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Chromosomal integrity and DNA damage in freeze-dried spermatozoa
(凍結乾燥した精子における染色体完全性とDNA損傷)

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Title:

Chromosomal integrity and DNA damage in freeze-dried spermatozoa

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Abstract

Freeze-drying technology may one day be used to preserve mammalian spermatozoa indefinitely without cryopreservation. Freeze-dried mouse spermatozoa stored below 4°C for up to 1 year have maintained the ability to fertilize oocytes and support normal development. The maximum storage period for spermatozoa increases at lower storage temperatures. Freeze-drying, per se, may reduce the integrity of chromosomes in freeze-dried mouse spermatozoa, but induction of chromosomal damage is suppressed if spermatozoa are incubated with divalent cation chelating agents prior to freeze-drying. Nevertheless, chromosomal damage does accumulate in spermatozoa stored at temperatures above 4°C. Currently, no established methods or strategies can prevent or reduce damage accumulation, and damage accumulation during storage is a serious obstacle to advances in freeze-drying technology. Chromosomal integrity of freeze-dried human spermatozoa have roughly background levels of chromosomal damage after storage at 4°C for 1 month, but whether these spermatozoa can produce healthy newborns is unknown. The safety of using freeze-dried human spermatozoa must be evaluated based on the risks of heritable chromosome and DNA damage that accumulates during storage.

Key words

Chromosome, Cryopreservation, DNA damage, Freeze-drying, Spermatozoa

Introduction

Cryopreservation with liquid nitrogen and storage at very low temperatures (−80°C) are used for many cell and tissue types and many purposes in a wide range of biological fields because these samples maintain many important characters and their genetic material is largely unaltered during cryopreservation and cryostorage. Nevertheless, sperm preservation methods that do not require liquid-nitrogen-based cryopreservation are needed for the following reasons. 1) Liquid nitrogen is not readily available in many countries and places (e.g., many developing countries, pacific islands, space stations). 2) These cryopreserved samples are often destroyed or damaged because low-temperature storage facilities fail due to human errors or loss of power. 3) These samples may be contaminated by pathogenic viruses that are stored in the same cryostorage facilities [1]. Although incidents of cross-contamination are rare in cryobanks, it is difficult to make sure that it has not occurred yet [2]. For these reasons, advances in sperm preservation techniques that do not require liquid nitrogen or deep-freezer storage may contribute to the safe preservation of the genome resources of mammalian species. 4) Potentially, freeze-dried spermatozoa may be transported anywhere without any refrigerants, such as dry ice [3, 4].

This review focuses on freeze-drying of mammalian spermatozoa, and particularly mouse and human spermatozoa. Recent progress and persistent problems associated with the methods used to maintain the integrity of DNA and chromosomes of the freeze-dried spermatozoa are discussed.

Participation of motionless spermatozoa in fertilization

A method for freeze-drying spermatozoa was published approximately a half century

ago. More recently, Polge et al. [5] reported that the majority of freeze-dried fowl spermatozoa were motile after rehydration, and in 1976, Larson and Graham [6] reported that some freeze-dried bull spermatozoa were motile after rehydration. Moreover, even motionless spermatozoa can fertilize oocytes and support normal development with advances in intracytoplasmic sperm injection (ICSI) [7]. These advances in ICSI led us to consider simple methods for mammalian spermatozoa preservation that do not require cryoprotectants [8] and the retrieval of sperm from frozen cadavers [9, 10]. Freeze-dried spermatozoa need not be motile after rehydration; in laboratory mice, zygotes generated using ICSI and freeze-dried sperm can develop into healthy, full-term offspring [3]. Moreover, mice derived from the freeze-dried spermatozoa gave rise to first- and second-generation progeny with stable genomes [11]. Many studies have investigated freeze-dried spermatozoa in mammalian species other than mice and humans. Most of these studies explored whether freeze-dried spermatozoa from cattle [12–15], dog [16], hamster [17], human [17–21], pig [22, 23], Rhesus monkey [24], rabbit [20, 25] or rat [26–29] could develop to the pronuclear stage, the blastocyst stage, and/or to live birth.

For assisted reproduction in most species, ICSI must be introduced and improved to ensure that sperm that become non-motile because of harsh isolation, preservation, or storage conditions can support normal development, although storage of freeze-dried mammalian spermatozoa has great potential as an alternative to traditional nitrogen-based cryopreservation.

Evaporative drying versus freeze-drying

Procedures used to freeze-dry mouse sperm usually include a freezing step (1 to 10

min) before the sublimation of water in the samples. A lyophilizer is generally used to sublimate the water. Glass ampoules containing the frozen sperm sample are connected to the lyophilizer, and a vacuum is applied at an inner pressure of approximately 1 m Torr for 12 h [3] or 0.04 mbar for 4 h [30]; alternatively, primary drying and subsequent secondary drying pressures (0.37 mbar and 0.001 mbar, respectively) are applied to the ampoules [31].

Evaporative drying is another method used to prepare dried spermatozoa. Reportedly, evaporative drying of mouse spermatozoa is an exceptional method for preparing dried sperm specimens that eliminates the initial freezing step of freeze-drying, which is likely to injure the spermatozoa [32–35]. This evaporative drying method has been used primarily for laboratory mice sperm, and the technique has not been optimized for other mammalian species. Sperm suspension is applied to a glass slide and dried for 5 min at room temperature under a stream of nitrogen gas; notably, the time required to dry the sample is much shorter than the sublimation time required for freeze-drying, which is at least 4 h. Moreover, the equipment required for the evaporative drying is simpler and cheaper than a lyophillizer [36]. It is unclear whether evaporative drying is superior to freeze-drying for preserving mammalian spermatozoa, and studies on the long-term maintenance of the dried spermatozoa preserved without cryostorage will address this question. Developmental competence of ICSI-derived zygotes varies between laboratories and/or person doing ICSI. However, assessment of the chromosomal (DNA) integrity in dried spermatozoa will give us significant information on the ability of the spermatozoa to produce normal live offspring. Dried spermatozoa must have high levels of chromosomal (DNA) integrity to support normal development of ICSI-derived zygotes.

Importance of chromosomal (DNA) integrity in freeze-dried spermatozoa

The freezing, drying, and exposure to vacuum necessary to prepare freeze-dried samples are harmful to spermatozoa. Chromosomal (DNA) damage is likely to be induced in the spermatozoa during each step. Therefore, two questions arise: 1) Can zygotes with paternally transmitted chromosome aberrations develop into live offspring? 2) Does the chromosomal damage generated in freeze-dried spermatozoa pose genetic risks to successive generations?

