研究科分　麻布大学雑誌　第11・12巻・2005年

ブタ凍結乾燥精子の特性およびその精子からの産子作製法に関する研究

Researches on characteristics of freeze-dried sperm and offspring production from the sperm in the pig

柏崎直巳，紫野正雄，猪股智夫

獣医学研究科

Naomi Kahiwazaki, Masao Shino and Tomo Inomata

Graduate School of Veterinary Science, Azabu University

Abstract. Successful offspring production from freeze-dried (FD) sperm has been reported in laboratory animals but not in pigs. The integrity of the DNA in the FD sperm is reported to affect embryogenesis. Release of endonucleases from the sperm is one of the causes of induction of sperm DNA fragmentation. We examined the effects of chelating agents, which inhibit the activation of such enzymes, on DNA fragmentation in FD sperm, and on the *in vitro* and *in vivo* development of porcine oocytes. Boar spermatozoa were sonicated and suspended in buffer supplemented with 1) 50 mM EGTA, 2) 50 mM EDTA, 3) 10 mM EDTA, or 4) no chelating agent. A fertilization medium (Pig-FM) was used as a control. Spermatozoa were put into a glass ampoule, precooled at − 40 ℃ for 6 h, and lyophilized for 6 h. The ampoules were stored at 4 ℃ and the contents rehydrated with distilled water. The rehydrated spermatozoa were then incubated in Pig-FM at room temperature. The rate of DNA fragmentation in the control group, as assessed by the TUNEL method, increased gradually as time after rehydration elapsed. However, the rates in all experimental groups did not increase, even at 180 min. These rates were all significantly lower ($P < 0.05$) than that of the control group. Sperm heads from all experimental and control groups were incubated for 0–60, 60–120, or 120–180 min and then injected into *in vitro*-matured porcine oocytes. The rate of blastocyst formation in the control group was significantly lower ($P < 0.05$) than those in the 50-mM EGTA and 10-mM EDTA groups incubated for 120–180 min. Finally, we transferred oocytes injected sperm from 50-mM EGTA or control groups incubated for 0–60 min into recipients. One of the two recipients of the 50-mM EGTA oocytes became pregnant but aborted on day 29 after transfer. The two recipients of the control oocytes became pregnant, and one miscarried two fetuses on day 39. The results suggested that fragmentation of DNA in FD boar sperm is one of the causes of decreased *in vitro* development of injected oocytes to the blastocyst stage. Supplementation with 50 mM EGTA in a freeze-drying buffer improves this ability. Furthermore, porcine oocytes injected with FD sperm heads were able to grow to 39-day fetuses.

Introduction

Long-term storage of spermatozoa cryopreserved in liquid nitrogen (− 196 ℃) has been carried out in mammals for almost half a century since the first report (cattle, Stewart, 1951; sheep, Salamon & Lightfoot, 1967; pig, Pursel & Johnson, 1975; Westendorf *et al.*, 1975; human, Bunge & Sherman, 1953). Storage of semen in

frozen form is as useful method of preserving the germplasm from genetic resources of important economic traits and genetic diversity. However, this method requires expenditure on liquid nitrogen and space for storing containers. It has long been expected that the adaptation of freeze-drying technology for sperm preservation should enable sperm storage at ambient temperatures or at 4 ℃. Freeze-drying has been used for preserving viruses, bacteria, yeasts, and fungi, because freeze-dried materials are easy to store and transport without special equipment (Day & McLellan, 1995). However, freeze-dried spermatozoa lose their motility even after rehydration (Kusakabe *et al.*, 2001), and intracytoplasmic sperm injection (ICSI) is required for successful fertilization or embryo development. Viable offspring have been produced by ICSI of freeze-dried spermatozoa in mice (Wakayama & Yanagimachi, 1998; Kusakabe *et al.*, 2001; Kaneko *et al.*, 2003a, b), rabbits (Liu *et al.*, 2004), and rats (Hirabayashi *et al.*, 2005). However, in pigs (Kwon *et al.*, 2004) and cattle (Keskintepe *et al.*, 2002), it has been reported only that oocytes resulting from ICSI with freeze-dried spermatozoa have developed to the blastocyst stage.

