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—Review—

Challenging Endeavour for Preservation of Freeze-Dried Mammalian Spermatozoa

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Abstract. Freeze-drying (lyophilization) has been proposed as an alternative method for sperm preservation to overcome the disadvantages of the current cryopreservation method such as the high maintenance cost of frozen stocks, the problems associated with transportation of frozen materials and the potential risk of total loss of the frozen stock. Since freeze-dried spermatozoa after rehydration lose their motility, which is an essential requirement to complete physiological fertilization, a relatively difficult microinsemination technique must be applied to rehydrated spermatozoa. Theoretically, it has been supposed that freeze-dried spermatozoa could maintain their functions and abilities to interact with the oocyte cytoplasm after prolonged storage at refrigerator temperature. However, sufficient yield of transferable blastocysts and production of live offspring derived from freeze-dried sperm samples are still subjects to be challenged and overcome in large domestic species.

Key words: Freeze-drying, Lyophilization, ICSI, Spermatozoa

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Although spermatozoa from many mammalian species can be cryopreserved in a frozen status and used for offspring production either via artificial insemination (AI) or *in vitro* fertilization (IVF), freeze-drying (lyophilization), a widely used method for dehydrating a vast range of materials including foodstuffs, pharmaceuticals, biotechnology products, vaccines, diagnostics and biological materials, has been proposed as an alternative method for sperm preservation.

Since freeze-dried (FD) spermatozoa after rehydration lose their motility, the essential requirement to complete physiological fertilization, a relatively difficult and specialized technique such as intracytoplasmic sperm injection (ICSI) must be applied to the FD spermatozoa. However, this tough working can overcome the potential disadvantages of sperm cryopreservation such as the high maintenance cost of frozen stock, the problems associated with transportation of frozen stock and the risk of accidental total loss of frozen stock. Advantages of sperm freeze-drying can be further emphasized when the spermatozoa from bulls with high economical importance are very sensitive to the process of cryopreservation (large variation in cryosensitivity among bulls) [1, 2]. In species whose spermatozoa are difficult to preserve by freezing, the significance of freeze-drying will be magnified. Under the increasing demands for preservation of huge numbers of mutant or transgenic strains, the significance is also notable. In addition, freeze-drying offers advantages over conventional heat-drying by minimizing damage and loss of activity in delicate heat-labile materials such as enzymes, hormones and vaccines [3, 4]. For parenteral products, the wet material can be accurately dispensed and sterilized by filtration just before filling into final containers, so the possibilities of particulation and bacterial contamination can be reduced [5]. The requirement of expensive equipment may be the only disadvantage of the freeze-drying.

In this review, the background of sperm preservation by freeze-drying (principles and history) is described. Thereafter, the approaches to improve the efficacy of sperm freeze-drying mainly in rodents and the recent progress in the characteristics of FD bull spermatozoa are summarized.

Principles of the Freeze-drying Process

Contrary to conventional dehydration, which depends on an evaporation phenomenon, freeze-drying depends on direct transition from a solid (ice) to vapor (gas) phase (sublimation phenomenon). In freeze-drying equipment, the liquid phase can be avoided by maintaining a sufficiently low temperature under low pressure (high vacuum), while providing enough energy (latent heat) to compensate for the phase transition. The subliming vapor is then 'trapped' into the condenser, which is held at a lower temperature than the drying material, to maintain a low vapor pressure surrounding the drying material. The difference in phase transition of water molecules between ambient and high vacuum conditions is shown in Fig. 1. Both the atmospheric pressure and temperature are among the critical factors that are essential for induction of sublimation. However, the low atmospheric pressure required to induce sublimation is highly variable among substances. In the case of carbon dioxide (CO_2), sublimation occurs from dry ice to CO_2 vapor even under an ambient atmospheric pressure of 1 atm.

