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Effects of Cooling, Cryopreservation and Heating on Sperm Proteins, Nuclear DNA, and Fertilization Capability in Mouse

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ABSTRACT In the present study, we used confocal microscopy and electrophoresis to study the effects of heating to 5 or 100°C or cooling to 4°C or −196°C on the stability of sperm proteins and DNA. We used intracytoplasmic sperm injection (ICSI) to determine the fertilizing capability of treated spermatoza. It was shown that sperm cryopreservation at −196°C or cooling at 4°C altered neither protein and DNA profiles nor the sperm fertilization capability, while the protein and DNA profiles of sperm heated at 100°C were irreversibly degraded and inactivated. The proteins of sperm were severely damaged while the nuclear DNA still maintained its integrity when heated to 58°C. Observation by laser confocal microscopy showed that after being heated to 58°C and 100°C, the nuclear of mouse sperm lost their ability to activate oocytes and they could not transform to male pronuclei though the membrane of some sperm could degrade and induce the formation of sperm asters in ICSI oocytes. The results indicate that the use of 58°C heating only causes the degradation of sperm proteins, while the 100°C heating elicits the irreversible degradation of both sperm proteins and nuclear DNA, and the damage of sperm proteins is primarily responsible for the observed decrease in sperm fertilizing capability. *Mol. Reprod. Dev. 72: 129–134, 2005.*

Key Words: sperm protein; nuclear DNA; cryopreservation; cooling; heating; fertilizing capability

INTRODUCTION

The mouse is a primary research animal in mammalian genetics and represents an excellent model for the study of embryonic development and human genetic diseases. The development of mouse sperm cryopreservation procedures (Tada et al., 1990; Nakagata and Takeshima, 1993; Nakagata, 1995, 2000; Songsasen et al., 1997; Thornton et al., 1999; Sztein et al., 2000) that allow a convenient, cost-effective storage, and availability of a large number of gametes from single males, is of particular importance for the maintenance of the large variety of unique inbred, mutant, recombinant, and rapidly increasing number of transgenic stocks. Live offspring were obtained after intracytoplasmic sperm injection (ICSI) with isolated sperm heads (Kuretake et al., 1996), spermatozoa frozen without cryoprotectant (Wakayama et al., 1998), as well as freeze-dried spermatozoa (Wakayama and Yanagimachi, 1998). Recently, Live pups were also obtained after ICSI with heated spermatozoa (Cozzi et al., 2001).

Regardless of the previous use of complete spermatozoa or individual sperm heads, and of the cryopreserved, air-dried or heated spermatozoa, to what extent and how the temperature affects sperm fertilizing capability is still an attractive topic and needs further clarification. In the present study, we used laser confocal microscopy, native polyacrylamide gel electrophoresis (native-PAGE) of sperm proteins, and agarose electrophoresis of nuclear DNA to assess the ability of mouse spermatozoa exposed to −196, 4, 58, and 100°C to support fertilization. We observed that, as did fresh spermatozoa, spermatozoa that had been cooled to 4°C or cryopreserved at −196°C were able to activate and fertilize metaphase II oocytes, whereas those that had been heated to 58 or to 100°C evidently lost their fertilizing capability. However, 58 and 100°C heating damages to proteins and DNA were not entirely identical.

MATERIALS AND METHODS

Unless otherwise noted, chemicals used in this research were obtained from Sigma (St. Louis, MO). All animals were maintained in accordance with the Animal Experiment Standard of State Key Lab of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences.

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Media

CZB medium (Chatot et al., 1989) supplemented with 5.55 mM D-glucose and 5 mg/ml BSA (fraction V) was used for oocyte and embryo culture. Modified CZB medium (CZB-HEPES) with 20 mM HEPES and reduced $NaHCO_3$ (5 M), and 0.1 mg/ml polyvinyl alcohol (30–70 kDa) was used for sperm, oocyte, and embryo collection, subsequent treatments, and ICSI. All cultures were performed in CZB at 37°C under 5% CO_2 in air. Isotonic NaCl solution supplemented with polyvinylpyrrolidone (PVP) was used to suspend spermatozoa prior to ICSI.