The ICSI procedure renders immotile spermatozoa able to fertilize. However, there is currently a debate about the risk of sperm with abnormalities achieving fertilization (completion to the male pronucleus), because physiological selection processes such as binding to the zona pellucida, acrosomal reaction, and fusion to the ooplasm, are bypassed (Lopes *et al.*, 1998; Sun *et al.*, 1997). DNA fragmentation in human spermatozoa is one of the causes of failure of embryonic development and successful pregnancy (Henkel *et al.*, 2004). This suggests that the presence of structurally intact DNA in the sperm is quite important for normal embryogenesis after fertilization.

It has been suggested that endonucleases are among the causes of DNA fragmentation in spermatozoa (Kusakabe *et al.*, 2001). Sperm endonucleases are released from plasma membrane-damaged spermatozoa during freeze-drying or freezing without cryoprotectant ((Kusakabe *et al.*, 2001) and activated with divalent cation such as Ca^{2+} and Ma^{2+} Sotolongo *et al.*, 2005). However, activation of endonucleases is inhibited by the addition of chelating agents such as ethylene glycol-bis [beta-aminoethyl ether]-*N,N,N',N'*-tetraacetic acid (EGTA) to freeze-drying buffer (Kusakabe *et al.*, 2001), with the result that chromosome stability is maintained and the rate of development to offspring after ICSI is improved in mice (Kaneko *et al.*, 2003a). However, this improved effect has been reported only in mice. No experiments have been designed in other species, including pigs, to clarify the relationship between sperm endonucleases and DNA fragmentation of freeze-dried spermatozoa.

We therefore examined the effect of the addition of chelating agents such as EGTA or ethylenediamine-*N,N,N',N'*- tetraacetic acid, disodium salt (EDTA) to freeze-drying buffer in preventing fragmentation of boar sperm nuclear DNA. Furthermore, we investigated the relationship between DNA fragmentation in spermatozoa and *in vitro* or *in vivo* development of porcine oocytes following injection with freeze-dried boar sperm heads.

Materials and Methods

Sperm Collection and Freeze-drying

Ejaculated semen was collected from a boar of the Landrace breed. It was centrifuged for 10 min at 600 × *g* and the supernatant was discarded. The sperm pellet was resuspended in Modena solution (Weitze. 1991). The spermatozoa were then sonicated for 1 min to isolate the sperm heads from the tails. The spermatozoa were centrifuged again for 2 min at 600 × *g* and were resuspended in four different types of freeze-drying buffer: 1) 50 mM EGTA (no. 346-01312; Dojindo, Laboratories, Kumamoto, Japan) was added to the basic solution [50 mM NaCl and 1 M Tris-HCl (Kusakabe *et al.*, 2001)] (50 mM EGTA); 2) 50 mM EDTA (no. 345-01865; Dojindo) was added to the basic solution (50 mM EDTA); 3) 10 mM EDTA and 0.117 mM sorbitol were added to the basic solution (10 mM EDTA); and 4) 0.15 mM sorbitol was added to the basic solution (non-chelated medium). As a control, the spermatozoa were resuspended in a pig fertilization medium (Pig-FM; Suzuki *et al.*, 2002). The osmolality and pH of the buffers

1–4 were 265 mOsm/kg and pH 8.0, respectively. Those of the control buffer were to 305 mOsm/kg and pH 7.4, respectively. The concentration of spermatozoa in all buffers was adjusted to 3×10^8/ml. Sperm from each group were placed as a 100-μl suspension into a glass ampoule (φ 8 mm × 150 mm, Nakayamashoji, Tsukuba, Japan). Each ampoule was then precooled at − 40 ℃ for 6 h and attached to a freeze-drying system (DuraDry μP, FTS Systems, California, USA) for 6 h. The ampoule was then closed by heat from a gas burner and stored at 4 ℃.

Rehydration and Incubation of Freeze-dried Spermatozoa

Freeze-dried sperm samples were rehydrated in 100 μl distilled water. The sperm suspension was centrifuged for 2 min at 600 × *g* and the supernatant was removed. The pelleted spermatozoa were resuspended and incubated in Pig-FM for 0–180 min, according to the experimental design, at ambient temperatures.

