Reprod Dev Biol 39(3) : 69-76 (2015)
http://dx.doi.org/10.12749/RDB.2015.39.3.69

ISSN : 1738-2432 (Print)
ISSN : 2288-0151 (Online)

Revolution of Dead-Cell: Production of New Generation by Intracytoplasmic Dried-Sperm Injection in Mammal

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ABSTRACT

In a conventional sense, dried-spermatozoa are all dead and motionless due to the lost of their natural ability to penetrate oocytes both *in vivo* and *in vitro*. However, their nuclei are completely able to contribute to normal embryonic development even after long-term preservation in a dried state when the dried-spermatozoa are microinjected into the oocytes. In this sense, dried spermatozoa must still be alive. Thus, defining spermatozoa as alive or dead seems rather arbitrary. Several drying method of sperm including freeze-drying, evaporative/convective-drying and heat-drying were represented in this review. Although the drying protocol reported here will need further improvement, the results suggest that it may be possible to store the male genetic resources.

(Key words : Dried-spermatozoa, Genetic integrity, Genetic resource, ICSI, Embryonic development)

INTRODUCTION

The storage of spermatozoa is an important step in assisted reproduction technology (ART). As sperm motility is essential for fertilization, preservation of motility is indispensable in storing sperm. At present, spermatozoa are usually stored in a frozen form, using a cryoprotectant with the need for liquid nitrogen. However, a method by which spermatozoa could be stored for a long time without the need for liquid nitrogen was developed, such technique would not only be of economical benefit but also facilitate long distance transport of spermatozoa. One of the possible techniques is a desiccation. In a conventional sense, dried-sperm are all considered dead cells due to lose their natural ability to penetrate oocytes both *in vivo* and *in vitro* if they lose their motility, but their ability to contribute to early embryonic developmental competence can be investigated using the technique of intracytoplasmic sperm injection (ICSI) which is already established-technique in many mammalian species for producing live offspring when their spermatozoa lack motility, causing infertility.

Micromanipulation technology has been applied in the field of human assisted fertilization. Methods such as zona drilling, partial zona dissection, sperm injection into the perivitelline space and ICSI have been planned and human pregnancies have been reported. ICSI is the most direct micromanipulation method, for which sperm motility is unnecessary. Since Hiramoto (1962) was the first to record embryological development following injection of sperm cells into the eggs of sea urchins, the technique was applied to amphibians (Brun, 1974). In the later studies, Uehara and Yanagimachi (1976, 1977) first reported that nuclei of hamster spermatozoa microinjected into hamster oocytes could transform into well-developed pronuclei, ICSI has been applied to a variety of mammalian species such rabbits (Keefer, 1989), cattle (Goto, 1993), swine (Iritani *et al*., 1992), humans (Palermo *et al*., 1992; Bourne *et al*., 1995), mice (Ahmadi *et al*., 1995; Kuretake *et al*., 1996; Wakayama and Yanigimachi, 1998) and sheep (Catt *et al*., 1996). Live offspring have been obtained following ICSI in humans (Palermo *et al*., 1992, 1993; Van Steirteghem *et al*., 1993a, b), mice (Kimura and Yanagimachi, 1995; Lacham-Kaplan and Trounson, 1995), cats (Pope *et al*., 1997, 1998), horses (Cochran *et al*., 1998), sheep (Gomez *et al*., 1998), cattle (Hamano *et al*., 1999), rhesus monkeys (Hewitson *et al*., 1998) and swine (Martin, 2000). Furthermore, normal fertilization can also be obtained by injection isolated sperm heads into oocytes in cattle (Keefer, 1989), rabbits (Bourne *et al*., 1995), humans (Mansour *et al*., 1995) and swine (Nakai *et al*., 2003). Normal offspring have been obtained by injection sperm heads isolated from their tails by sonication and then freed from the plasma membrane and the acrosome by detergent treatment in

* This study was financially supported by Chonnam National University, 2014.