Collection of Oocytes

Oocytes for microinjection were recovered from superovulated Kunming female mice (3–12-week old). Females were injected twice 48 hr apart with 7.5 IU pregnant mare serum gonadotropin (PMSG, Tianjin Hormone Factory, China) and 7.5 IU human chorionic gonadotropin (hCG, Tianjin Hormone Factory, China). Oocytes were collected from oviducts 13–14 hr after hCG injection and freed from the cumulus by a 3-min treatment with 0.1% hyaluronidase in CZB-HEPES. Cumulus-free oocytes were rinsed several times in CZB-HEPES and then incubated in CZB at 37°C under 5% CO_2 in air for up to 2 hr before microinjection.

Preparation of Spermatozoa

Spermatozoa were collected from caudae epididymides of outbred Kunming male mouse after squeezing them into a 500-μl drop of CZB-HEPES under mineral oil. Spermatozoa were allowed to disperse at 37°C for 30 min. The sperm samples from at least five mice were totally collected into a 10-ml centrifuge tube, were added to 5-ml CZB-HEPES medium, and were washed three times at 600*g* for 5 min in the same medium. Then, the suspensions were concentrated to 1.5 ml and were finally divided 200-μl aliquot into five 1.5-ml Eppendorf tubes. For cooling at 4°C, the Eppendorf tube including 200-μl sperm sample was cooled in a 4°C refrigerator for at least 30 min. For cryopreserved at −196°C, the Eppendorf tube including same volume sperm sample was first added to about 200-μl liquid nitrogen was sealed and then was sunk in liquid nitrogen tank for more than 30 min. For heating treatment, the samples were heated at 58°C for more than 30 min or heated at 100°C for more than 30 min in water bath. Finally, all treated samples were kept on ice until the sperm protein, and nuclear DNA were extracted or the fertilizing capability was assessed. Above all, treatments were repeated at least three times.

Preparation and Native-PAGE of Spermatozoa Protein Sample

Fresh, heated, cooled, and cryopreserved spermatozoa samples were, respectively, added to 1-ml CZB-HEPES without BSA and were centrifuged at 800*g* for 10 min at 4°C. The pellets were washed twice in 500-μl CZB-HEPES without BSA at 800*g* for 10 min at 4°C, and finally resuspensded in the same solution. The spermatozoa samples were frozen-thawed at least three times in −196°C liquid nitrogen, each time at least 1 hr, and were centrifuged at 12,000*g* for 5 min at 4°C. The supernatants were then recovered, divided in aliquots, and stored at −70°C until the electrophoresis analysis was carried out.

Native electrophoresis analysis (Gallagher, 1995) was performed in polyacrylamide gels using the Hofer miniVE vertical electrophoresis System (Amersham Biosciences, Piscataway, NJ). The running conditions were 15 mA stacking gel for 45 min and 30 mA separating gel for 5 hr. Once the bromophenol blue had reached the anode, the gels were fixed and stained by Coomassie brilliant blue R-250.

Extraction and Agarose Electrophoresis of Spermatozoa Nuclear DNA

Fresh, heated, cooled, and cryopreserved spermatozoa samples were added to 1-ml CZB-HEPES and were centrifuged at 800*g* for 10 min. The pellets were washed twice in 300-μl sperm lysis buffer containing 10-mM Tris-HCl (pH 8.0), 0.5M EDTA, 100g/l SDS, 0.1% proteinase K and 40-mM dithiothreitol, and incubated in a water bath for 2 hr at 58°C. The solutions cooled to room temperature were added to an equal volume of phenol/chloroform/isopropanol (25:24:1) and gently mixed to form two phases, then the two phases were separated by centrifugation at 5,000*g* for 15 min at room temperature. The upper aqueous phase was transferred into another centrifuge tube and was added to 1/10 volumes of sodium acetate buffer (3M NaAc, pH 6.0). Two volumes of ethanol were then added to the fresh tube at room temperature. The mixture was thoroughly mixed and centrifuged in the tube at 5,000*g* for 5 min at room temperature until the solution formed a precipitate. The precipitated sperm nuclear DNA was stored in 100-μl TE buffer (10 mM Tris-HCl, pH 7.2–7.6, 1 mM EDTA).