Chromosomal damage induced in male germ cells contributes to early post-implantation death [37]. While, induction of so-called “minor aberrations” [38, 39] may be rather a serious event from the view point of genetic toxicology. Marchetti et al. [40] suggested that mouse embryos with a small number (less than 4) of paternally transmitted chromosome aberrations experienced problems in later embryonic stages. Mouse zygotes with structural chromosome aberrations generated spontaneously via ICSI can develop into live offspring carrying chromosome alterations [41, 42].

The fate of the structurally aberrant chromosomes has been examined in somatic and germ cells. Stable structural chromosome aberrations, especially reciprocal translocations induced by gamma radiation, can persist in mouse bone marrow cells in vivo for at least 30 days after irradiation [43]. Unbalanced karyotypes with chromosome aberrations such as deletions or partial trisomy can be derived from chromatid-type aberrations generated in cultured human lymphocytes [44]. Embryos with reciprocal translocations originating from mouse spermatozoa exposed to mutagenic compounds could develop into live offspring [40]. The frequency of embryos with structural chromosome aberrations originating from mouse spermatozoa exposed to γ-ray-irradiation (2 and 4 Gy) was reduced at the 2-cell stage, but increased at the 4-cell

145 stage [45]. This observation indicated that DNA double-strand breaks persisted in
146 embryos through the first cell division and new chromosome aberrations formed in
147 subsequent divisions [45]. Freeze-dried spermatozoa with extremely damaged
148 chromosomes (multiple aberrations) may inhibit blastocyst formation and
149 post-implantation development after ICSI. Some zygotes with fewer chromosome
150 aberrations inherited from freeze-dried spermatozoa are supposed to have the ability to
151 develop into live offspring. In this case, some types of aberrant chromosomes will be
152 transmitted to daughter cells (blastomeres). In the other case, some chromatid-type
153 aberrations may be converted to another aberration type during subsequent cell
154 divisions. Live offspring produced from spermatozoa with severe chromosomal damage
155 induced during improper freeze-drying are at high risk for abnormal karyotypes and
156 specific types of genetic alterations (e.g., microdeletions). Thus, improvement of
157 chromosomal integrity in freeze-dried spermatozoa is necessary not only for efficient
158 production of live offspring, but also to maintain the genetic background of the animal
159 strains being propagated with freeze-dried spermatozoa. Moreover, freeze-dried human
160 spermatozoa must be free from *de novo* induction of chromosomal damage to prevent
161 genetic disorders and related diseases.
162
163 **Classification of chromosomal damage induced in freeze-dried spermatozoa**
164 Chromosomal integrity in spermatozoa is likely to be adversely affected by
165 freeze-drying per se and post-freeze-drying storage at room temperature. Types of
166 chromosomal damage induced in freeze-dried spermatozoa may be classified as primary
167 chromosome damage (PCD) or accumulated chromosome damage (ACD). PCD is
168 induced just after freeze-drying. In contrast, ACD arises during post-freeze-drying

storage [46]. The types of PCD and ACD reviewed here are DNA damage and/or aberrant chromatin remodeling [46, 47]. Currently, it is unknown whether PCD and ACD can include numerical chromosome aberrations because no studies have focused on these types of aberrations in embryos derived from freeze-dried spermatozoa. Speculation on the mechanism causing PCD could be as follows. Hamster, human, and mouse spermatozoa contain an endogenous nuclease that requires Ca^{2+} and Mg^{2+} for enzymatic activity [48, 49]. The spermatozoa seemed to have the endogenous nuclease to cleave their own DNA. Fragmentation of sperm DNA was induced after sperm were incubated overnight in a medium supplemented with a detergent, Triton X-100. The DNA fragments were similar in size to those generated by DNase I [49]. However, it is still unclear why the nuclease was activated by the detergent. Reportedly, DNA fragmentation was also observed in frozen-thawed human spermatozoa [49] and in sonicated mouse spermatozoa following storage in culture media [50]; both observations indicate that the nuclease was activated.

Moreover, the PCD were induced severely when spermatozoa that had been freeze-dried in a standard culture medium containing Ca^{2+} and Mg^{2+} were microinjected into oocytes [30]. Electron microscopic examination showed that the sperm plasma membrane was removed upon treatment with Triton X-100 [51] and ruptured after freeze-drying [3]. Therefore, nuclei in freeze-dried spermatozoa must be exposed to high concentration of Ca^{2+} via the ruptured plasma membrane because oocytes subjected to ICSI show normal Ca^{2+} oscillations [52]. Thus, it is likely that the Ca^{2+}- and Mg^{2+}-dependent nuclease would be activated following damage to the plasma membrane and the subsequent influx of cations into sperm nuclei.

Chromatin of mouse testicular spermatozoa is more vulnerable to freeze-drying than

chromatin of epididymal spermatozoa [53]. Induction of PCD in testicular spermatozoa can be suppressed by treating the spermatozoa with diamide, an oxidizing agent that forms disulfide bonds (–S-S–) from sulfhydryl (–SH) groups in sperm protamines [53]. In testicular spermatozoa, sperm DNA in SS-poor chromatin will be more exposed to the Ca^{2+}- and Mg^{2+}-dependent nuclease than DNA in SS-rich chromatin.

Suppression of PCD induction

In 2001, it was discovered that a simple chelating solution suppressed PCD during freeze-drying of mouse spermatozoa. In contrast, PCD was severe in mouse spermatozoa freeze-dried in culture medium without cryoprotection [30]. Presumably, the chelating agent is an active component of the solution. The solution is composed of 50 mM sodium chloride (NaCl), 50 mM EGTA (ethylenglycol-*bis*(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid), and 10 mM Tris-HCl (EGTA Tris-HCl buffered solution: ETBS, pH 8.2-8.4) and is usually used to suspend naked DNA preparation for molecular biology protocols. The EGTA presumably inhibits the activity of Ca^{2+}-dependent nuclease by chelating Ca^{2+}, and a modified version of the solution adjusted to pH 8.0 was also developed [54]. Exclusion of NaCl from the solution may improve the developmental competence of zygotes derived from the freeze-dried spermatozoa. One such solution is TE buffer [29, 39]. TE buffer consists of 1 mM EDTA (ethylenediamine tetraacetic acid) and 10 mM Tris-HCl. The efficacy of specific chelating solutions for freeze drying of spermatozoa probably differs for different animal species, and solutions optimized for different species are likely to differ in some components and the concentrations of shared components. Reportedly, mouse spermatozoa freeze-dried in TE buffer supported the development of more offspring

than those freeze-dried in ETBS [39]. In contrast, boar spermatozoa freeze-dried in the ETBS seemed to support in vitro development of embryos better than boar spermatozoa freeze-dried in EDTA-based solutions [23].

