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Spermatozoa cryopreservation alters pronuclear formation and zygotic DNA demethylation in mice

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ABSTRACT

This study was conducted to investigate the effects of spermatozoa cryopreservation on DNA demethylation in mouse zygotes. Global methylation was studied in zygotes fertilized with cryopreserved sperm by immunostaining, and relative transcript abundance of *Tet3*, a key gene responsible for zygotic DNA demethylation, was examined by real-time quantitative polymerase chain reaction. Fresh sperm group served as control. Results indicated spermatozoa cryopreservation decreased fertilization rate (68.2% vs. 86.9%; $P < 0.01$) and delayed pronuclear formation ($P < 0.05$), compared with the control group. The percentages of embryos developed to cleavage and blastocyst stages in the freezing group (52.9% and 66.8%, respectively) were lower ($P < 0.01$ and $P < 0.05$, respectively) than those of the control group (83.4% and 81.1%, respectively). Furthermore, embryos obtained from cryopreserved sperm had higher relative methylation levels ($P < 0.05$) and less *Tet3* mRNA concentrations ($P < 0.01$) in advanced pronuclear stages. Hence, we reported that spermatozoa cryopreservation disturbed the Tet3-mediated DNA demethylation progression in the zygotic paternal genome, which could be detrimental to the development of early mouse embryos, and most of the differences observed might be explained by delayed fertilization when using cryopreserved sperm.

1. Introduction

The first successful spermatozoa cryopreservation was reported in 1949 [1]. Since then, this technology has been used in numerous mammalian species including humans [2–4]. Despite recent considerable progress, spermatozoa cryopreservation is still far from perfection [5,6]. For example, although the viability and fertility of frozen/thawed sperm are acceptable, embryo implantation and normal fetus rates are significantly decreased [5,7,8]. Cryopreservation damages developmentally important organelles including plasma membrane [9], acrosome [10], and mitochondria [11], which may cause problems in embryonic development and fetal growth.

The sperm delivers genetic and epigenetic information to the oocyte. External factors can have limited effects on the nuclear structure and integrity, owing to the special chromatin packaging in spermatozoa [12]. No obvious defect in the DNA integrity of frozen/thawed sperm has been detected, and only slight structural damages can be found [7,8]. Nevertheless, structural damages can be restored through DNA repair mechanisms after fertilization, ensuring safe delivery of the paternal genetic information [13,14]. In addition to DNA sequences, epigenetic modifications in the genome are also crucial for embryonic development in mammals [15]. DNA methylation, one of the major epigenetic modifications, plays a crucial role in gene expression regulation, zygotic genome activation, and embryonic

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development [16]. DNA methylation is sensitive to environmental insults, and various forms of assisted reproductive treatments can alter the methylome and impact the embryonic development [17]. It is well established that cryopreservation can change the global genomic methylation and specific gene expression in oocytes and embryos [18–21]. However, little is known about the effect of spermatozoa cryopreservation on DNA methylation in zygotes.

The DNA methylation pattern changes dramatically during preimplantation development. To regain developmental totipotency, the zygotic genome must undergo DNA demethylation to erase the epigenetic memory inherited from the gametes. Usually, DNA demethylation is achieved passively in a replication-dependent manner through cell divisions. In the zygote, however, active DNA demethylation is observed, and this process has important implications for embryonic development [16,22]. In the paternal pronucleus (PN), 5-methylcytosine (5mC) is converted into 5-hydroxymethylcytosine (5hmC) by oxidation [23–25]. This reaction can be catalyzed by a family of dioxygenases, the ten-eleven translocation (TET) proteins [26,27]. Recently, works have established an essential role of TET3 in mouse zygotes. TET3 is specifically enriched in the paternal PN of the zygote, and its ablation by RNA knockdown or genetic deletion abolishes the generation of 5hmC to preclude complete DNA demethylation [24,25].