Actually, the freeze-drying protocol contains two different stages. During the first stage (main drying), latent heat is supplied to the frozen product under low atmospheric pressure while keeping its temperature above the critical temperature. Raising the

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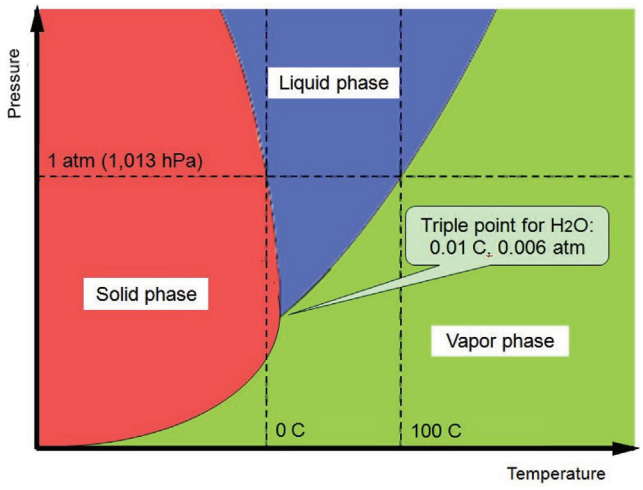

Fig. 1. Water phase transition. Normal phase transition: At sea level (where the pressure is equal to 1 atm), water molecules change from solid ice to liquid (melting) if their temperature is increased above the sea level freezing point (0 C) and then change to vapor (evaporation) if their temperature is increased above the sea level boiling point (100 C). Sublimation phase transition: If the water temperature is higher than the freezing point while the atmospheric pressure is maintained below 0.006 atm (6.1 hPa), the water molecules may be warmed enough to thaw. However, in the absence of enough pressure for liquid formation, the water molecules change directly to the vapor phase.

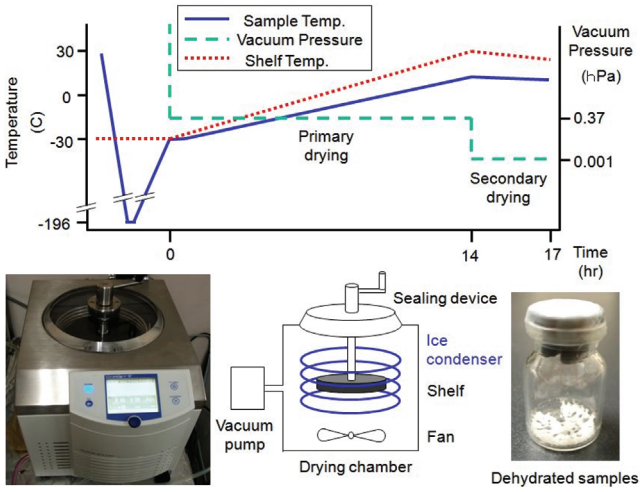

Fig. 2. Kinetics of vacuum pressure and shelf temperature programmed for a sperm freeze-drying protocol in a freeze-dryer machine (ALPHA 2–4 LSC; CHRiST, Germany). In the frozen sample-containing vessels placed on a temperature-controllable shelf in the drying chamber, the liquid phase can be avoided by maintaining a sufficiently low temperature under low pressure (high vacuum as 0.37 hPa) while providing enough energy (latent heat) to compensate for the phase transition. The subliming vapor is then 'trapped' onto the ice condenser, which is held at a lower temperature than the drying material, to maintain a low vapor pressure surrounding the drying material. Once the secondary drying process (0.001 hPa, 3 h) for induction of full dehydration of the sample is completed, the sample vessel is sealed with a rubber cap with or without backfilling a nonreactive gas such as nitrogen or argon. The dehydrated samples are shaded and preserved in a desiccator.

