Pronuclear formation of freeze-dried canine spermatozoa microinjected into mouse oocytes

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Abstract

Purpose The aim of the present study was to investigate the fertilizing capacity of fresh, frozen-thawed and freeze-dried canine spermatozoa.

Methods After canine spermatozoa were injected into mouse oocytes, the rates of oocyte activation, male pronuclear formation and chromosomal aberrations were investigated.

Results The rates of oocyte activation were comparable (90.6–100%), no matter the sperm type injected. The percentage of male pronuclear formation was higher ($P < 0.001$) in the freeze-dried spermatozoa (92.3%) than the fresh (61.5%) and frozen-thawed (69.2%) spermatozoa. However, the chromosomal damage in the oocytes injected with freeze-dried spermatozoa was higher (72.9%; $P < 0.001$) than with fresh (26.9%) and frozen-thawed (21.4%) spermatozoa.

Conclusions These data indicate using mouse oocytes that freeze-dried canine spermatozoa may potentially fertilize canine oocytes although chromosomal damage is frequently generated.

Keywords Dog · Chromosome · Freeze-drying · Intracytoplasmic sperm injection · Spermatozoa

Introduction

Cryopreservation of spermatozoa has enabled the cost effective preservation and long-distance transportation of genetic resources. As one of the cryopreservation methods in mammalian spermatozoa, freeze-drying has been applied in mouse [1–8], rat [9, 10], rabbit [11], bull [12], boar [13, 14] and human [15] spermatozoa. An advantage in freeze-drying is to store the spermatozoa without the use of liquid nitrogen for a long period. However, freeze-dried spermatozoon must be microinjected into an oocyte to be fertilized, because it is no longer motile. Since the chromosomal integrity of these spermatozoa is impaired [7], methodological optimization of freeze-drying is still an essential issue for successful sperm preservation [15–17].

Canine species are bred worldwide as companion animals and working dogs, including guide dogs for the blind. Assisted Reproductive Technologies (ARTs) in canines, e.g. artificial insemination [18, 19], in vitro fertilization [20, 21] and somatic cell nuclear transfer [22, 23], has been steadily progressing. ARTs seems to have led to the more efficient production of capable working dogs, and may be further advanced by successful preservation of canine spermatozoa. However, to the best of our knowledge, canine spermatozoa have never been freeze-dried. Activation of oocytes by sperm, subsequent formation of male pronucleus (MPN) and their chromosomal integrity can be considered as indicators for fertilizing ability of spermatozoa. Furthermore, these indicators can be monitored by microinjection into mouse oocytes in mouse [24, 25], whale [26] and human [27, 28] spermatozoa.

We report here that freeze-dried canine spermatozoa microinjected into mouse oocytes were able to activate oocytes and form MPN.

Capsule Canine spermatozoa can be freeze-dried with oocyte activating and male pronuclear forming capacity.

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Materials and methods

Reagents and media

All chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) unless specifically stated. The culture medium for mouse oocytes after intracytoplasmic sperm injection (ICSI) was Chatot-Ziomek-Bavister (CZB) [29] supplemented with 5.56 mM D-glucose and 4 mg/ml bovine serum albumin (fraction V; Sigma-Aldrich, St. Louis, MO). Mouse oocyte collection and microinjection were performed in a modified CZB supplemented with 20 mM Hepes-Na, 5 mM NaHCO₃, and 0.1 mg/ml polyvinyl alcohol (cold water soluble; Sigma-Aldrich) in place of bovine serum albumin (H-CZB). Sperm preparation was performed in modified Toyoda-Yokoyama-Hoshi (TYH) medium [30] supplemented with 20 mM Hepes, 5 mM NaHCO₃, and 0.1 mg/ml polyvinyl alcohol in place of bovine serum albumin (H-TYH). The pH value of both H-CZB and H-TYH was adjusted to approximately 7.4.

