm obtained by CF and DF methods. (**b**) Gene Ontology (GO) enrichment analysis of DEmiRs. (**c**) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEmiRs. Expression levels of miRNAs in frozen–thawed sperm were measured by qRT-PCR of (**d**) hsa-let-7b-3p, (**e**) hsa-miR-34b-5p, and (**f**) hsa-miR-516b-5p. Data are presented as mean ± standard error of the mean. $^{*}P < 0.05$, $^{***}P < 0.001$. CF: conventional freezing; DF: droplet vitrification freezing; qRT-PCR: quantitative real-time polymerase chain reaction.

offspring.[70] This effect was blocked by coinjection of a selective inhibitor of miR-212/132 into oocytes. This suggested that the phenotype was induced by changes in the sperm RNA composition in the corresponding father. MiR-212/132 was identified as a factor involved in this process.

In this context, environmental stress induces changes in hsa-miR-181a-5p expression in human sperm. This knowledge can be used to study the potential effects of environmental factors on germ cell programming and offspring development.[71]

Among the eight common DEmiRs identified in this study, miR-92b-3p is also associated with embryo implantation. It promotes the proliferation and migration of trophoblast cells (PTr2 line) through its target gene, phosphofructokinase-M (*PFKM*), and therefore regulates embryo implantation.[72]

Among DEmiRs that were not co-DEmiRs between CF and fresh sperm samples, miR-542-5p is associated with embryo implantation. It inhibits the proliferation and migration of trophoblastic cells in an embryo, induces apoptosis of trophoblastic cells, and blocks embryo implantation.[73] In this context, oligoasthenospermia is associated with poor embryonic development.[74,75] Among DEmiRs that were not co-DEmiRs between fresh and DF sperm, miR-132-5p (downregulated expression) is associated with teratospermia and oligozoospermia.[76] In this context, downregulation of sperm miR-1973 is associated with oligospermia.[77] Among DEmiRs between sperm frozen by CF and DF methods, miR-34b-5p (expressed in spermatoplasm) is associated with oligospermia.[78]

The number of DEmiRs was slightly higher in DF/fresh sperm than that in CF/fresh sperm. However, few specific DEmiRs affected early embryonic development and intergenerational inheritance (except for the co-DEmiRs mentioned above), indicating minimal differences between these two freezing methods in terms of their effect on miRNAs associated with early embryonic development and intergenerational inheritance.

The mechanisms underlying miRNA alterations in mature sperm remain elusive. However, changes in miRNA expression following sperm cryopreservation may be attributed to diminished levels owing to freezing-induced microenvironmental damage. They may also be attributed to the regulatory effect of circular RNA, which acts as a miRNA sponge.[79,80]