TUNEL Assay

DNA fragmentation in spermatozoa was assessed by using a detection kit (*In situ* Cell Death Detection Kit, Fluorescein, Roche Applied Science, Tokyo, Japan). In accordance with the manufacturer's instructions the spermatozoa were centrifuged, fixed, and permeabilized. After two washings with 200 μl PBS, spermatozoa were incubated for 15 min at 37 ℃ in 50 μl of labeling solution containing TdT enzyme and dUTP. Spermatozoa were washed twice with PBS and observed under a standard inverse microscope (Olympus IX71, Tokyo, Japan) equipped with appropriate standard fluorescence facilities for green fluorescent protein (GFP) dye at a magnification of × 200 (Figure 1). For each group, the fluorescence of three independent samples with 300 cells each was evaluated.

Oocyte Collection and *In Vitro* Maturation (IVM)

Protocols for the use of animals were approved by the Animal Care Committee of the National Institute of Agrobiological Sciences and Azabu University, Japan. Ovaries were obtained from prepubertal cross-bred gilts (Landrace, Large White, and Duroc breeds) at a local slaughterhouse and transported to the laboratory at 35 ℃. Cumulus–oocyte complexes (COCs) were collected from follicles 2–6 mm in diameter in TCM 199 (with Hanks' salts; Sigma Chemical, St. Louis, MO) supplemented with 10 % (v/v) fetal bovine serum (Gibco, Life Technologies, Grand Island, NY), 20 mM Hepes (Dojindo Laboratories, Kumamoto, Japan), 100 IU/ml penicillin G potassium (Sigma), and 0.1 mg/ml streptomycin sulfate (Sigma). Maturation culture was performed as reported previously (Kikuchi *et al.* 2002, Nakai *et al.* 2003, 2006). In brief, about 40 COCs were cultured for 20–22 h in four-well dishes (Nunclon Multidishes; Nalge Nunc International, Rochester, NY), each well contains 500 μl of maturation medium. The medium was a modified North Carolina State University (NCSU)-37 solution (Petters & Wells 1993) containing 10 % (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 μM β-mercaptoethanol, 1 mM dibutyl cAMP (dbcAMP; Sigma), 10 IU/ml eCG (PMS 1000 Tani NZ; Nihon Zenyaku Kogyo, Koriyama, Japan), and 10 IU/ml hCG (Puberogen 1500 U; Sankyo, Tokyo, Japan). The COCs were subsequently cultured for 24 h in maturation medium without dbcAMP and without hormones. Maturation culture was carried out at 39 ℃ under conditions of CO_2, O_2, and N_2 adjusted to 5 %, 5 %, and 90 %, respectively (5 % O_2). After maturation culture, cumulus cells were removed from the oocytes by treatment with 150 IU/ml hyaluronidase (Sigma) and gentle pipetting. Denuded oocytes with the first polar body were harvested under a stereomicroscope and served as IVM oocytes.

Procedure of Sperm Head Injection and Oocyte Stimulation

Two solutions were prepared for ICSI; 1) for oocytes, a modified NCSU-37 without glucose but supplemented with 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, 4 mg/ml BSA, 50 μM β-mercaptoethanol (IVC-PyrLac; Kikuchi *et al.* 2002) and supplemented with 20 mM Hepes of which the osmolarity was adjusted to 285 mOsm/kg (IVC-PyrLac-Hepes; Nakai *et al.*, 2003, 2006), and 2) for sperm, IVC-PyrLac-Hepes supplemented with 4 % (w/v) polyvinyl pyrrolidone (MW 360,000; Sigma) (IVC-

PyrLac-Hepes-PVP). Sperm heads were injected as described previously (Nakai *et al.* 2003, 2006). Immediately before ICSI, the sperm suspension was again centrifuged for 2 min at 600 × *g* and resuspended in IVC-PyrLac-Hepes-PVP. About 20 oocytes were transferred into a 20-μl drop of IVC-PyrLac-Hepes. The solution containing the mature oocytes was placed on the cover of a plastic dish (Falcon 35-1005; Becton Dickinson and Company, Franklin Lakes, NJ). A small volume (0.5 μl) of the freeze-dried sperm head suspension was transferred to a 2-μl drop of IVC-PyrLac-Hepes-PVP, which was prepared close to the drops used for the oocytes. All drops were covered with paraffin oil (Paraffin Liquid; Nakarai Tesque, Inc., Kyoto, Japan). A single sperm head was aspirated from the suspension into an injection pipette, and the pipette was moved to the drop containing the oocytes. The sperm head was injected into the ooplasm by using a Piezo-actuated micromanipulator (PMAS-CT150; Prime Tech Ltd, Tsuchiura, Japan). One hour after the injection, the sperm head-injected oocytes (20 oocytes) were transferred to an activation solution consisting of 0.28 M D-mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.1 mg/ml BSA and washed once. They were then stimulated with a direct current pulse of 1.5 kV/cm for 20 μs by using a somatic hybridizer (SSH-10; Shimadzu, Kyoto, Japan).