The samples of DNA (each 2 μl) with 0.20 volume of 6× gel-loading buffer were slowly loaded into the slots of the submerged 0.8% agarose gel with 0.5 μg/ml ethidium bromide. After migrated through the gel in due time, sperm nuclear DNA samples were examined under UV illumination.

ICSI Procedure and In Vitro Culture of Zygotes and Embryos

Injection of sperm heads into mouse oocytes was performed according to a previous method (Kimura and Yanagimachi, 1995) using a piezo-driven micropipette. Immediately before injection, 0.25 ml of cryopreserved, heated or fresh sperm suspension was mixed with 5 ml of 12% PVP-saline medium. A single spermatozoon was drawn tail first, and a few piezo-pulses were applied in the neck region to separate the head from the flagella. The injection of the sperm head into the oocyte was performed immediately, then the zygotes were cultured in fresh CZB medium at 37°C in 5% CO_2/air.

Laser Confocal Microscopy of ICSI Oocytes

The ICSI oocytes and embryos were collected and fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4) for 40 min at room temperature (RT). Fixed samples were permeabilized by transferring into PBS supplemented with 0.1% (w/v) Triton-X100 and 0.3% BSA for 30–40 min (RT). After washing twice in PBS containing 0.01% Triton-X100, samples were incubated in block solution (PBS containing 1% BSA) for 1 hr at RT. The microtubules were localized by incubation for 1 hr at RT with a fluorescein isothiocyanate-labeled mouse monoclonal antibody against α-tubulin, which was diluted 1:100 in blocking solution. Nuclear status of samples was evaluated by staining with 10 μg/ml PI in PBS for 10 min. Following extensive washing, samples were mounted on slides with antifluorescence-fade medium (1,4-diazobicyclo[2,2,2]-octane, DABCO). Finally, the samples were observed under a Leica confocal laser-scanning microscope (TCS-4D, Bensheim, Germany).

Data Analysis

Results expressed as percentage in different groups were analyzed by Chi-square (χ^2). Statistical difference was considered when $P < 0.05$.

RESULTS

Protein Differences in Native-PAGE Among Fresh, Frozen-Thawed, and Heated Sperm

The profiles of native-PAGE indicated that the total proteins in mouse sperm cryopreserved at −196°C (lane 1), cooling at 4°C (lane 2), and fresh sperm (lane 3) were not degraded and maintain their inherent properties, while those in the sperm heated at 58°C (lane 4) and 100°C (lane 5) were degraded irreversibly (Fig. 1). The intensity of protein degradation in the sperm heated at 100°C was more evident than that in 58°C-treated sperm. In particular, 100°C heating not only resulted in much more small subunits than 58°C heating but also engendered the sediments of larger protein in the interspace between stacking and separating gel.

Nuclear DNA Differences Among Fresh, Frozen-Thawed, and Heated Sperm

The profiles of agarose electrophoresis indicated that the nuclear DNA of mouse fresh sperm (lane c), sperm cryopreserved at −196°C (lane a), cooling at 4°C (lane b), and heated at 58°C (lane d) were not denatured and maintained their inherent properties while those in the sperm heated at 100°C (lane e) were degraded irreversibly (Fig. 2), suggesting that the nuclear DNA in the former four groups of spermatozoa could be fully transmitted to zygotes except for the sperm heated at 100°C.

Fertilization and In Vitro Development of Oocytes After ICSI

Fertilization did not occur after microinjecting all sixty-nine 100°C-heated spermatozoa into oocytes, nor did the ninety-seven 58°C-heated spermatozoa among which only one oocyte was artificially activated. The fertilization rates among fresh sperm, −196°C-cryopreserved sperm and 4°C cooled sperm were not statistically different (89.9%, 94.7%, and 93.6%, $P > 0.05$) while those of 58- and 100°C-heated sperm were significantly lower ($P < 0.05$), only 1.3% and 0%, respectively, as shown in Table 1.