temperature above this level results in undesired processing defects during freeze-drying, while maintaining the product temperature below the critical temperature may result in unacceptable slow progress of drying. Since the critical temperature is variable due to the product formulation, determination of the critical temperature of the formulation prior to freeze-drying may be helpful in order to adapt the suitable conditions for its successful and safe processing in a reasonable time frame [6]. Approximately 90% of the total water in the sample (essentially all of the free water and some of the bound water) can be removed by sublimation during this first stage. The remaining bound water, which limits the structural integrity of the product, can be removed during the subsequent second stage (final drying). Since any physicochemical interaction between the water and the bulk dried material needs to be broken before the water is removed, a desorption process, which requires more energy than sublimation, is induced. Therefore, in the final drying stage, a higher temperature is applied to the freeze-drying material under lower atmospheric pressure than those typically used in the main drying stage. The container of the FD product must be sealed appropriately either under vacuum or after backfilling with a nonreactive gas such as nitrogen or argon. As long as the package is secure, the FD product can be preserved theoretically on a shelf for years without deterioration. One example of a freeze-drying process (dehydration program, equipment and FD sperm sample), which was proven to be effective for long-term storage of rat spermatozoa [7], is shown in Fig. 2.

Research History of Sperm Freeze-drying

The idea of applying freeze-drying to sperm cells appeared as early as 1949 when it was reported that 50% of the FD fowl spermatozoa regained their motility after rehydration [8], followed by an another attempt in human spermatozoa [9]. Successful recovery of motile bull spermatozoa after freeze-drying and rehydration was first reported by Leidl [10] and was later reported by several groups [11–14]. However, the reproducibility of the earlier reports [15–17] was questionable. Although application of AI with FD bull spermatozoa resulted in successful pregnancy [13], the FD spermatozoa failed to recover their motility after short-term storage or excessive dehydration [10, 11, 18]. Since recent studies indicated that fully dried spermatozoa have completely damaged plasma membranes [19, 20], the earlier studies may have dealt with incompletely dehydrated sperm samples. The research in the field of sperm freeze-drying was suspended until ICSI using nonmotile spermatozoa was proven to be practical [21].

In the new series of research, no attention has been paid to the motility of FD spermatozoa after rehydration because even upon application of ICSI with motile spermatozoa, immobilizing the spermatozoa before ICSI is essential. Research has been conducted to refine the freeze-drying protocol, including (1) the principle conditions during freeze-drying (temperature, vacuum pressure or

Table 1. Achievements in freeze-drying of mammalian spermatozoa

Species	Highest achievement	References
Mouse	Live offspring	[19, 22–25]
Rat	Live offspring	[7, 26, 27]
Hamster	Live offspring	[28]
Rabbit	Live offspring	[20]
Cattle	Blastocyst	[29–31]
Pig	Blastocyst and pregnancy	[32, 33]
Monkey	Pronuclear zygote	[34]

period of drying), (2) the composition of the freeze-drying solution and rehydration solution and (3) temperature and period for storage of FD spermatozoa, to maintain the functionality of FD spermatozoa (see the next paragraph). The parameters examined for sperm functionality were chromosome integrity, DNA integrity, sperm-borne oocyte-activating factor (SOAF) activity to induce calcium oscillations or meiosis resumption of oocytes, microtubule-organizing center (MTOC) activity relating to centrosome function and ability to participate in *in vitro* or *in vivo* embryonic development. As summarized in Table 1, successful production of live offspring derived from ICSI with FD spermatozoa (FD-ICSI) has been reported in the mouse [19, 22–25], rat [7, 26, 27], hamster [28] and rabbit [20]. By applying FD-ICSI, blastocysts were obtained in large domestic species such as cattle [29–31] and pigs [32, 33], and pronuclear-stage zygotes were obtained in monkeys [34].