In the present study, we investigated the 5mC and 5hmC distribution patterns and the *Tet3* mRNA abundances in mouse zygotes fertilized with cryopreserved sperm to determine the potential changes in zygotic global genomic methylation due to spermatozoa cryopreservation.

2. Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich. All animals were maintained and handled in accordance with the requirements of the Institutional Animal Care and Use Committee of the China Agricultural University.

2.1. Oocyte collection

Oocytes were collected from 8- to 12-week-old Kunming female mice (Academy of Military Medical Sciences, Beijing, China) as described previously [28]. Briefly, females were induced to superovulate with 10 IU eCG (Ningbo Hormone Products Company, Ningbo, China) followed by 10 IU hCG (Ningbo Hormone Products Company) 48 hours later. Cumulus–oocyte complexes were collected from oviducts at 14 hours after hCG treatment and recovered in M2 medium supplemented with 3 mg/mL BSA. Cumulus cells were dispersed with 0.1% hyaluronidase, then the cumulus-free oocytes were rinsed thoroughly and placed in drops of human tubal fluid medium (HTF; Millipore, MA, USA), which were covered with mineral oil for up to 1 hour in an incubator (at 37 °C in an atmosphere of 5% CO_2 and at maximum humidity) before IVF.

2.2. Spermatozoa preparation, freezing, and thawing

Cauda epididymides were excised from 10- to 15-week-old Kunming male mice, and spermatozoa were collected by squeezing them into drops of cryopreservative medium (R18S3, 18% raffinose and 3% skim milk [Becton Dickinson and Company, NJ, USA] in distilled water). To allow even dispersion, spermatozoa were incubated for 10 minutes at 37 °C.

Sperm freezing and thawing procedures were carried out as described previously with minor modifications [29]. The sperm suspension was divided into 10-μL aliquots and loaded into 0.25-mL plastic straws (IMV Technologies, L'Aigle, France). The straws were heat sealed, placed in liquid nitrogen vapor for 10 minutes, and then immediately plunged into liquid nitrogen before being stored for at least 1 week.

Cryopreserved sperm were thawed in a water bath at 37 °C for 5 minutes. Once thawed, spermatozoa were transferred into HTF medium and incubated for 30 minutes before insemination.

2.3. IVF and embryo culture

The IVF procedures using fresh or frozen/thawed sperm were performed as described previously [29,30]. Briefly, spermatozoa were isolated from the cauda epididymides and capacitated by incubation for 1.5 hours in pregassed HTF medium before introducing to HTF-containing mature oocytes. Meanwhile, the frozen/thawed sperm were used for insemination in the same way.

At 4 hours postfertilization (hpf), the eggs were removed from the fertilization drop, washed in KSOM medium (Millipore), transferred to drops of KSOM medium covered with mineral oil, and then cultured in an incubator.

Samples were collected at several time points corresponding to embryo developmental period. The zygotes were recovered individually at 4, 6, 8, and 10 hpf for pronuclear morphometric analysis and immunostaining. The oocytes, zygotes of PN3 to PN4 stages or 2-cell embryos were harvested at 0, 8, or 24 hpf for real-time quantitative polymerase chain reaction (RT-qPCR). The proportions of the fertilized zygotes, 2-cell embryos, and blastocysts were assessed at 10, 24, and 96 hpf, respectively.

2.4. Pronuclear morphometric analysis

The pronuclear morphologies of cells were examined on an inverted IX70 microscope (Olympus, Tokyo, Japan) immediately after staining with propidium iodide. The classification of zygotic PN1 to PN5 stages was performed according to previous studies, in which the pronuclear morphology and postfertilization timing were taken into consideration [24,31].

2.5. Immunostaining

The immunostaining analysis was carried out as described with modifications [32]. Immunostaining reagents for washing, blocking, antibody dilution, and mounting were purchased from Beyotime (Shanghai, China).

After brief washing, the embryos were fixed in 3.7% paraformaldehyde for 40 minutes and permeabilized with 0.5% Triton X-100 for 10 minutes. Subsequently, the cells were denatured with 4N HCl for 10 minutes and