EDTA is a standard supplement in cell culture media. EDTA binds a wide range of divalent cations, including Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, but EGTA preferentially binds Ca^{2+}. Moreover, EGTA is not an effective chelating agent for Zn^{2+}. In human spermatozoa, Zn^{2+} is presumed to play an important role in stabilizing sperm chromatin structure at ejaculation [55, 56]. Although EGTA chelates Ca^{2+} and, therefore, prevents activation of endogenous Ca^{2+}-dependent nuclease in spermatozoa, it does not affect the zinc status of sperm chromatin. In fact, fertile sperm donors have higher zinc content in their sperm chromatin than infertile men [55].

Mouse spermatozoa can be suspended in modified ETBS and kept in a refrigerator for 1 week before freeze-drying. The modified ETBS (50 mM EGTA + 100 mM Tris-HCl), unlike the original ETBS, does not contain NaCl [21]. Mouse spermatozoa suspended in the original ETBS lose mobility after incubation at 37°C for 10 min, whereas the majority of spermatozoa suspended in the modified ETBS maintain mobility after incubation at 37°C for 10 min. The modified ETBS allows for efficient collection of many motile spermatozoa. The modified ETBS seemed to be less toxic to mouse spermatozoa than original ETBS, and the modified ETBS can be used for freeze-drying of mouse cumulus and ES cells [57].

Interestingly, induced PCD is very severe in mouse spermatozoa that had been briefly suspended in modified ETBS just before freeze-drying. However, the level of PCD decreases as the pre-freeze-drying incubation time in modified ETBS is increased (up to 1 week at 4°C) (Fig. 1) [21].

Types of chromosome aberrations associated with PCD

PCD is observed in mouse spermatozoa freeze-dried in modified CZB medium [58, 59]. The frequency of each type of chromosome aberration in spermatozoa freeze-dried in CZB corresponds roughly to that in spermatozoa freeze-dried in modified ETBS without pre-freeze-drying incubation (Fig. 1). Most of the PCD is due to chromosome breaks (csb). The frequency of zygotes with other types of aberrations (e.g., chromosome exchange (cse), including dicentric chromosomes, ring chromosomes, and reciprocal translocation.) might be underestimated because the reciprocal translocations are not readily detected with conventional staining methods. The frequency of zygotes with csb decreases as the duration of the pre-freeze-drying incubation increases; consequently, overall levels of PCD decrease (Fig. 1).

Recently, Bignold [60] proposed mechanisms for the induction of clastogen-induced structural chromosome aberrations. The model invoked a failure in DNA-enzyme tethering during existence of enzyme-created DNA strand breaks. According to the model, formation of chromosome aberrations initiates with DNA double-strand breaks (DSBs) created by DNA-repair enzyme before DNA synthesis.

It is important to determine whether the chromosome breaks associated with PCD formed from DSBs generated in sperm DNA directly by freeze-drying. The origin of PCD can be assessed using single cell gel electrophoresis assay (comet assay) [46]. The comet assay is a well-known technique used to detect DNA damage in situ. The standard comet assay includes an alkali treatment of cells embedded in agarose gel on glass slides; this alkali treatment unwinds DNA, and the cells are then subjected to electrophoresis in an alkaline solution (pH 13 or higher). A modified version, the comet assay with the A/N protocol, consists of alkaline DNA unwinding and electrophoresis at

neutral pH [61–63]. The alkaline comet assay reveals single-strand breaks (SSBs), DSBs, and alkaline-labile sites (ALS) in DNA in somatic and germ cells [64–66]. Another version, called the neutral comet assay, reveals primarily DSBs; the electrophoresis is performed at neutral pH and without the alkali pre-treatment [67, 68].

The comet assay with the A/N protocol revealed significant DNA migration in freeze-dried spermatozoa indicating PCD, but the neutral comet assay did not reveal any damage [46]. According to hypothetical explanation for the induction of PCD, endogenous nuclease like DNase I, mentioned previously, might create "nicks" (i.e., SSB) in sperm DNA before or just after microinjection of freeze-dried spermatozoa into the oocytes. Immediately after SSB creation, an as-yet unidentified enzyme would convert the SSBs to DSBs before DNA synthesis. Enzymes such as single-strand-specific nuclease (e.g., S1 nuclease) might cleave single-stranded DNAs at the SSBs (Fig. 2).

Accumulated chromosome damage (ACD)

Chelating solutions used for freeze-drying sperm play an important role in suppressing the induction of PCD. Mouse spermatozoa freeze-dried in the modified ETBS seemed to have better chromosomal integrity than those freeze-dried in the original ETBS when stored at 4°C and 25°C for up to 3 months (Fig. 3). Unfortunately, neither the original ETBS nor modified ETBS seem to inhibit the accumulation of DNA damage in freeze-dried mouse spermatozoa stored at room temperatures. DNA damage in the freeze-dried spermatozoa, i.e. accumulation of as-yet unknown DNA modifications during storage, is referred to as accumulated chromosome damage (ACD). Identification of the causes of ACD should lead to vast improvement in the

developmental competence of oocytes injected with freeze-dried spermatozoa stored at room temperature.

Recent findings [46] suggest that chromosome breaks were the most common type of ACD (Fig. 4). However, when mouse oocytes were injected with spermatozoa freeze-dried in original ETBS, the frequency of chromatid exchange (the number of chromatid exchanges per zygote) increased after storage of sperm for 1 to 4 months at 22–24°C. Induction of chromatid exchanges seemed to be enhanced by heat-stress (50°C, 1 to 5 days) (Fig. 4). DNA damage in sperm induced by heat-stress was not detected using the neutral comet assay, but it was detected using the comet assay with the A/N protocol [46]. The type of damage induced directly in sperm DNA would be not the DSBs that are responsible for the induction of chromosome breaks resulting in chromosomal aberrations. SSBs or other types of DNA lesions, not including DSB, were probably associated with the formation of chromatid exchanges after DNA replication (Fig. 2b). These chromatid exchanges, also called quardriradials, are thought to form by the rejoining of two SSBs generated in two different chromosomes. Most of the SSBs created in sperm DNA will convert to DSBs via repair and/or replication enzymes present in the oocytes cytoplasm [69] to form the chromosome breaks that cause ACD. However, higher temperatures may induce steric alterations in the sperm chromatin or DNA [70, 71]. These hypothetical steric alterations in sperm DNA or chromatin could interfere with the binding of specific proteins (or enzymes) that are required for chromosome condensation [72] and with the conversion of SSBs to DSBs. The chromatid exchanges would form from the SSBs that persisted until the DNA replication stage (Fig. 2) [46].