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mice (Kuretake *et al*., 1996; Ahmadi and Ng, 1997; Kimura *et al*., 1998; Ward et al., 2003). In this review, not only several methods on the sperm drying but also the history of fertilization using sperm dried by each drying method are described.

FERTILIZATION BY FREEZE-DRIED SPERM

Regarding freeze-dried spermatozoa, numerous studies have been published so far and summarized in Table 1. Freeze-drying is a process in which frozen material is dried through the sublimation of ice (Polge *et al*., 1949). The first successful fertilization in mammals using freeze-dried spermatozoa was reported by Yushchenko (1957) who obtained 12 normal rabbit offspring after artificial insemination of freeze-dried spermatozoa. Since the early days of research on the preservation of mammalian spermatozoa, there has been an interest in storing sperm in a dry state. Sperm from humans (Sherman, 1954), hamsters (Uehara and Yanagimachi, 1976), cattle (Jeyendran *et al*., 1981; Keskintepe *et al*., 2002), mice (Wakayama and Yanagimachi, 1998), rabbits (Liu *et al*., 2004), swine (Kwon *et al*., 2004) have all been preserved by freeze-drying with varying levels of success. These reports suggest that spermatozoa have lost their motility and membrane integrity but presumable have genetic integrity. The association of DNA with the sperm-specific basic proteins, protamines, serve to condense chromatin and render the sperm nuclei structurally stable and genetically inactive. Protamine disulfide bonds are known to confer unusual stability upon mammalian sperm nuclei (Perreault *et al*., 1988). Yanagida *et al*. (1991) examined the thermostability of mammalian sperm nuclei using ICSI method and reported that morphologically matured mammalian sperm nuclei do not lose the capability to form pronuclei or to synthesize DNA even when exposed to high temperature. The methods used in freeze-drying have varied. In general these techniques require elaborate protocols of freezing and vacuum drying or purchase of expensive freeze-drying equipment (Meryman *et al*,. 1963). One of the most important factors during the drying process is residual moisture content. Buitink *et al*. (1998) gave strong evidence that moisture content may affect storage of desiccated cells. Crowe *et al*. (1998) showed that preservation of liposomes with sugars required no residual water because sugars alone provided adequate stability. Gordon *et al*. (2001) implied that moisture content may affect viability of human mesenchymal stem cells, but no correlation between viability and moisture content was demonstrated. Plasma membrane integrity of mouse fibroblasts dropped off rapidly for moisture content <15%, thereby compromising cellular survival.

More recently, it has been reported that freeze-dried spermatozoa are able to produce normal blastocyst and live offspring when injected into oocyte in many mammalian species (see Table 1). These reports suggest that spermatozoa have lost their motility, acrosome integrity or plasma membrane integrity but have genetic integrity unmarred by freeze-drying. Let's see how to dry the sperm sample. Freeze-drying of sperm was performed as reported by many researchers (Keskintepe *et al*., 2002; Ward *et al*., 2003; Kwon *et al*., 2004; Liu *et al*., 2004; Watanabe *et al*., 2009; Li *et al*., 2009; Choi *et al*., 2011; Kaneko *et al*., 2014). Briefly, aliquots of 100−250 μl of the final sperm suspension in various solutions was transferred into eppendorf tubes or glass lyophilization vials, and the tubes or vials were directly plunged into liquid nitrogen for 20 sec −5 min. The tubes or vials were then placed in a precooled (−80℃) freeze-flask and the flask was attached to a freeze-drying system. The inside pressure of freeze-flask was varied. After various times under lyophilization, the flask was removed from the system and the tubes or vials were closed with or without removing air and firmly sealed and stored at 4℃, room temperature, −80℃ or below for various period. The freeze-dried sperm samples were rehydrated by adding 100−250 μl of milli-Q water, and immediately used for ICSI. In earlier report, it is interesting that the majority of the oocytes that survived the injection of head isolated from freeze-dried sperm was activated and fertilized normally (Wakayama and Yanagimachi, 1998). Perhaps, oocyte activation was induced by sperm-borne oocyte-activating molecules rather than by gamete membrane ligand receptor interactions. The majority of fertilized oocytes developed to blastocyst stage and developed to normal offspring when transferred to surrogate mother, and well grew normally (Wakayama and Yanagimachi, 1998; Liu *et al*., 2004; Kaneko *et al*., 2007). It is now clear that mammalian spermatozoa can retain their genetic integrity. Many similar reports have been published so far (see table 1). Recently, it has been shown that oocytes injected with long-term preserved drying sample can be fertilized. Oocytes injected with freeze-dried spermatozoa which was stored at 4℃ for 1 or 3 years developed to the blastocyst stage and developed to live offspring when transferred to surrogate mother (Kaneko and Nakagata, 2006; Hochi *et al*., 2008; Kaneko *et al*., 2009; Kaneko and Serikawa, 2012b). In addition, the fertility of freeze-dried sperm was maintained for 5 years without deterioration (Kaneko and Serikawa, 2012b).