Animals

(C57BL/6 J×DBA/2)F1 (B6D2F1) mice (CLEA Japan, Inc., Tokyo, Japan) were used to collect the oocytes. Also, spermatozoa were recovered from a Labrador Retriever in our colony. Animal care and all experiments were performed according to the Guiding Principles for the Care and Use of Research Animals of Obihiro University of Agriculture and Veterinary Medicine.

Preparation of oocytes and spermatozoa for ICSI

B6D2F1 female mice, 7–11 weeks of age, were super-ovulated by i.p. injection of 10 IU equine chorionic gonadotrophin (Asuka Pharmaceutical, Tokyo, Japan) followed by injection of 10 IU human chorionic gonadotrophin (Asuka Pharmaceutical) 48 h later. The oocytes recovered from the oviducts between 14 and 16 h after human chorionic gonadotrophin injection were denuded of their cumulus cells by treatment with 0.1% (w/v) bovine testicular hyaluronidase (Sigma-Aldrich) in H-CZB. The denuded oocytes were repeatedly rinsed in CZB medium and kept at 37°C under 5% CO₂ in the same medium until ICSI.

Sperm collection, freezing and freeze-drying procedures

The ejaculated spermatozoa were collected by digital manipulation [31] into a sterile tube (Corning, NY, USA). The first and third fractions (seminal plasma) of the ejaculates were discarded. The sperm-rich second fraction of the ejaculates was used for the experiments. One each of

the ejaculates was subjected to a cryopreservation, freezing or freeze-drying, as described below.

Spermatozoa were frozen by using the commercially available semen extender, AndroMed (Minitüb, Tiefenbach, Germany), following the supplier recommended methods. Briefly, the semen was diluted with AndroMed and cooled to 4°C for 3 h. Then, the sperm suspension was loaded into a 0.25 ml straw, and kept in an atmosphere of liquid nitrogen vapor, i.e. placed horizontally 6 cm above the surface of liquid nitrogen in a closed styrene foam box (21.0 cm×13.0 cm×13.0 cm), for 15 min. The straws were plunged into liquid nitrogen and stored up to 3 months. When thawing, the straws were immersed into water bath at 37°C and immediately used for ICSI. Percentage of motile spermatozoa after thawing was 46.2%.

Freeze-drying was performed as reported by Kawase et al. [5]. Briefly, the spermatozoa were placed in EGTA Tris-HCl-buffered solution (50 mM EGTA, 50 mM NaCl and 10 mM Tris-HCl, pH 8.0) [17] and kept at 37°C for 10 min. The sperm suspensions (250 µl) were transferred into the amber vacuum vial for freeze-drying (V-2B; Nichiden-rika Glass, Kobe, Japan). The vials were plunged into liquid nitrogen for 5 min and then transferred to a programmable freeze-dryer (BETA2–16; Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) that had been precooled to –30°C. The freeze-drying conditions consisted of primary drying at a pressure of 0.37 mbar and secondary drying at a pressure of 0.001 mbar. Since the freeze-dried spermatozoa can be kept indefinitely at –80°C or below [4], the vials were stored at –80°C for up to 1 month. The freeze-dried spermatozoa were rehydrated by adding 250 µl of milli-Q water, and immediately used for ICSI.

ICSI procedure

Before injection, a batch of 15 oocytes was transferred into a droplet (5 µl) of H-CZB, which had been prepared beside a sperm-containing droplet in the ICSI chamber covered with paraffin oil (Merck Japan, Tokyo, Japan). A spermatozoon was aspirated into the injection pipette tail first in H-TYH containing 10–12% polyvinyl pyrrolidone (molecular weight: 360000; Nacalai Tesque, Kyoto, Japan), and the tail was cut at the mid-piece by applying a few piezopulses. The tail-cut spermatozoon was individually injected into a mouse oocyte according to the method of Kimura and Yanagimachi [32]. The ICSI series of experiments was finished within 1 h of sperm preparation. Thereafter, the injected oocytes were washed with CZB and transferred into a droplet (30 µl) of the same medium covered with paraffin oil at 37°C under 5% CO₂ in air for cultivation.