Developmental competence of mouse spermatozoa preserved without cryostorage

The maximum time that freeze-dried spermatozoa can be stored and maintain the ability to support normal development of embryos, fetuses, and live offspring was estimated by several groups. Freeze-dried mouse spermatozoa can be stored indefinitely at −80°C without deterioration [73]. Li et al. [34] estimated that 90% of mouse spermatozoa preserved by evaporative drying lost the ability to produce offspring after storage at −80°C for 173 weeks (3.6 years) or storage at 4°C for 20 weeks (5 months). In contrast, other groups demonstrated that freeze-dried spermatozoa had no decline in the ability to support post-implantation development of zygotes during at least 1 year of storage at 4°C [74]. After 1.5 years of storage at 4°C, freeze-dried sperm were used to generate a sufficient number of healthy progeny to establish a breeding colony [74]. In addition, mouse spermatozoa freeze-dried in modified ETBS retained the ability to support development of normal fetuses when preserved at 4°C for up to 12 months (Fig. 5a) [21]. Nonetheless, there is no evidence that freeze-dried spermatozoa can be preserved indefinitely at 4°C. Freeze-dried spermatozoa deteriorate to a greater or lesser degree with increasing storage time, though the time that sperm maintain their integrity in storage differs between the protocols used to dry the spermatozoa (e.g., pressure and time for vacuuming, medium for suspending spermatozoa, size of vials, lyophillizer).

How long can freeze-dried spermatozoa be preserved at room temperature? Freeze-dried spermatozoa stored for 1 month at 25°C were less able to support development of normal live offspring than those stored for 3 months at 4°C [3]. Moreover, freeze-dried mouse spermatozoa stored at 24°C for 5 months did not produce offspring [75]. Kawase et al. [73] estimated that mouse oocytes injected with

freeze-dried mouse spermatozoa stored at 25°C for 1 month seldom developed into blastocysts. Most mouse spermatozoa preserved by evaporative drying and stored at 22°C for 1 month lost the ability to support the development of blastocysts [33, 34]. In contrast, a low proportion (11%) of 2-cell embryos derived from mouse spermatozoa freeze-dried in modified ETBS and stored for 12 months at 25°C developed into normal day-18 fetuses (Fig. 5b). Further improvement in the solutions used during freeze-drying may help in preventing or delaying the decline of chromosomal integrity.

Some studies analyzed the developmental competence of oocytes injected with unfrozen spermatozoa stored at room temperature. Mouse epididymal spermatozoa stored for 7 days at 22°C in TYH medium [76] lost motility, plasma membrane integrity, and acrosome integrity [77]. However, some oocytes fertilized in vitro by the spermatozoa stored for up to 3 days did develop into normal fetuses [77]. Van Tyuan et al. [78] reported that developmental competence of mouse oocytes microinjected with mouse spermatozoa stored at 27°C in KSOM medium containing amino acids and BSA [79] declined as the sperm storage period increase to 15 days, at which point the developmental competence reached zero. Spermatozoa taken from whole cauda epididymidis that had been preserved for 1 year at room temperature in powdered NaCl could activate oocytes [80]; however, most of these spermatozoa failed to support the development of zygotes into morula or blastocysts when the sperm were stored for 1 week to 1 month after isolation [80]. Sperm deterioration was never stopped by freeze-drying and any of the methods mentioned above other than freeze-drying.

Based on these studies, the dehydration, freeze-drying, and evaporative drying methods used to preserve mouse spermatozoa do not effectively prepare the spermatozoa for more than 1 month of storage at room temperature.

Heat-resistant nature of sperm-born oocyte-activating factor(s) in freeze-dried mouse spermatozoa

What do unfrozen spermatozoa lose during storage at ambient or higher temperatures? The majority of spermatozoa preserved in ETBS for 9 days at room temperatures (22–24°C) lose the ability to activate oocytes [81]. Some spermatozoa preserved in KSOM medium with amino acids and BSA and stored at 27°C maintain the ability to activate oocytes for a few weeks [78]. Perry et al. [82] demonstrated that mouse spermatozoa suspended in NIM medium lose the ability to activate oocytes if the spermatozoa are incubated at temperatures over 44°C for 30 min. Mouse spermatozoa incubated at 56°C (a temperature that inactivates HIV) for 30 min cannot activate oocytes [83]. In contrast, freeze-dried mouse spermatozoa heated continuously at 50°C for up to 7 days maintained the ability to activate oocytes [73]. Liu et al. [52] demonstrated that most oocytes microinjected with freeze-dried bovine spermatozoa heated at 56°C for 15 min exhibited a normal pattern of calcium oscillations. Sperm-borne oocyte-activating factor(s) (SOAF) [51] are likely to acquire heat resistance after freeze-drying, but we have no information on the inner temperatures of the glass ampoules that are vacuum-sealed after freeze-drying. At room temperature, the SOAF in freeze-dried spermatozoa may not be destroyed even after long-term preservation (>5 months) [39]. The most likely candidate for the SOAF is protein-based and sperm-specific, phospholipase C zeta [84–86]. Higher order structures composed of protein molecules should readily denature as temperatures rise. Thus, the characteristic of water-free sperm sample preserved under a vacuum is not well established.

Gamma-ray-resistance of freeze-dried mouse spermatozoa

It will be necessary to examine the effect of physical circumstances such as ultraviolet light, ionizing and non-ionizing radiation to deteriorate freeze-dried spermatozoa. We reported that chromosomes of mouse spermatozoa freeze-dried in ETBS were more resistant to γ-ray-irradiation (up to 8 Gy) than those of the spermatozoa suspended in ETBS [87]. This means that no significant difference of chromosomal integrity was observed between freeze-dried spermatozoa that had been exposed to γ-ray-irradiation and those that had not been exposed to the irradiation [87]. The resistance to ionizing irradiation may be a very important to maintaining the integrity of mammalian genomes during long-term storage of gametes as sperm preservation techniques advance.