Results in the literature demonstrated that the process of freeze-drying does not affect genetic integrity even though the cell membrane such as acrosomal membrane was damaged because the media including EGTA or trehalose adequately protected sperm during freeze-drying by preserving the viability of their nuclei (McGinnis *et*

Table 1. Final destination of freeze-dried spermatozoa

Species	Final destination	References
Mouse	Live offspring	Wakayama and Yanagimachi (1998)
Mouse	Live offspring	Kusakabe *et al.* (2001)
Mouse	Live offspring	Kaneko *et al.* (2003a)
Mouse	Live offspring	Kaneko *et al.* (2003b)
Mouse	Live offspring	Ward *et al.* (2003)
Mouse	Zygote	Kusakabe and Kamiguchi (2004)
Mouse	Blastocyst	Kawase *et al.* (2005)
Mouse	Zygote	Liu *et al.* (2005)
Mouse	Live offspring	Kaneko and Nakagata (2005)
Mouse	Live offspring	Kaneko and Nakagata (2006)
Mouse	Pregnancy	Kawase *et al.* (2007a)
Mouse	Pregnancy	Kawase *et al.* (2007b)
Mouse	Pregnancy	Kusakabe *et al.* (2008)
Mouse	Live offspring	Ono *et al.* (2008)
Mouse	Live offspring	Li *et al.* (2009)
Mouse	Pregnancy	Kawase *et al.* (2009)
Mouse	Zygote	Watanabe *et al.* (2009)
Mouse	Zygote	Kusakabe and Tateno (2011)
Mouse	Sperm	Kawase and Suzuki (2011)
Mouse	Live offspring	Kaneko and Serikawa (2012a)
Cattle	Sperm	Meryman and Kafig (1963)
Cattle	Sperm	Gravance *et al.* (1998)
Cattle	Blastocyst	Keskintepe *et al.* (2002)
Cattle	Zygote	Martins *et al.* (2007a)
Cattle	Blastocyst	Martins *et al.* (2007b)
Cattle	Zygote	Abdalla *et al.* (2009a)
Cattle	Zygote	Abdalla *et al.* (2009b)
Cattle	Blastocyst	Hara *et al.* (2011)
Cattle	Blastocyst	Hara *et al.* (2014)
Rat	Live offspring	Hirabayashi *et al.* (2005)
Rat	Live offspring	Kaneko *et al.* (2007)
Rat	Sperm	Yamashiro *et al.* (2007a)
Rat	Live offspring	Hochi et al. (2008)
Rat	Live offspring	Kaneko et al. (2009)
Rat	Live offspring	Kaneko and Serikawa (2012b)
Pig, cattle	Sperm	Pfaller *et al.* (1976)
Pig	Blastocyst	Kwon *et al.* (2004)
Pig	Blastocyst	Men *et al.* (2013)
Horse	Pregnancy	Batellier *et al.* (2001)
Horse	Sperm	Scherzer *et al.* (2009)
Horse	Live offspring	Choi *et al.* (2011)
Human	Sperm	Morris (2006)
Human	Sperm	Gianaroli *et al.* (2012)
Monkey	Zygote	Sanchez-Partida *et al.* (2008)
Monkey	Sperm	Tollner *et al.* (2011)
Deer	Sperm	Esteso *et al.* (2006)
Dog	Sperm	Yamashiro *et al.* (2007b)
Kangaroo	Sperm	Czarny *et al.* (2009)
Tilapia	Live offspring	Poleo *et al.* (2005)
Rabbit	Live offspring	Liu *et al.* (2004)
Wild animal	Zygote	Kaneko *et al.* (2014)