Factors Affecting Efficacy of a Freeze-drying Protocol

(1) Temperature, vacuum pressure and drying period: These conditions during freeze-drying have a great impact on the functions of FD spermatozoa, as interaction among all these factors regulates the kinetics and degree of drying. Prolongation of the freeze-drying period for boar spermatozoa from 4 to 24 h gradually reduced the ability of FD spermatozoa to participate in embryonic development *in vitro*, particularly when the FD spermatozoa were stored at +25 C [32]. Recently, Kawase *et al*. [35] claimed that the vacuum pressure is important during the primary dehydration process for mouse spermatozoa based on the DNA integrity and embryonic development *in vitro* and *in vivo* (0.37 hPa has been recommended rather than 1.03 or 0.04 hPa). The dehydration program reported for mouse spermatozoa [35] was successfully applied to rat spermatozoa with a few minor modifications (Fig. 2) [7].

(2) Freeze-drying solution: Wakayama and Yanagimachi [19], who first reported the successful birth of FD-ICSI-derived mouse offspring, applied two conventionally used culture media (CZB and DMEM) to the freeze-drying solution. On the other hand, Kusakabe *et al*. [22] recommended the use of a solution composed from 10 mM Tris-HCl, 50 mM NaCl and 50 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Addition of the divalent cation chelator (EGTA) was expected to slow down or prevent the endonuclease-mediated chromosomal damage by repressing the activity of the enzyme released from membrane-damaged spermatozoa. Increasing the pH of this solution to 8.0 or addition of DMSO or vitamin E acetate improved its protective ability [24, 36]. Preincubation of mouse but not human spermatozoa in similar solution for 1–6 days at +4 C increased the resistance of the spermatozoa to freeze-drying [37]. A solution containing 1 mM ethylenediaminetetraacetic acid (EDTA) instead of EGTA was found to maintain chromosomal integrity and participation in embryonic development of mouse and rat FD spermatozoa stored for 1 year at +4 C [7, 38]. Supplementation of trehalose, a disaccharide accumulated in numerous anhydrobiotic animals during the drying stage [39], showed a similar protective function to that of EGTA [30]. Trehalose may bind to the sperm membrane and make it more stable, or the high glass transition state of trehalose may put the dried cells in a static glassy state that inhibits the cellular, chemical, biological and physical processes [40]. The time elapsed after rehydration until utilization of FD spermatozoa for ICSI may be a possible factor affecting the efficacy of FD spermatozoa because some cytoplasmic active components may be released from the completely damaged plasma membrane of FD spermatozoa into the postrehydration medium. Chromosomal integrity and participation in embryonic development of boar FD spermatozoa for up to 2 h after rehydration can be maintained by addition of EDTA or EGTA to freeze-drying solution [33].

(3) Temperature and storage period of FD spermatozoa: It is the most preferable that the FD spermatozoa can be stored for a long period at an ambient or refrigerator temperature without loss of their functions. While Wakayama and Yanagimachi [19] reported that the ability of mouse FD spermatozoa to activate the oocytes or to participate into embryonic development *in vitro* or *in vivo* was not affected by storage either at +4 or +25 C for up to 3 months, Kwon *et al*. [32] observed that the ability of boar FD spermatozoa to participate into embryonic development *in vitro* was more hampered after 1 month storage at +25 C than +4 C and that porcine oocytes injected with FD spermatozoa stored for 3 months at +4 C did not develop *in vitro*. An adverse effect of 3-month storage at +4 C on occurrence of DNA damage was also observed in mouse spermatozoa [41]. Similarly, rat FD spermatozoa stored for 1 year at +25 C exhibited more frequent chromosomal abnormalities than those stored at +4 C [7]. Regardless of the successful offspring production from FD spermatozoa stored for 1–2 years at +4 C in the mouse [25, 38], rat [7] and rabbit [20], it has been reported, based on the theory of accelerated degradation kinetics, that FD mouse spermatozoa need to be kept at lower than –80 C for prolonged storage [41].

Functional Integrity of FD Bull Spermatozoa

FD spermatozoa undergo exposure to and freezing in cryoprotectant-free and calcium-free buffer, complete dehydration, long-term storage and rehydration, which may adversely affect either structural or functional sperm characteristics. A few early physiological events at the pronuclear stage have been examined in detail.