In Vitro Culture of Sperm Head-injected Oocytes

Sperm head-injected oocytes before and after electrical stimulation were cultured *in vitro*. Two types of *in vitro* culture (IVC) medium were prepared (Kikuchi *et al.* 2002). The first was IVC-PyrLac. The second contained 5.55 mM glucose, as used in the original NCSU-37 medium reported, and was also supplemented with 4 mg/ml BSA and 50 μM β-mercaptoethanol (IVC-Glu). IVC-PyrLac was used from Day 0 (the day of injection and electrical stimulation) up to Day 2. The medium was changed once, to IVC-Glu, on Day 2, and this medium was used for subsequent culture. IVC was carried out at 38.5 ℃ under 5 % O_2.

Assessment of Embryonic Development

Embryos cultured for 6 days were mounted on glass slides and fixed in 25 % (v/v) acetic acid in ethanol, stained with 1 % (w/v) orcein in 45 % (v/v) acetic acid, and examined under a phase-contrast microscope. We examined the rate of blastocyst formation and mean number of cells per blastocyst.

Statistical Analysis

Acrosome status was examined and analyzed by the χ^2-test (Stat View; Hulinks, Tokyo, Japan). The percentage of spermatozoa with DNA fragmentation was scored. Embryonic development to the blastocyst stage (rate of blastocyst formation and mean number of cells per blastocyst) was evaluated. The percentage data were arcsine transformed (Snedecor & Cochran 1989). The data were subjected to analysis of variance (ANOVA) using the General Linear Model procedure and were then analyzed by Duncan's multiple range test (Statistical Analysis System institute, Cary, NC, USA).

Experimental Design

Experiment 1: Assessment of acrosome status in freeze-dried spermatozoa

We assessed acrosome status after freeze-drying. The acrosome status of frozen and thawed spermatozoa (Kikuchi *et al.*, 1998; Ikeda *et al.*, 2002) was also investigated. Rehydrated freeze-dried spermatozoa from the Pig-FM group and the frozen and thawed spermatozoa were placed on to glass slides and air-dried. The specimens were stained by a triple-stain technique (Talbot & Chacon. 1981; Kikuchi *et al.*, 1998; Ikeda *et al.*, 2002). The stained samples were examined under a microscope with a 100 × objective lens. Two types of spermatozoa were identified: 1) acrosome-intact cells and 2) acrosome-free cells (Figure 2). Three preparations were made for each sperm sample, and about 100 spermatozoa were observed in each preparation under the light microscope.

Experiment 2: Effect of chelating agents on DNA fragmentation after rehydration of freeze-dried spermatozoa

We examined the influence of chelating agents (EDTA and EGTA) on spermatozoal DNA fragmentation. After rehydration of the spermatozoa, the proportion with DNA fragmentation in each group was assessed by the TUNEL method as soon as the sperm had been centrifuged and resuspended in Pig-FM. Fresh ejaculated spermatozoa were also assessed. Furthermore, to examine the influence of duration of incubation after rehydration on the effects of chelating agents on sperm DNA fragmentation, spermatozoa in each group were incubated in Pig-FM at room temperature for 0, 60, 120, or 180 min after rehydration. The proportions of spermatozoa with DNA fragmentation were then examined.

Experiment 3: IVC of freeze-dried sperm head-injected oocytes

We examined the influences of time elapsed after rehydration and chelating agents on the *in vitro* developmental ability of oocytes injected with freeze-dried sperm heads from each group. Spermatozoa from each group were incubated in Pig-FM at room temperature for 0–60, 60–120, or 120–180 min and then injected into IVM oocytes. The oocytes were electrically stimulated 1 h after the injection, cultured *in vitro* for 6 days as described above, and fixed. Three replicated trials, using a total of 55–134 oocytes, were carried out for each group.