Chromosomal integrity of freeze-dried human spermatozoa

To preserve the fertility of male patients undergoing cancer treatments; patients' spermatozoa are often cryopreserved in liquid nitrogen before chemo- and radiation therapies. Potentially, some spermatozoa can be freeze-dried and stored as a secondary stock to be used in the case of failure of the cryostorage facility. However, pilot studies to determine the proper protocol for freeze-drying human spermatozoa require many oocytes from laboratory animals, and there is little or no report on the relationship between developmental competency and chromosomal (DNA) integrity of freeze-dried human spermatozoa. Rudak et al. [88] directly analyzed human sperm chromosomes following in vitro fertilization of golden hamster oocytes with the fresh spermatozoa. In 1976, Uehara and Yanagimachi [19] demonstrated that freeze-dried human spermatozoa retain the ability to form sperm and oocyte pronuclei after injection into hamster

oocytes. Katayose et al. [17] demonstrated that freeze-dried human and hamster spermatozoa stored at 4°C between 6–12 months retain the ability to form sperm pronuclei after injection into hamster oocytes. In contrast, freeze-dried spermatozoa stored at 25°C retain the ability to form pronuclei for no more than 2 weeks of storage. These experiments indicated that pronuclear formation could be used as an assay in studies investigating the effects of preservation techniques and storage temperatures on damage accumulation in freeze-dried human spermatozoa. Hoshi et al. [20] reported that there was no significant difference between sperm pronuclear formation rates in hamster oocytes injected with freeze-dried (85%) and non-freeze-dried human spermatozoa (89%). These findings indicated that human oocytes injected with freeze-dried human spermatozoa may have the potential to develop into embryonic stages past the pronuclear stage. In contrast, freeze-dried human spermatozoa deteriorate within 2 weeks of their preservation when stored at ambient temperatures and are unable to support pronuclear formation.

To analyze human chromosomes without confusing chromosomes of mouse oocytes, freeze-dried human spermatozoa were injected into enucleated mouse oocytes [21]. In the protocol followed to freeze-dry the spermatozoa, a semen sample is allowed to liquefy at 37°C for 30 min, and then a 0.5 ml aliquot is carefully placed at the bottom of a small test tube containing 2 ml of modified ETBS pre-warmed to 37°C. Under these conditions, most mouse and human spermatozoa swim into the modified ETBS and remain motile for 10 min following the initiation of swimming. In contrast, mouse spermatozoa that swim into original ETBS stop moving within 10 min [30]. Therefore, modified ETBS may be superior to original ETBS for collection of the motile human spermatozoa prior to freeze-drying. Furthermore, the pre-freeze-drying incubation in

modified ETBS is unnecessary for suppressing the induction of PCD in freeze-dried human spermatozoa. It may be that EGTA penetrates human sperm nuclei more readily than mouse sperm nuclei.

Chromosome analysis of enucleated oocytes injected with human spermatozoa freeze-dried without pre-freeze-drying incubation demonstrated that 86–92% of the sperm-injected oocytes reached metaphase of the first mitosis [21]. These rates are similar to the rate (89.4%) previously reported for enucleated mouse oocytes injected with fresh human spermatozoa [89]. Of the sperm-injected oocytes reached metaphase, 91.1% possessed normal chromosome constitution [21]. This level of chromosomal integrity is almost same as background levels (roughly, 86–95%) reported for IVF using golden hamster oocytes and fresh human spermatozoa [90–93] and for ICSI of morphologically normal spermatozoa obtained from fertile or healthy men into mouse oocytes [94–97]. Moreover, results of multicolor multi-chromosome FISH analysis in human spermatozoa indicated that advanced male age increases the frequency of structural chromosome aberrations in sperm nuclei [98]. The overall mean frequency of spermatozoa with aberrations is 5.8%, and the aberrations are limited to structurally unbalanced rearrangements. Therefore, PCD induced in human spermatozoa is negligible as long as the spermatozoa are freeze-dried properly.

Freeze-drying and human spermatozoa with large vacuoles

The morphology of the freeze-dried spermatozoa may be important when selecting a spermatozoon for ICSI. The presence of large vacuoles in human spermatozoa has been noted for many years. In 1973, Bedford et al. [99] found a vacuole-like structure in human sperm heads that decondensed upon treatment with SDS and DTT. Berkovitz et

al. [100] reported that microinjection of vacuolated spermatozoa into oocytes reduced the pregnancy rate and was associated with early spontaneous abortion. Perdrix et al. [101] demonstrated that numerical chromosome aberrations and chromatin condensation defects occurred more frequently in teratozoospermic spermatozoa with large vacuoles than in those without large vacuoles. In addition, the presence of the large vacuoles in spermatozoa seems to be correlated with DNA fragmentation [102]. Human spermatozoa without large vacuoles can be selected in real time for assisted fertilization using morphologically-selected sperm for injection; this procedure has been named intracytoplasmic morphologically-selected sperm injection (IMSI). Reportedly, IMSI can also be used to select spermatozoa without aneuploidy [103]. In contrast, frequency of day 2 embryos derived from human spermatozoa showed no significant difference between conventional ICSI and IMSI [104]. Moreover, the presence of large vacuoles in sperm was correlated with induction of structural chromosome aberrations or DNA damage in fertile donors or fertile patients [105].

While some reports suggested that the vacuolated spermatozoa were correlated with chromosomal abnormalities in sperm, there is no direct evidence that large vacuoles partially or exclusively cause the chromosomal or DNA damage. It is unknown whether levels of PCD and ACD are higher in freeze-dried spermatozoa with large vacuoles than those with small or no vacuoles. In addition, it may be important to determine how the chromosomal integrity of freeze-dried human spermatozoa from fertile donors differs from freeze-dried spermatozoa from infertile patients and how chromosomal integrity differs between semen samples consisted of vacuole-rich and vacuole-poor sperm populations.

Conclusion

Examination of the chromosomal integrity of freeze-dried human and mouse spermatozoa may play an important role in improving the developmental competence of zygotes derived from these spermatozoa. PCD in mouse spermatozoa induced by freeze-drying can be suppressed by suspending the spermatozoa in chelating solutions prior to freeze-drying. In contrast, no current method can suppress ACD in freeze-dried mouse spermatozoa during post-freeze-drying storage, especially during storage at room temperature. Mouse fetuses were produced using freeze-dried mouse spermatozoa stored at 25°C for up to 1 year; however, increases in the rate of implantation loss indicated that ACD in spermatozoa occurred during storage. Moreover, the fetuses produced with freeze-dried spermatozoa subjected to long-term storage may have higher risks of genetic alterations. A better understanding the causes of ACD is an important first step in suppressing or preventing ACD in freeze-dried spermatozoa.