Observation of Oocyte Activation After ICSI by Laser Confocal Microscopy

In order to assess the activation of mouse oocytes injected with sperm exposed to different temperatures, the PI staining of DNA and immunochemical staining of α-tublin were examined by laser scanning confocal microscopy. Results showed that after sperm cryopreserved at −196°C and cooled at 4°C were injected into oocytes, oocytes were activated and fertilization events occurred normally (Fig. 3 A1–A3 and B1–B3). However, heating at 58 and 100°C could hamper the sperm heads from activating the oocytes as indicated by the failure of second meiosis resumption and spindle rotation. Although sperm head could decondensed and sperm asters could form, the injected sperm heads failed to form normal male pronucleus. Two normal spindles or

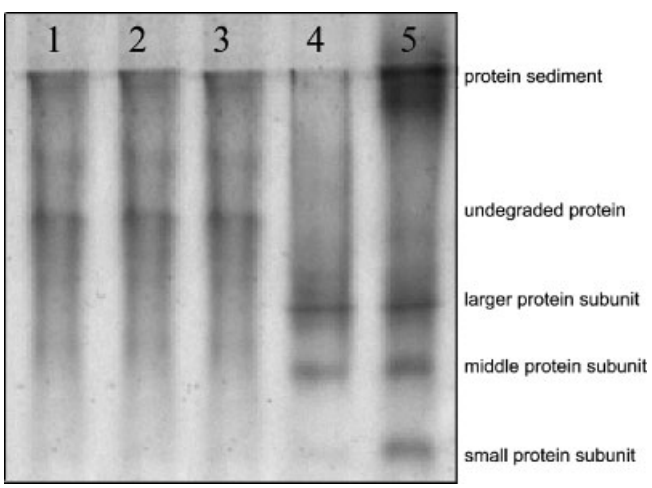

Fig. 1. Expression profile of mouse sperm protein in native-PAGE. From the first to the fifth lane (shown as number **1–5**): Protein extracts from mouse spermatozoon cryopreserved at −196°C, cooling at 4°C, fresh, heated at 58 and 100°C.

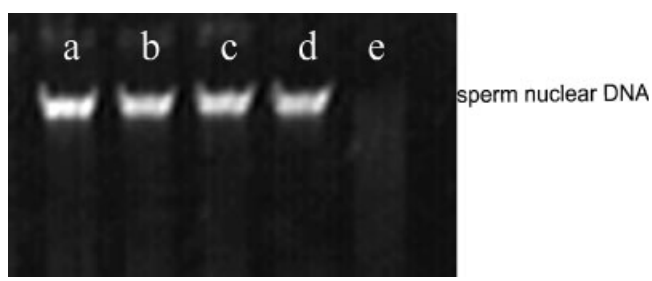

Fig. 2. Electrophoresis analysis of mouse sperm nuclear DNA. From the first to the fifth lane (shown as **a–e**): Nuclear DNA from mouse spermatozoon cryopreserved at −196°C, cooling at 4°C, fresh, heated at 58 and 100°C.

TABLE 1. Development of Mouse Oocytes After Sperm Injection

Sperm	No. injected oocytes	No. surviving oocytes (%)	No. oocytes (%)	
			2 PN	Two-cell embryo
Fresh	65	62 (95.4)[a]	58 (93.6)[a]	55 (88.7)[a]
Cryopreserved at −196°C	93	89 (95.7)[a]	80 (89.9)[a]	77 (86.5)[a]
Cooling at 4°C	80	75 (93.8)[a]	71 (94.7)[a]	68 (90.7)[a]
Heated at 58°C	79	75 (94.9)[a]	1 (1.3)[b]	0[b]
Heated at 100°C	67	63 (94.0)[a]	0[b]	0[b]

Values in the same column with different superscripts (a and b) are significantly different ($P < 0.05$).
PN, pronucleus.

spindle-like structures were observed in the oocytes injected with heat-exposed sperm heads (Fig. 3D1–D3, E1–E3).

DISCUSSION

During mammalian fertilization, once spermatozoon enters into oocyte and loses its membrane, the sperm-borne oocyte-activating factor (SOAF) elicits the activation of oocyte and supports the full development. SOAF originates from sperm head submembrane matrices, and comprises discrete, heat-sensitive (SOAF-I), and-stable (SOAF-II) components. The related data suggest that multiple sperm components are required to induce oocyte activation via SOAF-mediated activation process (Perry et al., 2000).