(1) Calcium oscillation-inducing ability: The earliest function of sperm cells at fertilization is to induce repetitive increases of calcium ion concentration in the ooplasm, the so-called calcium oscillations. The calcium oscillations are responsible for the initiation and completion of many postfertilization events including resumption of the second meiosis [42] and exocytosis of cortical

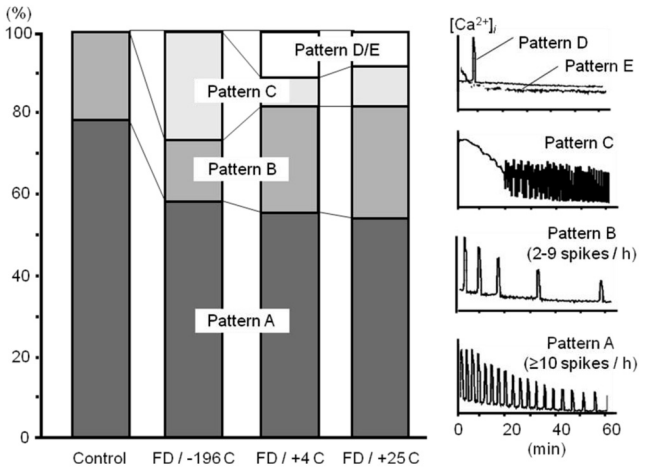

Fig. 3. Ability of bull spermatozoa freeze-dried and stored for 1 year at 3 different temperatures to induce calcium oscillations in mouse oocytes [47]. B6D2F1 mouse oocytes preloaded with 22 μM Ca^{2+}-sensitive fluorescent dye (fluo-3-acetoxymethylester) for 30 min were microinjected with bull sperm heads within 20 min of preloading with fluo-3. Injected oocytes were transferred to a glass slide chamber, and the kinetics of intracellular calcium ion ($[Ca^{2+}]i$) were monitored for 1 h at 20-sec intervals under a confocal laser-scanning microscope. The $[Ca^{2+}]i$ responses were classified into 5 patterns (A to E).

granules [43]. In bovine IVF, calcium oscillations were found to last until pronuclear migration and nuclear envelop breakdown [44, 45]. Liu *et al*. [46] reported that bull spermatozoa freeze-dried under a single vacuum pressure and rehydrated immediately after freeze-drying have similar calcium oscillation-inducing ability to nondried spermatozoa in mouse oocytes. When bull spermatozoa were freeze-dried and then stored for 1 year, an interspecies microinsemination assay using the rehydrated sperm cells indicated a slight influence of freeze-drying [47]. Repetitive increases of intracellular calcium ion concentration were recorded in the majority of injected mouse oocytes, with the exception of a few oocytes injected with FD spermatozoa stored at +4 or +25 C that exhibited a single increase or no response (Fig. 3). After homologous ICSI, the proportion of bovine oocytes resuming meiosis and developing to the pronuclear stage was higher in the control group than those in all the FD groups [47]. Although the routine bovine ICSI technique includes application of exogenous stimuli for oocyte activation [48, 49], stronger stimuli may be required when FD bull sperm are used for bovine embryo production by ICSI.

(2) Active DNA demethylation: In the early stages of embryonic development, the gametic methylation marks are erased (demethylation) and replaced with the embryonic marks that are important for embryonic development and acquirement of toti- or pluripotency. The paternal genome is subjected to replication-independent, genome-wide active demethylation during the first few hours after fertilization, while the maternal genome maintains its methylation level until the beginning of the mitotic division stage, which is when both the maternal and paternal genomes undergo replication-dependent, passive demethylation with each mitotic cycle [50, 51]. Although the active demethylation process of the paternal genome is considered to be controlled by a function of an ooplasmic factor(s), this process may be facilitated either by a sperm factor(s) or by male pronuclear chromatin composition [52]. There is agreement that bovine paternal genomes undergo active demethylation [53, 54], but the extent of the demethylation process varies considerably. Abdalla *et al*. [55] reported that the bovine paternal genome rapidly demethylated within 10 h after IVF and 6 h after ICSI and that, at least, the freeze-drying and/or storage process have no adverse effect on demethylation of the paternal genome. The demethylation extent in pronuclear-stage bovine zygotes, as assessed quantitatively by the overall average of the relative methylation (RM; male/female) in an immunostaining protocol against 5-methylcytosine, was moderate with $0.4 \leq RM < 0.6$ [55].