al., 2005; Martins *et al.*, 2007). Embryos obtained by injection of freeze-dried spermatozoa stored for short-term and long-term period can be develop to the stage of blastocyst and live offspring.

FERTILIZATION BY EVAPORATIVE-, CONVECTIVE- OR AIR-DRYING SPERM

Because of its ease of application and cost-effectiveness, evaporative/convective or air drying is an attractive alternative to traditional or simplified methods at ambient temperature for preserving mammalian spermatozoa (see Table 2). Briefly, 20 μl aliquots of the sperm suspension were placed on a sterile glass slide. The slide was then placed into the drying chamber where a continuous stream of compressed ultrapure-grade nitrogen gas through the chamber. The length of drying time was varied. After drying, a silicone isolater was placed around the dried sperm and covered with a sterile glass coverslip. Afterward, spermatozoa were vacuum-sealed using a vacuum sealer and stored at 4℃, ambient temperature or −80℃ for various period. The evaporative/convective drying sperm samples were rehydrated by adding 20 μl of milli-Q water, and immediately used for ICSI. On the other hand, for air-drying, semen was diluted and resuspended in solution. Smear was made on a sterile glass slide under laminar flow and left to dry for 15−20 min at room temperature. The dried sperm sample stored at 5℃ for various short period (Alonso *et al.*, 2014). Live fetuses and normal offspring were produced, providing the feasibility of the evaporative or convective drying procedure (Bhowmick *et al.*, 2003; McGinnis *et al.*, 2005; Li *et al.*, 2007a, 2007b, 2009; Liu *et al.*, 2012, 2014). The final moisture content (0.5-7%) of convective dried mouse spermatozoa did not affect the rate of blastocyst formation of injected oocytes as much as the rate of drying, and mouse sperm could survive desiccation by rapid convective drying to anhydrobiotic levels (≤0.1 g H_2O/g dry weight, 4.5% RW) with storage over-night at 4℃. When moisture content remained above −0.2 g H_2O/g dry weight, spermatozoa dried and stored at 4℃ retained high level of developmental potential to blastocysts even when stored for three months.

Results have been shown that the dried spermatozoa are capable for inducing fertilization and development of pre-implantation embryos when sperm were evaporatively/convectively dried and microinjected into normal viable oocyte. In particular, embryos derived by ICSI using evaporatively/convectively dried spermatozoa were transferred into surrogate mother to develop to healthy live-born offspring.

Table 2. Final destination of evaporative-, convective-, vacuum- and air-drying spermatozoa

Species	Final destination	References
Mouse	Pregnancy	Bhowmick *et al.* (2003)
Mouse	Pregnancy	McGinnis *et al.* (2005)
Mouse	Live offspring	Li *et al.* (2007a)
Mouse	Live offspring	Li *et al.* (2007b)
Mouse	Blastocyst	Elmoazzen *et al.* (2009)
Mouse	Live offspring	Liu *et al.* (2012)
Mouse	Live offspring	Li *et al.* (2009)
Mouse	Live offspring	Liu *et al.* (2014)
Monkey	Sperm	Meyers (2006)
Monkey	Blastocyst	Meyers *et al.* (2009)
Monkey	Blastocyst	Klooster *et al.* (2011)
Horse	Blastocyst	Alonso *et al.* (2014)
Human	Sperm	Lung and Bahr (1972)
Sheep	Blastocyst	Hollinshead *et al.* (2004)