The results of our laser confocal microscopy demonstrate that sperm motility and plasma membrane integrity are not essential for ICSI-mediated fertilization. Similar to fresh spermatozoa, the spermatozoa cryopreserved at −196°C or cooling at 4°C could activate mouse oocytes and supported the development of ICSI zygotes (shown in Fig. 3). It suggests that the sperm proteins, such as membrane proteins and SOAF in sperm cryopreserved at −196°C, were not denatured, therefore the fertilizing capability of the liquid nitrogen frozen spermatozoa was similar to that of noncontacting sperm. Similar to a previous study (Parrington et al., 1996), either sperm-borne proteins responsible for oocyte activation are resistant to such an increased physical shock or the amount of intact elements is sufficient to induce equivalent oocyte activation.

The other study (Moreira et al., 2003) indicates that the difference between sperm donor strains does not affect their fertilizing capability but the later embryonic development, and for inbred C57CBAF1 mouse sperm contacting with liquid nitrogen, this detrimental effect was immediately visible at the second cell stage and the subsequent in vitro embryo development to blastocyst stage was severely restricted. Those researchers suppose that the quick vaporization and physical expansion of the liquid nitrogen might possibly induce higher levels of DNA fragmentation in the contacting sperm (Moreira et al., 2003). Contrary to this hypothesis, in the present study, the profiles of total protein and nuclear DNA electrophoreses (Figs. 1 and 2) indicate that both protein and nuclear DNA in fresh, cooled, and cryo-preserved sperm is not denatured but maintain their inherent properties. Our results suggest that the intactness of sperm protein and nuclear DNA exposure to liquid nitrogen is not strongly related to the difference of mouse strain, and at least, the SOAF of Kunming mouse could functionally activate the oocyte while its sperm nuclear DNA forms the male pronucleus. In addition, Perry et al. (2000) have found that after injected independently into oocytes, either SOAF-Is or 48°C-heated sperm heads without membrane failed to generate Ca^{2+} oscillations. However, co-injection of SOAF-Is and 48°C-heated heads induced oscillations, mirroring their synergistic ability to activate oocytes. In the present study, the total proteins are degraded irreversibly when heated at 58 and 100°C so that the heated sperm could not normally activate oocyte as indicated by the failure of second meiosis resumption, male pronucleus formation, and cleavage.

In our study, the sperm nuclear DNA heated at 58°C is not damaged severely, while that heated at 100°C are almost completely denatured. If artificial activation for oocyte is exerted, the mouse spermatozoon heated at 58°C and microinjected into the oocyte can support full embryonic development leading to the production of a normal, healthy pup (Cozzi et al., 2001). In addition, the thermostability of mammalian sperm genome is linked to the extensive disulfide bonds cross-linking nuclear protamines (Yanagida et al., 1991). In mammals, during postmeiotic maturation of the spermatid, elongation, and condensation of the nucleus occur with the replacement of the somatic cell DNA-binding proteins, the histones, by the small and more basic sperm-specific protamines (Yanagimachi, 1994). In the resulting mature spermatozoon, the DNA is condensed and tightly packaged into a nucleoprotamine complex. The mouse sperm DNA stability probably reflects its high concentration in protamines; more than 98% of nuclear proteins are protamines (Balhorn et al., 1977).

Most mammalian sperm nuclei are moderately heat resistant and the sperm nuclei of mouse are sensitive to high temperature. After artificial activation, the early cleavage rate was not affected except for the subsequent embryonic development (Cozzi et al., 2001). Without oocyte artificial activation, although some of sperm nuclei were decondensed after exposure to 100°C, none could form male pronuclei (Fig. 3). However, the