(3) Microtubule-organizing center (MTOC): Since an interphase network of microtubules and the mitotic bipolar spindle are nucleated from the centrosome, the centrosome is considered to be MTOC [56]. During fertilization in mammals (except for rodents), the centrosome brought into the oocyte by the spermatozoon plays a critical role in assembly of the microtubule network that brings both male and female pronuclei to the center of the newly formed zygote, as reported in humans [57], rabbits [58] and cattle [59]. Reports on the MTOC function of FD spermatozoa are only available for rabbits [20] and primates [60]. In those reports, sperm preservation by freeze-drying had no adverse effects on the frequency of ICSI oocytes forming the sperm-aster [20, 59]. In the bovine, formation of sperm-asters in FD-ICSI oocytes 7 h post insemination (Fig. 4) occurred at a similar rate when compared to control ICSI oocytes [31]. Among the oocytes exhibiting sperm aster formation, the extent of the microtubule network was also comparable between the FD-ICSI and control ICSI groups. However, the MTOC of ICSI oocytes was not as functional as that of IVF oocytes in regard to aster formation and the microtubule network assembly, suggesting that the ICSI technique *per se* needs further improvements in the bovine.

Production of Bovine Blastocysts by FD-ICSI

The ability of FD spermatozoa to participate in embryonic development (best under *in vivo* conditions but alternatively under *in vitro* conditions) must be the most reasonable parameter to be examined. The success rate, assessed by *in vitro* and *in vivo* development after ICSI, is highly variable among mammalian species, with the highest success rate in humans [61] and mice [21]. Successful production of an ICSI-derived calf was reported in 1990 [62]. While the blastocyst yield from IVF-derived bovine zygotes is approximately 30–40%, the yield from ICSI-derived bovine zygotes is variable (5–40%). So far, it seems that piezo-assisted sperm injection followed by an activation treatment with ethanol plus cycloheximide or 6-dimethylaminopurine, which either interfere with the resynthesis or activation of MPF, is likely to achieve the best blastocyst yield *in vitro* and full-term development after transfer into recipient cattle [48, 49]. The routine piezo-ICSI procedure for bull spermatozoa is shown in Fig. 5. Bovine oocytes appear dark because the ooplasm contains lipid droplets at a very