Freeze-drying may become available for preserving human spermatozoa in the future. Currently, however, we lack sufficient information on ACD in freeze-dried human spermatozoa. Moreover, whether chromosomal integrity of freeze-dried human spermatozoa differs between the spermatozoa with and without vacuoles is unknown. Therefore, we need to consider that freeze-dried spermatozoa stored for long-term periods may increase the risk of genetic alteration transmittable to newborns.

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Legends of figures

Figure 1

Primary chromosome damage (PCD) induced after freeze-drying of mouse spermatozoa. Spermatozoa freeze-dried in modified CZB (mCZB) and modified ETBS (mETBS) were stored at 4°C for up to 63 days [30] or up to 14 days [21], respectively. The PCD decreases as pre-freeze-drying incubation time in the mETBS increases. Abbreviations, csb: chromosome break; cse: chromosome exchange; ctb: chromatid break; cte: chromatid exchange. Aberrations such as chromosome fragmentation and multiple aberrations (10 or more aberrations per zygote) that could not be counted were excluded from the data set.

Figure 2

Schematic diagrams of hypothetical explanations of primary chromosome damage (PCD) and accumulative chromosome damage (ACD) in freeze-dried mouse spermatozoa. (a) A DNA single strand break (SSB) was probably created by enzymatic action before or immediately after intracytoplasmic sperm injection (ICSI) and resulted in initiation of PCD. (b) Unidentified DNA lesions other than SSBs may accumulate in DNAs of freeze-dried spermatozoa during their storage. Some of SSBs created at lesions may not be converted to DNA double strand breaks (DSBs). The SSBs that persisted until DNA replication stage may be responsible for the formation of chromatid-type aberrations.

Figure 3

Chromosomal integrity of zygotes derived from mouse spermatozoa freeze-dried in

839 ETBS (a) and modified ETBS (b). Frequency of zygotes with normal chromosome
840 constitution was expressed as the integrity per freeze-dried sample. Freeze-dried
841 spermatozoa were preserved at 4°C (○) or room temperatures of 22–24°C (●) (a) and at
842 4°C (○) or 25°C (●) (b).
843
844 Figure 4
845 Accumulated chromosome damage (ACD) in freeze-dried mouse spermatozoa. The
846 spermatozoa freeze-dried in ETBS and modified ETBS (mETBS) were stored at
847 room temperature or at 50°C. When using mETBS, the spermatozoa were
848 freeze-dried after pre-freeze-drying incubation in mETBS at 4°C or 25°C for 3 to 7
849 days [46]. Abbreviations, csb: chromosome break; cse: chromosome exchange; ctb:
850 chromatid break; cte: chromatid exchange. Aberrations such as chromosome
851 fragmentation and multiple aberrations (10 or more aberrations per zygote) that could
852 not be counted were excluded from the data set.
853
854 Figure 5
855 Post-implantation development of mouse ($B6D2F_1$) oocytes microinjected with
856 mouse ($B6D2F_1$) spermatozoa freeze-dried after pre-freeze-drying incubation in
857 modified ETBS at 4°C for 5 to 7 days (a) [21] and 25°C for 4 to 7 days (b)
858 (unpublished). Post-freeze-drying samples were stored for 3 and 12 months at the same
859 temperatures as the pre-freeze-drying incubation. The embryos were transferred into
860 CD-1 females (albino) on the first day of pseudopregnancy after being mated with
861 vasectomized CD-1 males (albino). Number of implants (white bars) is consistent with

862 total number of normal fetuses (black bars) and resorption sites examined on 14-day or
863 18-day gestation.
864 *Significantly different ($P < 0.05$) from the data obtained from the spermatozoa
865 preserved for 3 months by χ^2 comparison.
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Figure 1

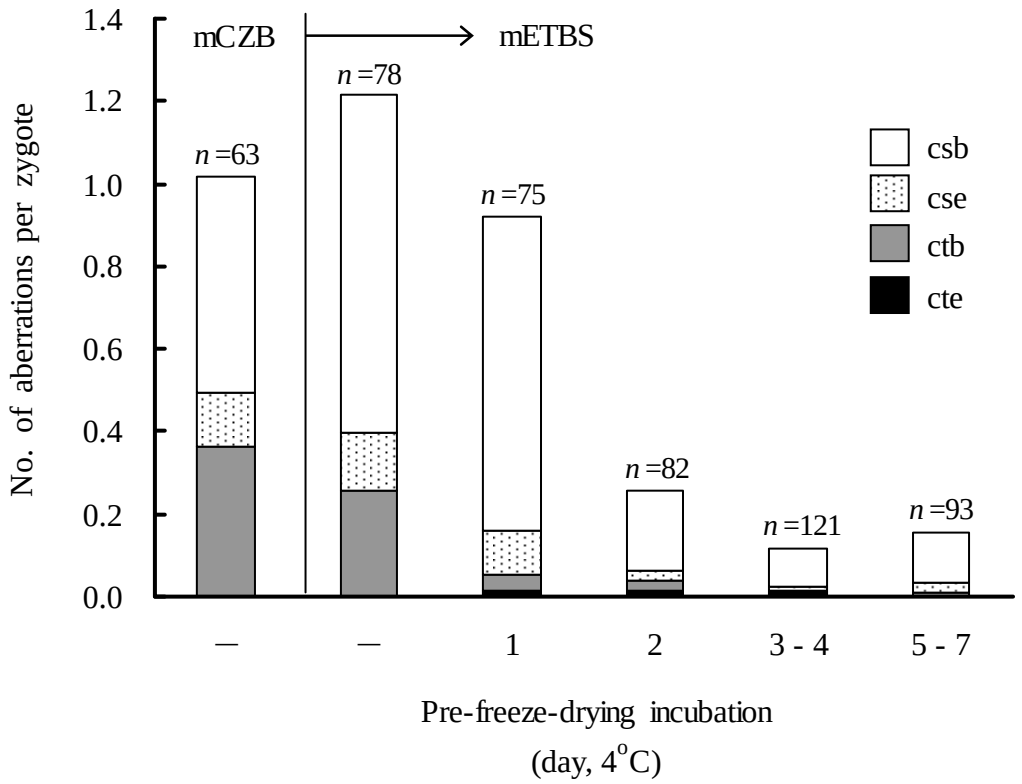

mCZB
mETBS
n =63
n =78
n =75
n =82
n =121
n =93
csb
cse
ctb
cte
No. of aberrations per zygote
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
—
—
1
2
3 - 4
5 - 7
Pre-freeze-drying incubation
(day, 4°C)