FERTILIZATION BY HEAT-DRIED SPERM

Very little information is available on intracytoplasmic heat-dried sperm injection in mammalian and summarized in Table 3. Heat treatment does not appear to irrevocably damage spermatozoa. Sperm nuclei isolated from hamster, mouse and human spermatozoa heated to 90℃ for 30 min were able to form pronuclei when injected into hamster oocytes (Yanagida *et al*., 1991) and rabbit spermatozoa heated to 60℃ for 30 min and then injected into rabbit oocytes could support early embryonic development of the six- to eight-cell stage (Hoshi *et al*., 1992). More recently, mouse spermatozoa heated at 56℃ for 30 min were shown to support full embryonic development (Cozzi *et al*., 2001). Thus, mammalian spermatozoa appear to be highly resistant to nonphysiologically high temperatures. This ability of sperm nuclei to withstand drying and high temperatures give a chance to investigate whether spermatozoa could withstand drying by heating. This is much simpler and less expensive than either freeze-drying or evaporative/convective drying and would have useful applications in the preservation of male genomes of both laboratory and farm animals.

Based on the previous research background, microinjection process using heat-dried sperm has been tried for the first time as far as we know (Lee *et al*., 2006, 2013). In these cases, 100 μl aliquots of the sperm suspension were transferred to 2 ml vial bottles. The bottles then were heated in a dry oven at 50, 56, 90 and 120℃ for various times. After heating, the bottles were closed quickly with rubber caps without removing air or exchanging air with nitrogen gas, firmly sealed with parafilm, and stored at 4℃ for 7days to 12 months or 25℃ for 7 to 10 days. Heat-dried sperm samples were rehydrated by adding 100 μl aliquots of sterile distilled water to the bottles and then sonicated to separate heads from tails. Finally, the isolated sperm heads were microinjected into the oocytes. Residual water content resulted less than about 0.31 g H_2O/g dry weight and more than 80% of spermatozoa dried at lower temperature and stored at room temperature for 7−10 days appeared to be morphologically normal after rehydration. In particular, the proportion of acrosomal membrane damage was significantly higher in heat-dried spermatozoa than in unheated control. When oocytes injected with heads from heat-dried spermatozoa, the proportion of chromosomal damage was significantly increased in heat-dried spermatozoa than in unheated control, indicating chromosomes are damaged by the process of heat-drying. However, the ability of oocytes injected with heat-dried spermatozoa to develop to the blastocyst stage was not inhibited. The conceived recipient delivered live offspring when two-cell embryos derived from oocytes injected with heat-dried spermatozoa were transferred.

Table 3. Final destination of heat-dried spermatozoa

Species	Final destination	References
Cattle	Blastocyst	Lee and Niwa (2006)
Rat	Live offspring	Lee *et al.* (2013)

These results demonstrated that mammalian oocytes such as bovine and rat can be fertilized with heat-dried spermatozoa and that the fertilized oocytes can develop at least to the blastocyst stage. In addition, oocytes fertilized with heat-dried spermatozoa can also produce a full-term offspring.

CONCLUSION

Through the past in a span of twenty, numerous scientific reports have shown that sperm dried by various drying method are capable of producing normal embryonic development after microinjection into oocyte. Moreover, many researchers have made various improvements to the practical aspects of the drying process, with beneficial effects on the portion of embryo development. However, more work is needed to establish optimal systems such as practical application in preserving and transporting genetic resources. There still remain many areas which need to be studied, because it is essential to assure long-term preservation over several decades or centuries.

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(Received: August 12 2015/ Accepted: August 18 2015)