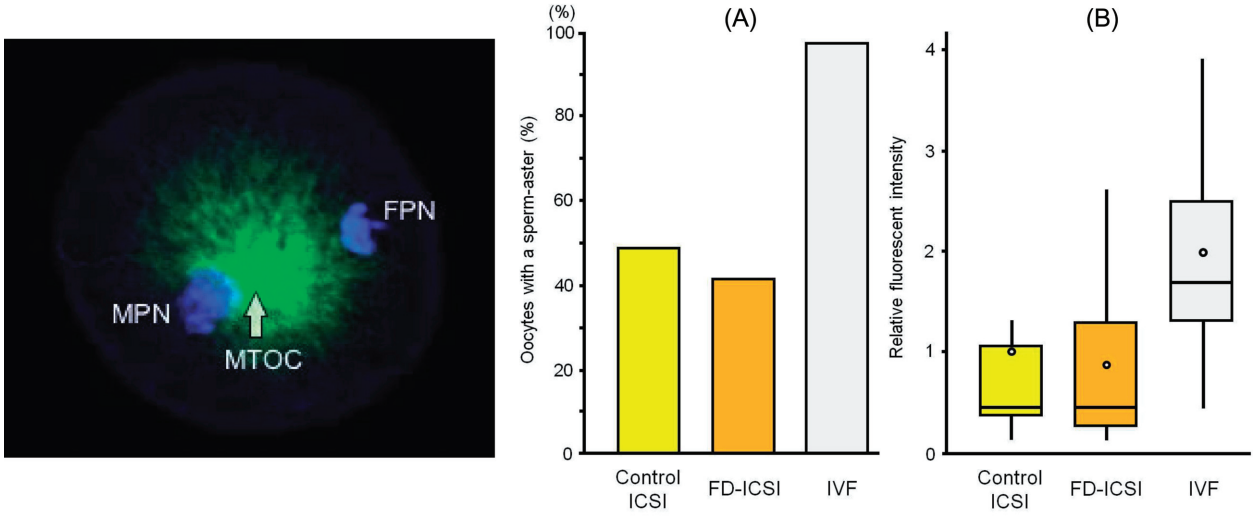

Fig. 4. Function of the microtubule-organizing center in FD bull spermatozoa [31]. (Left panel: Photograph) Seven hours after insemination, the oocytes were differentially immunostained by anti-tubulin (green) and DAPI (blue) for visualizing microtubule network assembly and the position of nuclear materials, respectively. MPN: Male pronucleus. FPN: Female pronucleus. MTOC: Microtubule-organizing center. (A: center panel, bar graph) The proportion of oocytes injected with FD spermatozoa and formed a sperm-aster was slightly lower than that of control ICSI oocytes. (B: right panel, box plot graph) Once the sperm-aster formed, the size of the microtubule network was comparable between FD-ICSI and control ICSI oocytes. However, the MTOC in bovine ICSI oocytes was not as functional as that in IVF oocytes in regard to the rate of aster formation and the size of the microtubule network.

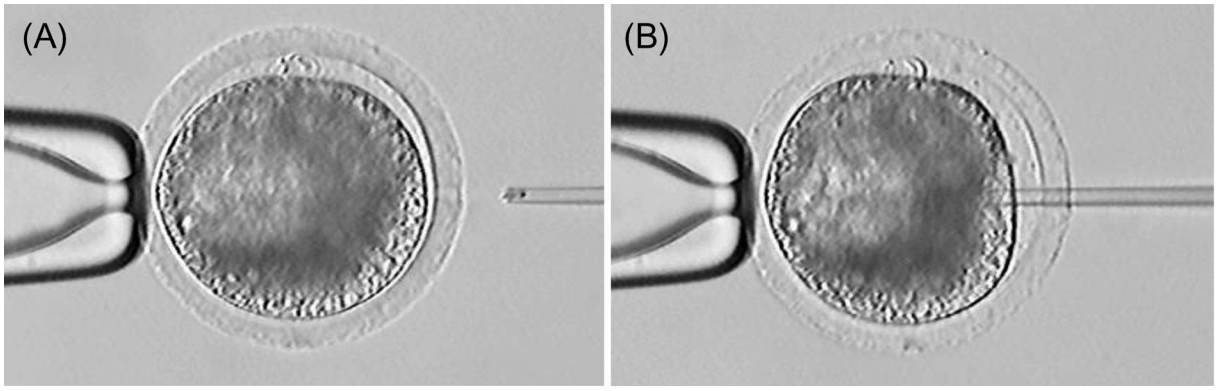

Fig. 5. Piezo-ICSI procedure in the bovine. (A) Single spermatozoon is immobilized by applying several piezo pulses to the midpiece before being aspirated tail first into a blunt-ended injection pipette with an outer diameter of 7–9 μm. An oocyte is held with a holding pipette with the polar body located at either the 6 or 12 o'clock position. The zona pellucida is drilled by several piezo pulses, and the spermatozoon is repositioned to the tip of the injection pipette. (B) The injection pipette is advanced mechanically deep into the center of the oocyte, stretching the oolemma extensively. Upon application of a single piezo pulse, the oolemma is punctured at the pipette tip. The spermatozoon is gently injected into the ooplasm, and then the injection pipette is withdrawn.