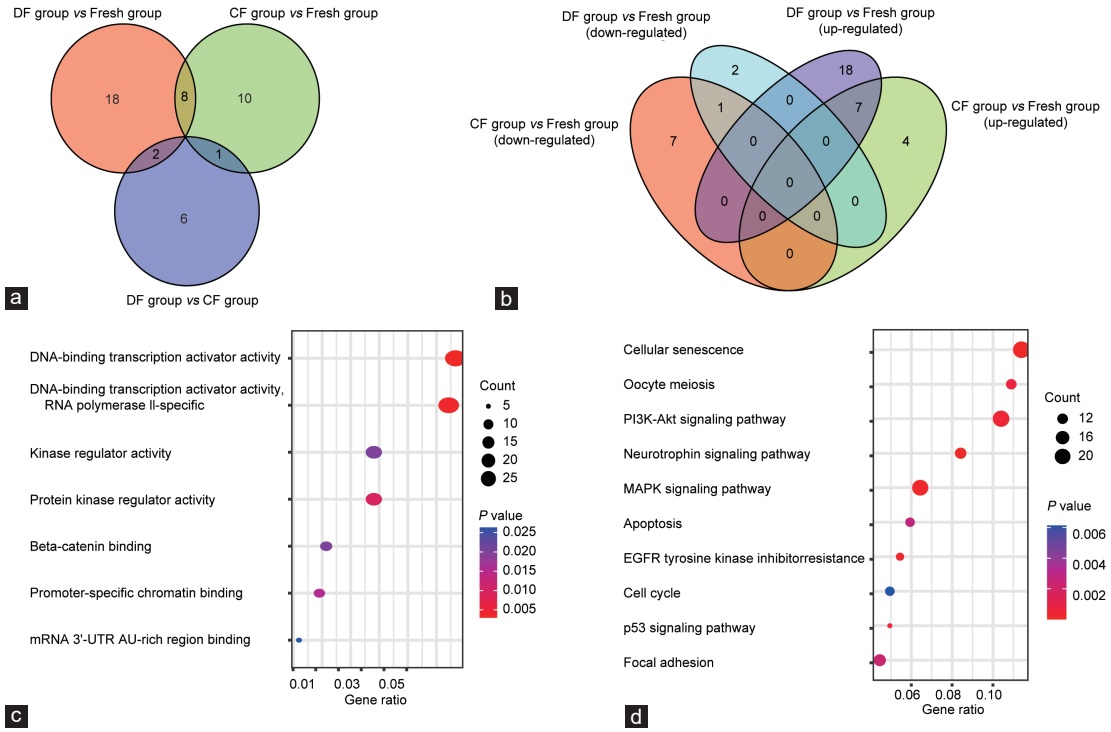

Figure 6: Analysis of eight common miRNAs in the intersection of the two groups of DEmiRs (CF sperm *vs* fresh sperm, and DF sperm *vs* fresh sperm). (**a**) Venn diagram of common DEmiRs (three sets). (**b**) Venn diagram of common DEmiRs (four sets). (**c**) Gene Ontology (GO) enrichment analysis of common DEmiRs. (**d**) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of common DEmiRs. CF: conventional freezing; DF: droplet vitrification freezing.

Circular RNAs are abundant in human sperm,[81] and exposure to stress caused by a freeze–thaw cycle may enhance miRNA release. Our findings suggested that cryopreservation induced modifications in the miRNA expression profiles of spermatozoa. This may cause sperm to carry altered miRNAs to oocytes and subsequently affect embryonic development and paternal epigenetic inheritance by altering mRNA expression.

Notably, it is possible that cryopreservation-related alterations in sperm miRNA expression were conducive to embryonic development. Embryo cryopreservation may ameliorate certain epigenetic abnormalities resulting from IVF or intracytoplasmic sperm injection.[82] An improved success rate of embryo transfer during the thawing cycle may be attributed to the stress response triggered by freezing and subsequent resuscitation.[83] In view of the multiple variables, precise prediction of the effect of sperm cryopreservation on embryo development and offspring health remains a challenge.

CONCLUSION

The differences between CF using the osmotic protectant glycerin and DF using a nonpermeable protectant were minimal in terms of miRNA expression related to embryo development and epigenetics and classical semen parameters (sperm density, morphology, motility, and DFI). This finding supports the safety of glycerin as a cryoprotectant and suggests that DF using a nonpermeable protectant can be employed as a clinical approach for freezing sperm, particularly in cases of intracytoplasmic sperm injection. The latter offers certain advantages, including rapid one-time freezing of multiple sperm balls, thawing of individual balls, and lower space requirements for freezing.

Because sperm freezing is widely employed in ART for treating human infertility, miRNA transcript detection has emerged as a noteworthy strategy to assess freezing methods in addition to conventional semen parameters such as sperm density, morphology, motility, and DNA fragmentation rate. This study compared the differences in miRNA expression profiles before and after freezing among sperm samples frozen using CF or DF methods, which is more meaningful in the case of similar cryopreservation effects. The identification of *in vitro* influences on sperm epigenetics-associated genomes represents an area of particular interest in contemporary research on ART, where miRNAs may assume an increasingly prominent role.

AUTHOR CONTRIBUTIONS

LXZ, XC, and HSG participated in the study design and manuscript preparation. LXZ conducted experiments and analyzed data. JM and YDZ participated in sample collection and experiments. RFG supervised sample collection. GYM participated in data analysis and figure preparation. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant No. 81771586).

Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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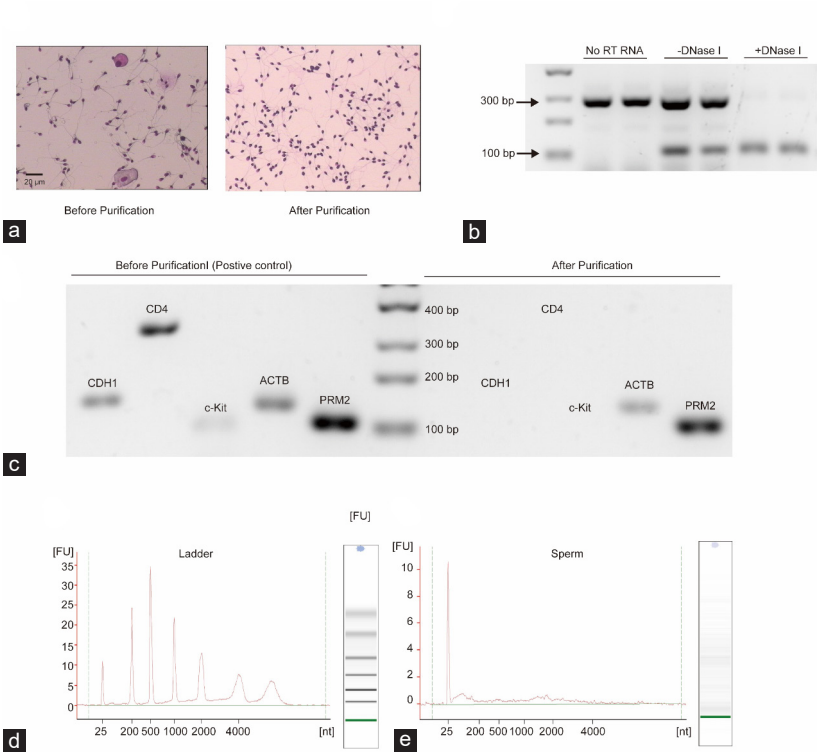

Supplementary Figure 1: Isolation and size distribution of highly purified sperm RNA. (**a**) Morphology of collected sperm under ordinary optical microscope (diff-quik staining). (**b**) The 226-bp PCR product of PRM2 indicated any DNA contamination, while a single 104-bp PCR product of PRM2 was from sperm RNA. (**c**) Biomarkers of leukocytes (CD4), androgone (c-KIT), and epithelial cells (CDH1) were unable to be amplified from RNA extracted from sperm samples, while the positive sperm (PRM2) and control markers (ACTB) were detected. Electrophoretic size distribution of RNAs in (**d**) ladder and (**e**) human mature sperm analyzed by an Agilent Bioanalyzer.

Supplementary Table 1: Characteristics of participants (mean±standard error of the mean)

Age	*Sperm total number ($\times 10^6$)*	*Motility (%)*	*Progressive motility (%)*	*Sperm morphology normal rate (%)*
31.5±0.5	231.3±26.9	52.9±1.9	45.1±2.0	4.2±0.1

Supplementary Table 2: Primer sequences for microRNA-cDNA and quantitative reverse transcription polymerase chain reaction

Genes	*Primer sequences*
hsa-miR-132-3p-F	AGCCAGCGTAACAGTCTACAGC
hsa-miR-132-3p-RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGACCA
hsa-miR-223-3p-F	GCGCGTGTCAGTTTGTCAAAT
hsa-miR-223-3p-RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGGGT
hsa-miR-375-3p-F	AACCGGTTTGTTCGTTCGGCT
hsa-miR-375-3p-RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACGC
hsa-miR-4516-F	ATATTATGGGAGAAGGGT
hsa-miR-4516-RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCCCG
hsa-miR-363-3p-F	CAGTAGCGAATTGCACGGTATCC
hsa-miR-363-3p-RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACAGA
hsa-miR-516b-5p-F	ATGCGCGCATCTGGAGGTAAG
hsa-miR-516b-5p-RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAGTG
hsa-let-7b-3p-F	GCGCGCTATACAACCTACTGC
hsa-let-7b-3p-RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGAAG
hsa-miR-34b-5p-F	AAGCGCCTTAGGCAGTGTCATT
hsa-miR-34b-5p-RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAATCA
miRNA-reverse	ATCCAGTGCAGGGTCCGAGG
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT