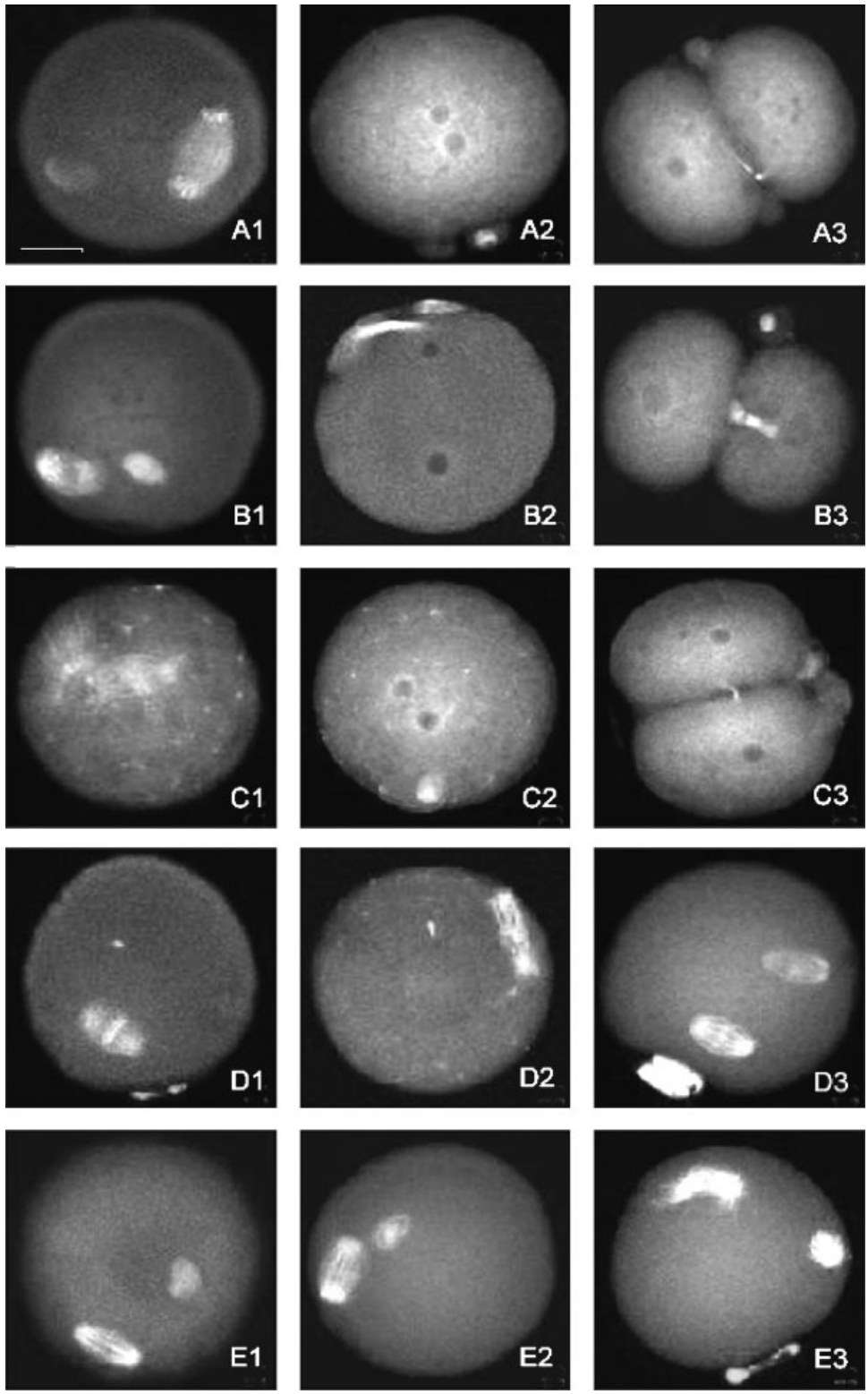

Fig. 3. Observation of cell cycle progression of ICSI oocytes as indicated by microtubule organization and nuclear configuration by laser confocal microscopy. Changes in oocytes microinjected with −196°C-cryopreserved sperm (**A1–A3**), 4°C-cooled sperm (**B1–B3**), fresh sperm(**C1–C3**), 58°C-heated sperm (**D1–D3**), and 100°C-heated sperm (**E1–E3**) were shown. Scale bar: 20 μm.

aetiology of ooctye unactivation is not entirely identical, although both 58 and 100°C heating harm the formation of pronuclei in our results. The present study indicates that the damage of sperm protein and/or nuclear DNA could be responsible for the observed decrease in sperm fertilizing capability. The sperm protein could be primarily responsible for the oocyte activation in the precondition of sperm nuclear DNA intactness.

In general, our ICSI studies show that sperm fertilization capability is more resistant to cooling/cryopreservation than to heating. Under low temperatures, neither sperm proteins nor nuclear DNA was damaged, whereas at high temperature, both sperm proteins and nuclear DNA are damaged, thus leading to the inability of sperm to activate and fertilize the oocytes.

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