Fig. 1 Mouse oocytes injected with frozen-thawed canine spermatozoa at 6 h after ICSI. Arrowheads indicate morphologically normal oocytes. Only morphologically normal oocytes are examined to evaluate the fertilizing capacity of canine spermatozoa. Bar=50 μ m

Chromosome preparation of one-cell oocytes

Six hours after ICSI, the rate of normal morphological oocytes was observed. The deformed oocytes were discarded and morphologically normal oocytes (Fig. 1) were transferred to CZB containing 0.02 μ g/ml vinblastine sulfate to inhibit the first cleavage division. At 19–21 h after ICSI, they were treated with 0.5% protease (Kaken Pharmaceuticals, Tokyo, Japan) in Ca^{2+} - and Mg^{2+} -free Dulbecco's phosphate buffered saline to digest the zona pellucida. Then, they were maintained in hypotonic solution consisting of equal volumes of 1% (w/v) sodium citrate and 30% (v/v) fetal calf serum (Gibco-BRL, Grand Island, NY) for 10 min at room temperature. These samples were prepared by the gradual-fixation/air drying method [33]. The slides were conventionally stained with 2% Giemsa (Merck) in buffered saline (pH 6.8) for 10 min.

Fig. 2 Freeze-dried spermatozoon **a** and the oocytes fertilized with freeze-dried spermatozoa **b**. Arrowheads indicate male or female pronucleus. Bar=10 μ m

Statistical analysis

All experiments were repeated three to five times. The chi-square test or Fisher's exact probability test was used for analyses. Differences were considered significant when the *P* value was less than 0.05.

Results

Freeze-dried spermatozoa are represented in Fig. 2a. The results of oocyte activation, MPN formation and chromosomal integrity are summarized in Table 1. When fresh, frozen-thawed and freeze-dried spermatozoa were injected into mouse oocytes, the proportions of morphologically normal oocytes after 6 h of ICSI significantly changed ($P < 0.001$) to 29.6, 59.1 and 100% of oocytes, respectively. Almost all oocytes (90.6–100%) were activated successfully, no matter which of the three sperm types was injected. On the other hand, the rate of MPN formation was significantly higher ($P < 0.001$) in the freeze-dried spermatozoa (92.3%; Fig. 2b) than the fresh (61.5%) and frozen-thawed (69.2%) spermatozoa. The chromosomal integrity was analyzable in a range of 86.4 to 93.4% (Fig. 3). Chromosomal damage was significantly increased ($P < 0.001$) in the freeze-dried spermatozoa compared with the fresh and frozen-thawed spermatozoa (Table 1).

Discussion

It is well known that the fertilizing capacity of mammalian sperm can be assessed using rodent oocytes [34–36]. Moreover, the spermatozoan chromosomes in a variety species can be easily visualized using these oocytes in combination with ICSI techniques [25–28]. Therefore, the present study carried out an assessment of

Table 1 Fertilizing capacity of fresh, frozen-thawed and freeze-dried canine spermatozoa microinjected into mouse oocytes

Type of sperm	No. examined*	Morphologically normal oocytes (%)**	No. fixed†	Activated oocytes (%)	Status of paternal nuclei (%)			No. MPN analyzed	Normal chromosome (%)
					MPN‡	DSH	CSH		
Fresh	108	32 (29.6) ^a	26	25 (96.2)	16 (38.5) ^a	10 (38.5) ^a	0 (0)	15	11 (73.3) ^a
Frozen-thawed	235	139 (59.1) ^b	117	106 (90.6)	81 (69.2) ^a	34 (29.1) ^a	2 (1.7)	70	55 (78.6) ^a
Freeze-dried	179	179 (100) ^c	169	169 (100)	156 (92.3) ^b	13 (7.8) ^b	0 (0)	144	39 (27.1) ^b

MPN, male pronucleus, DSH, decondensed sperm head, CSH, condensed sperm head

^{a-c} Within a column, values without a common superscript differ ($P < 0.001$)

* No. of surviving oocytes after sperm injection

** The oocytes were observed at 6 h after ICSI

† Only morphologically normal oocytes were fixed 19–21 h after ICSI

‡ The paternal nuclei in the pronuclear and first mitotic stages were judged as MPN

the fertilizing capacity of canine spermatozoa using mouse oocytes.