Experiment 4: Transfer of freeze-dried sperm head-injected oocytes

To evaluate the *in vivo* developmental ability of oocytes injected with freeze-dried sperm heads, we transferred oocytes injected with a rehydrated sperm, which had been incubated in Pig-FM or 50 mM EGTA for 0–60 min, to the oviducts of two synchronized recipients for each group (84–124 oocytes per a recipient). For the 50-mM EGTA group, we also transferred parthenogenetic oocytes with the sperm-injected oocytes to one of the recipients to increase the chance of pregnancy (Kawarasaki *et al.*, personal communication). Parthenogenetic embryos were generated by electro-stimulation with a direct current pulse of 2.2 kV/cm for 30 μs and incubated in IVC-PyrLac-Hepes supplemented with 10 μg/ml cytochalasin B (Sigma) at 37 ℃ for 3 h. Estrus in the recipient nonpregnant gilts was synchronized by an injection of 1000 IU of eCG and, 72 h later, an injection of 500 IU of hCG, as described previously (Kikuchi *et al.*, 1999; Kashiwazaki *et al.*, 2001; Nakai *et al.*, 2003). The sperm-injected or parthenogenetic oocytes were transported to the operating room at 37 ℃. Three hours after stimulation, the oocytes were transferred to both oviducts of the estrus-synchronized recipient gilts, in which ovulation was confirmed. Pregnancy was diagnosed in the recipients by using an ultrasound pregnancy detector (Medeta System Ltd., Arundel, West Susex, UK) at day 30 after oocyte transfer.

Results

Experiment 1

The percentage of sperm without acrosomes in the freeze-dried spermatozoa (86.7 %) was significantly ($P < 0.05$) higher than that in the frozen thawed spermatozoa (14.0 %). Almost all the spermatozoa after freeze-drying had their original head shape, regardless of the acrosomal findings.

Experiment 2

The percentage of sperm with DNA fragmentation in the Pig-FM group was significantly higher ($P < 0.05$) than in Fresh group, 50 mM EGTA, 50 mM EDTA, and 10 mM EDTA groups. The percentage of spermatozoa with DNA fragmentation in the Pig-FM group increased gradually with incubation time. On the other hand, the rates were not significantly different among other groups. The rate of DNA fragmentation in the Pig-FM group at 180 min (12.2 %) was significantly higher ($P < 0.05$) than those in the other groups (0.7 %–4.1 %).

Experiment 3

At 120–180 min, the percentage in the Pig-FM group (6.0 %) was significantly lower ($P < 0.05$) than those in the 50-mM EGTA group and the 10-mM EDTA group

Table 1. *In vivo* development of porcine oocytes after injection with freeze-dried sperm head.

Sperm[a]	Trial	No. of oocytes transferred	Pregnancy[b]
Pig-FM	1	124	+
	2	113	+
50 mM EDTA	1	100	−
	2	84[c]	not done

[a]Sperm was incubated for 0–60 min after rehydration.
[b]Pregnancy was diagnosed on the 30th day after the oocytes transfer.
[c]Parthenogenetic oocytes were also transferred to increase the chance of pregnancy.

(23.1 % and 22.6 %, respectively). At 120–180 min, the number in the 10-mM EDTA group (17.8 cells) was significantly lower ($P < 0.05$) than that in the 50-mM EGTA group (33.1 cells). The 50-mM EGTA group had the highest number, regardless of the incubation time after rehydration.

Experiment 4

The results of transfer of the embryos generated after freeze-dried sperm head injection to the recipients are shown in Table 1. In the Pig-FM group, both recipients were judged as being pregnant on day 29 after transfer. One of the pregnant recipients miscarried two fetuses on day 39 after the transfer. One seemed to have normal fetal development before the miscarriage. In the 50-mM EGTA group, one recipient miscarried on day 29 after oocyte transfer (we confirmed that the placenta had been excreted). The other was not diagnosed as being pregnant on day 29.