high concentration. Centrifugation of bovine oocytes makes it possible to localize the cytoplasmic lipids, and the operator can visualize the tip of the injection pipette inside the clarified area of the cytoplasm. Wei and Fukui [63] reported that the proportions of bovine oocytes that were successfully injected, survived or showed two pronuclei were higher when centrifuged oocytes were used in conventional ICSI. However, in our laboratory, application of oocyte centrifugation before piezo-ICSI did not improve the proportions of bovine oocytes surviving the ICSI procedure, cleaving or developing into blastocysts [64]. Characteristic phenomena after bovine ICSI such as failure of oocyte activation [65–68] and compromised sperm chromatin remodeling [69, 70] may be a possible reason for a low success rate in development of bovine ICSI oocytes into blastocysts.

Two publications concerning FD-ICSI in bovine species are available [29, 30]. Both groups used bull spermatozoa that were freeze-dried under a constant vacuum pressure without any control of the temperature and then stored the spermatozoa at +4 C for up

Table 2. An attempt at producing bovine blastocysts by FD-ICSI

FD protocol	No. of oocytes Injected	Cultured	PB-extruded	Cleaved	Developed to ExB
Single step	164	143 (87.2%)	108 (75.5%)	63 (44.1%)	2 (1.4%)
Two-step	193	176 (91.2%)	130 (73.9%)	63 (35.8%)	1 (0.6%)
Nondried control	161	135 (83.9%)	98 (72.6%)	91 (67.4%)	29 (21.5%)
IVF control	—	145	ND	113 (77.9%)	62 (42.8%)

Single step FD: 0.37 hPa for 14 h. Two-step FD: 0.37 hPa for 14 h + 0.001 hPa for 3 h. PB: Second polar body. ExB: Expanded blastocysts. ND: Not determined. The majority of the data was referenced from our recent report [31] .

to 3 months, with blastocyst yields of 19─30% (including some parthenotes). Abdalla *et al*. [71] determined an alternative activation regimen for bovine ICSI oocytes (5 μM ionomycin for 5 min immediately after ICSI plus 7% ethanol for 5 min 4 h after ICSI; blastocyst yield, approximately 30%) with one advantage, as this regimen supported development of only very few parthenotes to the blastocyst stage. FD bull spermatozoa were prepared either by the original 2-step freeze-drying protocol or the shorter 1-step protocol without the secondary dehydration process and stored for 7 days at +4 C until FD-ICSI (Table 2). When the above-mentioned activation regimen was applied, the cleavage rates of FD-ICSI oocytes were ~50% lower than that of control ICSI oocytes [31]. Regardless of the protocol for FD sperm preparation, the blastocyst development rate after FD-ICSI was much lower than that after the control ICSI, and thus, further improvement of the freeze-drying protocol for bull spermatozoa is required.

Conclusion

Mouse and rat spermatozoa were practically preserved at a refrigeration temperature (+4 C) for a long term (>6 months) after a two-step rehydration protocol for freeze-drying, with acceptable offspring rates after FD-ICSI. The freeze-drying protocol employed slightly reduced the ability of the bull spermatozoa to induce calcium oscillation, but it had no adverse effect on the active demethylation dynamics of the paternal genome and the microtubule assembly for formation of sperm-asters during the early stage after fertilization. However, FD bull spermatozoa participated very little in embryonic development to the blastocyst stage, even after improvement of blastocyst yield from ICSI oocytes by applying exogenous activation stimuli with ionomycin and ethanol. The detailed conditions during the freeze-drying process, which were found effective in the rodent species, need to be optimized for bull spermatozoa.

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