As shown in Table 1, freeze-dried canine spermatozoa were as capable of fertilization as fresh and frozen-thawed spermatozoa. When freeze-dried canine spermatozoa were injected into mouse oocytes, a majority of the nuclei transformed into the MPN. The value was obviously higher than in the other groups. A possible reason is that the plasma membrane of the freeze-dried spermatozoa was completely destroyed [7, 11, 15], which could lead to a more rapid contact between the sperm nuclei and the ooplasm.

Mouse oocytes are hypersensitive to the acrosome enzyme in spermatozoa: mouse oocytes injected with acrosome-intact bull and boar spermatozoa or acrosomal enzyme(s) undergo deformation and never reach the 2-cell stage [37]. In our preliminary experiments, paternal nuclei in all of the deformed oocytes at 19–21 h after ICSI entered arrest at the time of decondensation or pronuclear forma-

tion. They never reached the first mitotic stage (data not shown), suggesting that acrosomal contents in spermatozoa are disturbed the progress of fertilization. Therefore, the deformed oocytes were discarded and the remainder, morphologically normal oocytes, was utilized for the assessment of the fertilizing capacity (Fig. 1). Kimura et al. [24] demonstrated that sperm perinuclear material contained a necessary substance (sperm-borne oocyte activating factor: SOAF) to activate the oocytes, and that the SOAF activity could be analyzed using mouse oocytes. As shown in Table 1, it seems that the SOAF activity in canine spermatozoa was not species-specific, and was maintained even when the spermatozoa were freeze-dried. However, a portion of the mouse oocytes injected with freeze-dried and sonicated bull spermatozoa did exhibit an abnormal pattern of calcium oscillations [12]. For a conclusive assessment of the successful oocyte activation, the calcium oscillation pattern induced by freeze-

Fig. 3 Chromosomes spread from freeze-dried canine spermatozoa injected into mouse oocytes. **a** Normal chromosome set. The haploid chromosome number of canine spermatozoa is 39. Minor **b** and multiple **c** chromosome aberrations. Arrows indicate the chromosomal or chromatid fragmentations. The arrowhead represents reciprocal translocation. Bar=10 μ m

dried canine spermatozoa remains a subject for future investigation.

As shown in Table 1, the chromosomal integrity in fresh canine spermatozoa was relatively low (73.3%) compared with mouse (91.6% [25]) and human (95.7% [28]) spermatozoa. In our preliminary experiment, when frozen-thawed canine spermatozoa were injected into the double-volume (artificially fused) mouse oocytes, the rates of morphologically normal oocytes and MPN formation improved to 87.5 and 91.8%, respectively. Since mouse oocytes are hypersensitive to acrosomal enzyme, the size (volume) of the cytoplasm of the mouse oocyte might be an important factor for an assessment of fertilizing capability in canine spermatozoa.

The freeze-drying procedure generated chromosomal aberrations in canine spermatozoa (Table 1). Since frozen-thawed spermatozoa did not increase chromosomal damage, one of the reasons for the increased damage is likely to be demembranization in freeze-dried spermatozoa. Sperm nuclei with a defective plasma membrane can easily come into contact with endonuclease, leading to the generation of chromosomal nicks. In the mouse, the chromosomes in 30–60% of the freeze-dried spermatozoa were impaired [3, 7, 15], while the percentage of chromosomally damaged canine spermatozoa was 72.9% in the present study. The chromosomal damage of freeze-dried mouse spermatozoa can be decreased by modifying the pH value of the storage media [17], and by pre-treatment with diamide [3] and EGTA [15]. Therefore, it is expected that optimized conditions will subsequently be established for successful freeze-drying of canine spermatozoa.

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