Discussion

Intactness of sperm DNA is important for embryonic development. Fatehi *et al.* (2006) suggested that DNA fragmentation in spermatozoa reduces the rate of blastocyst formation of ICSI oocytes in humans and that structural integrity of sperm DNA is important for embryonic development. Our study suggested, in pigs, that DNA fragmentation decreases ability to develop *in vitro* to the blastocyst stage. This was emphasized by the fact that the rate of blastocyst formation from the use of longer-incubated (120–180 min) sperm in the Pig-FM group, whose rate of DNA fragmentation was significantly higher than those of the other groups, was lower than in the other groups. It also suggested that the level of DNA fragmentation that occurred in spermatozoa during freeze-drying treatment depended on the solution used for treatment, because fragmentation was not observed in fresh ejaculated sperm. Sperm DNA fragmentation is caused by the action of endonucleases (Kusakabe *et al.*, 2001) or oxidative stress (Twigg *et al.*, 1998). The endonucleases are released from plasma membrane-damaged spermatozoa after freeze-drying or freezing procedures (Kusakabe *et al.*, 2001). Therefore, we examined the influence on sperm DNA fragmentation of supplementation with two kinds of chelating agents in freeze-drying buffers. The percentage of spermatozoa with DNA fragmentation in the Pig-FM group was significantly higher than in the 50 mM EGTA, 50 mM EDTA, 10 mM EDTA, or Fresh groups. These results suggested that the addition of EGTA or EDTA to sperm diluents is effective in protecting sperm DNA during freeze-drying treatment.

When EGTA or EDTA was added to sperm diluent, the proportions of spermatozoa with DNA fragmentation did not increase during incubation after rehydration. This is presumably because these agents remain on the surface of the sperm after washing and replacement of sperm diluent with Pig-FM. It is also possible that, when whole or part of the sperm membrane is damaged, agents that do not have membrane permeability—such as the agents used in here—can enter the sperm through the damaged area, thus preventing fragmentation of the sperm DNA. On the other hand, the proportion of sperm with DNA fragmentation gradually increased with time in the Pig-FM group, because no agent was included. However, in the non-chelated group, which did not have a chelating agent either, the proportion of sperm with DNA fragmentation did not increase. Although this phenomenon is not easily explained, the reason could be as follows. We added sorbitol to this diluent for non-chelated group to adjust the osmotic pressure. Sorbitol is used as a cryoprotectant for human sperm and protects the sperm membrane (Alvarez & Storey, 1993). As mentioned above, endonucleases are

released from sperm with damaged membranes (Sotolongo *et al.*, 2005) and easily injure the DNA (Ward *et al.*, 2003). These facts suggest that sperm DNA fragmentation might have been inhibited by supplementation with sorbitol in the non-chelated group because the integrity of the sperm membrane was maintained during freeze-drying.

We evaluated chelating agents and treatments for their suitability in freeze-drying of porcine spermatozoa. The percentages of sperm with DNA fragmentation in the 50-mM EDTA and 10-mM EDTA groups were almost the same as in the 50-mM EGTA group. These results suggest that EDTA and EGTA are equally effective in inhibiting endonucleases. However, the rates of blastocyst formation and the mean number of cells per blastocyst in the 50-mM EDTA and 10-mM EDTA groups were lower than those in the 50-mM EGTA group. The explanation for this discrepancy has not yet been clarified; however, there seems to be a possibility that a different mechanism participates in the selection of ions, depending on the agent. Divalent cations such as Ca^{2+} or Mg^{2+} play an important part in the control of metabolism in cells (Rubin, 1975). Considering the possibility that EGTA or EDTA remains on the surface of the sperm even after washing and replacement of sperm diluent with Pig-FM, an agent or agents may enter the ooplasm in association with the sperm head. EDTA chelates both ions easily; in contrast, EGTA chelates only Ca^{2+} (Sanui & Pace, 1967; Azuma *et al.*, 2002). An imbalance of these ions in sperm-injected oocytes may cause the discrepancy between the DNA fragmentation and developmental effects of the two agents. The influences of divalent cations on the metabolism and developmental ability of embryos should be further investigated. EGTA is a suitable chelating agent for adding to freeze-drying buffers for boar sperm, as reported in a previous study in mice (Kusakabe *et al.*, 2001).

In the embryo transfer experiment, we obtained two fetuses of day 39 day in the 0–60-min Pig-FM group, but in the 50-mM EGTA group one recipient aborted on day 29. Kure-bayashi *et al.* (2000) has already confirmed that parthenogenetic porcine diploid embryos have the ability to develop up to day 29 after transfer. Our results suggest that the aborted fetuses in the Pig-FM group might have developed from fertilization by ICSI, but that the abortion in the 50-mM EGTA group might have been caused by implantation of only the parthenogenetic embryos. However, in this group, the origin of the embryos (oocytes after ICSI or oocytes activated parthenogenetically) could not be confirmed from our results. These facts suggest that a short period (0–60 min) of incubation of boar sperm after freeze-drying, even without a chelating agent, enables fertilization and *in vivo* development to fetuses. After incubation for 0–120 min, there were no significant differences in DNA fragmentation and blastocyst development between Pig-FM and 50-mM EGTA groups; this might have been the reason behind our result that fertilization and fetal development to day 39 were obtained by chance only in the Pig-FM group. However, more careful attention needs to be paid to the effect of chelating agents on fetal development after embryo transfer.