Figure 2

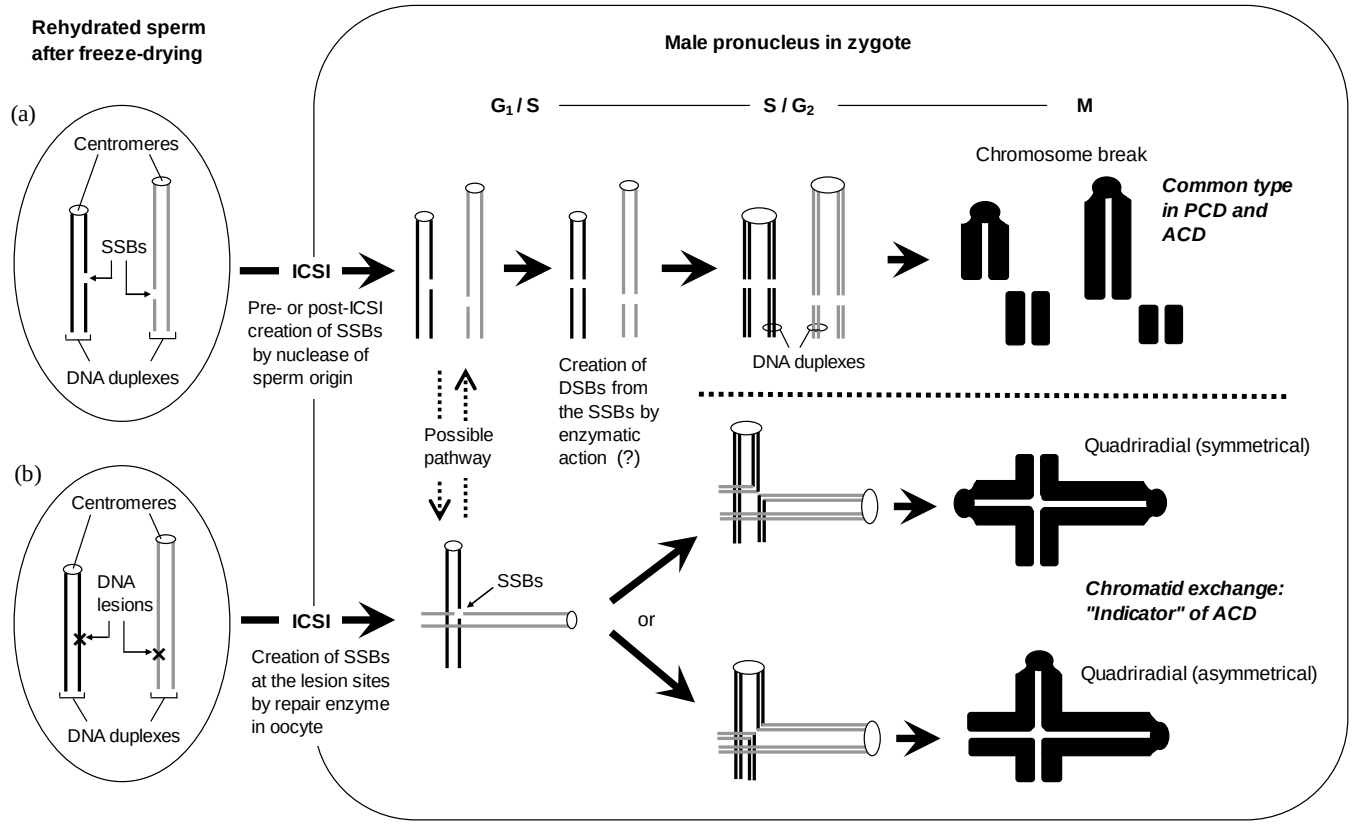

Rehydrated sperm after freeze-drying
Male pronucleus in zygote
(a)
Centromeres
SSBs
DNA duplexes
ICSI
Pre- or post-ICSI creation of SSBs by nuclease of sperm origin
G1 / S
S / G2
M
Chromosome break
Common type in PCD and ACD
Creation of DSBs from the SSBs by enzymatic action (?)
DNA duplexes
Possible pathway
(b)
Centromeres
DNA lesions
DNA duplexes
ICSI
Creation of SSBs at the lesion sites by repair enzyme in oocyte
SSBs
or
Quadriradial (symmetrical)
Chromatid exchange: "Indicator" of ACD
Quadriradial (asymmetrical)