F: forward; RT: reverse transcription; miRNA: microRNA

Supplementary Table 3: Literature list concerning differentially expressed microRNAs related to early embryonic development and paternal epigenetics

DEmiRs	*Change relative to fresh sperm*	*Embryonic development-related research*	*Epigenetic-related research*
Common DEmiRs between the two freezing methods and fresh sperm			
hsa-miR-1273g-3p	Up	*CDH1, Kras, IGF1, CDK19*, and *ARPP19* are related to early embryonic development[59–63]	
hsa-miR-132-3p	Up	MiR-132-3p downregulated in sperm is related to the low-quality embryo rate[67]	MiR132-3p, miR132-5p, miR212-3p, and miR212-5p are upregulated in sperm and hippocampus of mice, which significantly enhances long-term memory and cognitive functions in their offspring hippocampus[70]
hsa-miR-181a-5p	Up		Expression of hsa-miR-181a-5p in sperm is linked to the environment and mental stress[71]
hsa-miR-212-5p	Up		Same as above, hsa-miR-132-3p[70]
hsa-miR-375	Up	Sperm miR-375 plays a positive regulatory role during the embryonic cleavage stage[66]	MiR-375-3p is upregulated in mouse sperm with traumatic stress, mediating paternal epigenetics[69]
hsa-miR-4516	Up	*SOX10* and *CDK2AP2* are related to early embryonic development[64,65]	
hsa-miR-92b-3p	Up	Hsa-miR-92b-3p promotes the proliferation and migration of trophoblast cells, thereby regulating embryo implantation[72]	
DEmiRs between conventional freezing methods and fresh sperm			
miR-542-5p	Up	MiR-542-5p suppresses the proliferation and migration of trophoblastic cells, induces apoptosis of trophoblastic cells, and blocks embryo implantation[73]	
DEmiRs between droplet vitrification freezing and fresh sperm			
miR-132-5P	Up	Downregulation of miR-132-5P expression in sperm is associated with teratospermia and oligozoospermic[76]	Same as above, hsa-miR-132-3p[70]
miR-1973	Up	Downregulation of miR-1973 in sperm is associated with oligospermia[77]	

DEmiRs: differentially expressed microRNAs

Supplementary Table 4: Sperm purification effect-reverse transcription-polymerase chain reaction primer sequence list

Genes	*Primer sequences*	*Annealing temperature (°C)*	*Size of the products (bp)*
PRM2-F	5'-GGATCCACAGGCGGCAGCATCGCT-3'	60	104/226
PRM2-R	5'-GCATGTTCTCTTCCTGGTTCTGCA-3'		
C-KIT-F	5'-TACAACGATGTGGGCAAGA-3'	55	111
C-KIT-R	5'-TACGAAACCAATCAGCAAAG-3'		
CD4-F	5'-TTCAACTGTAAAGGCGAGTG -3'	57	322
CD4-R	5'-CGGATTGACTGCCAACTCT -3'		
CDH1-F	5'-CACCTTCCATGACAGACCC-3'	60	156
CDH1-R	5'-AACGCATTGCCACATACAC-3'		
ACTB-F	5'-TAGTTGCGTTACACCCTTTC-3'	56	153
ACTB-R	5'-GTCACCTTCACCGTTCCAG-3'		

PRM2: protamine 2; *C-KIT*: KIT proto-oncogene receptor tyrosine kinase; *CD4*: cluster of differentiation 4; *CDH1*: E-cadherin; *ACTB*: beta-actin; F: forward; R: reverse