Figure 3

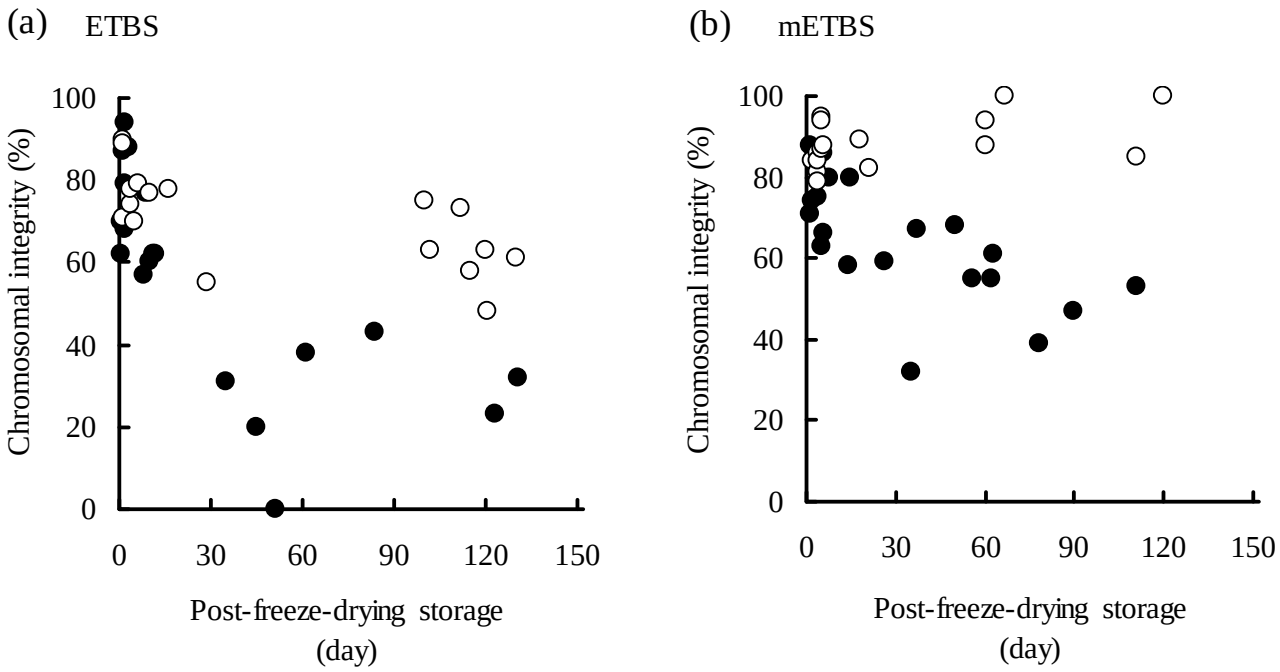

(a) ETBS
Chromosomal integrity (%)
100
80
60
40
20
0
0
30
60
90
120
150
Post-freeze-drying storage (day)
(b) mETBS
Chromosomal integrity (%)
100
80
60
40
20
0
0
30
60
90
120
150
Post-freeze-drying storage (day)

Figure 4

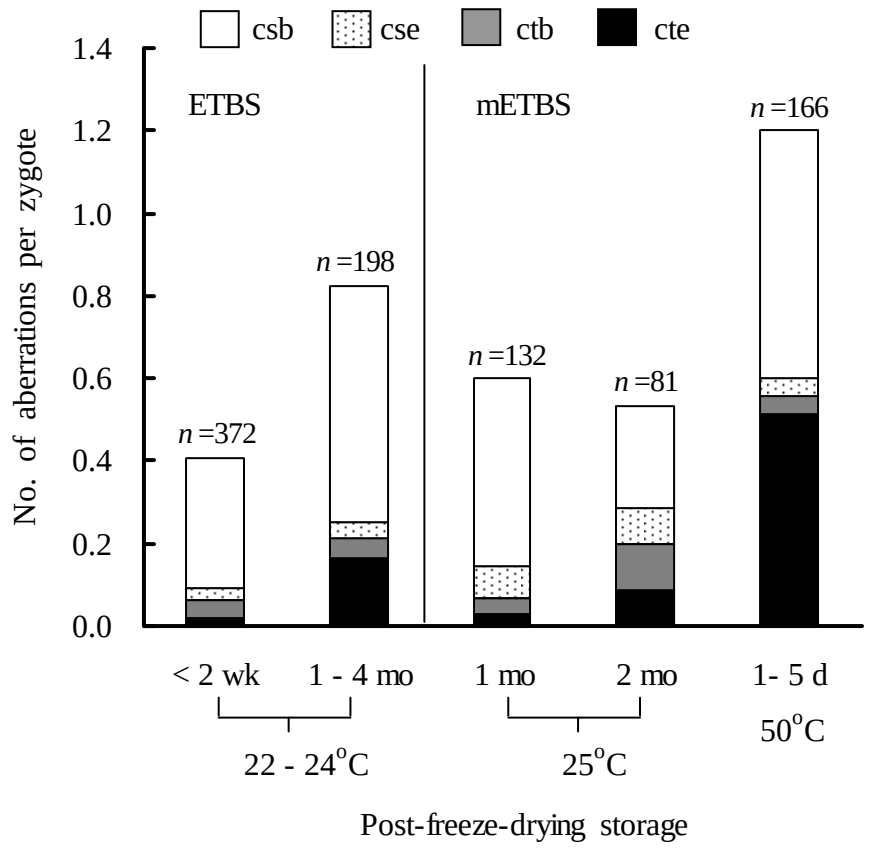
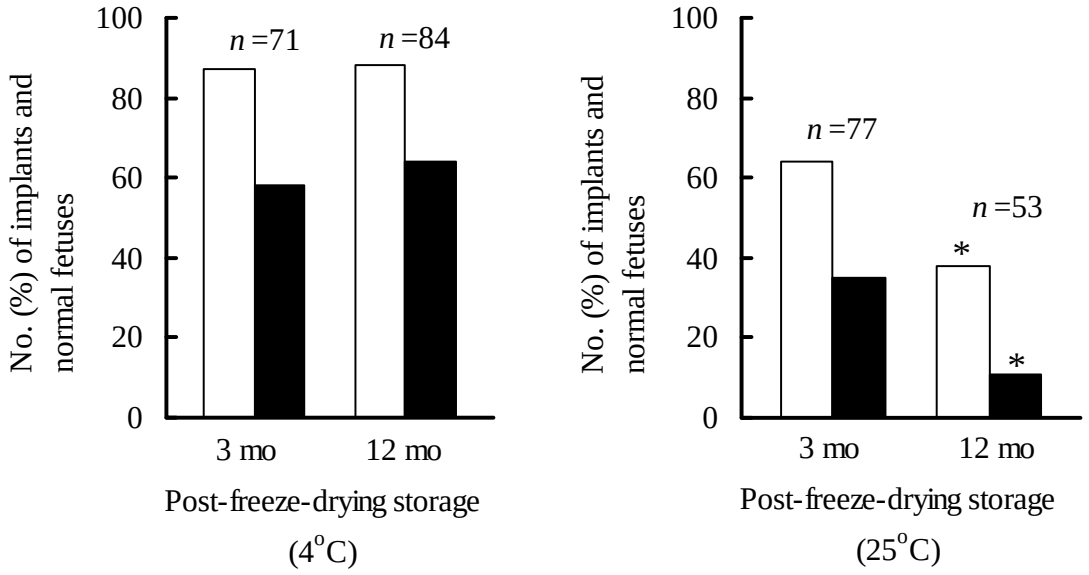

csb
cse
ctb
cte
ETBS
mETBS
No. of aberrations per zygote
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
n =372
n =198
n =132
n =81
n =166
< 2 wk
1 - 4 mo
1 mo
2 mo
1- 5 d
22 - 24°C
25°C
50°C
Post-freeze-drying storage

Figure 5


100
80
60
40
20
0
No. (%) of implants and normal fetuses
n =71
n =84
3 mo
12 mo
Post-freeze-drying storage
(4°C)
n =77
n =53
*
*
(25°C)