In conclusion, our results suggest that fragmentation of DNA in freeze-dried sperm decreases the developmental ability of injected oocytes. EGTA would be suitable chelating for addition to freeze-drying buffer. Furthermore, our results clearly suggest that oocytes injected with sperm heads have the competence to grow to day 39 after oocyte transfer. However, there is still a problem in obtaining viable offspring from freeze-dried boar spermatozoa.

References

Alvarez, J.G. & Storey, B.T. (1993). Evidence that membranes stress contributes more thatn lipid peroxidation to sublethal cryodamege in cryopreserved human sperm: glycerol and other polyols as sole cryoprotectant. *J. Andol.* 14, 199-209.

Azuma, T., Kondo, T., Ikeda, S., Imai, H. & Yamada, M. (2002). Effects of EDTA saturated with Ca^{2+} (Ca-EDTA) on pig, bovine and mouse oocytes at the germinal vesicle stage during maturation culture and the involvement of chelation of Zn^{2+} in pronuclear formation induction by Ca-EDTA. *Reproduction* 124, 235-240.

Bunge, R.G. & Sherman, J.K. (1953). Fertilization capacity of frozen human spermatozoa. *Nature* 172, 767-768.

Day, J.G. & McLellan, M.R. (1995). Cryopreservation and freeze-drying protocols. In *Methods in Molecular Biology vol. 38*, eds S. Salzberg, D. Searls, & S. Kasif.

Fatehi, A.N., Bevers, M.M., Schoevers, E., Roelen, B.A., Colenbrander, B. & Gadella, B.M. (2006). DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apotosis after the first cleavages. *J. Androl.* 27, 176-188.

Henkel, R., Hajimohammad, M., Stalf, T., Hoogendijk, C., Mehnert, C., Menkveld, R., Gips, H., Schill, W. & Kruger, T. (2004). Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil. Steril.* 81, 965-972.

Hirabayashi, M., Kato, M., Ito, J. & Hochi, S. (2005). Viable offsprong derived from oocytes intracytoplasmically injected with freeze-dried sperm heads. *Zygote* 13, 79-85.

Ikeda, H., Kikuchi, K., Noguchi, J., Takeda, H., Shimada, A., Mizokami, T. & Kaneko, H. (2002). Effect of preincubation of cryopreserved porcine epididymal sperm. *Theriogenology* 57, 1309-1318.

Kaneko, T., Whittingham, D.G. & Yanagimachi, R. (2003 a). Effects of pH value of freeze-drying solution on the chromosome integrity and development ability of mouse spermatozoa. *Biol. Reprod.* 68, 136-139.

Kaneko, T., Whittingham, D.G., Overstreet, J.W. & Yanagimachi, R. (2003 b). Tolerance of the mouse sperm nuclei to freeze-drying depends on their disulphide status. *Biol. Reprod.* 69, 1859-1862.

Kashiwazaki, N., Kikuchi, K., Suzuki, K., Noguchi, J., Nagai, T., Kaneko, H. & Shino, M. (2001). Development in vivo and in vitro to blastocysts of porcine oocytes matured and fertilized in vitro. *J. Reprod. Dev.* 47, 303-310.

Keskintepe, L., Pacholczyk, G., Machinicka, A., Norris, K., Curuk, M.A., Khan, L. & Brackett, B.G. (2002). Bovine blastocyst development from oocytes injected with freeze-dried spermatozoa. *Biol. Reprod.* 67, 409-415.

Kikuchi, K., Onishi, A., Kashiwazaki, N., Iwamoto, M., Noguchi, J., Kaneko, H., Akita, T. & Nagai, T. (2002). Successful piglet production after transfer of blastocysts produced by a modified in vitro system. *Biol. Reprod.* 66, 1033-1041.

Kikuchi, K., Nagai, T., Ikeda, H., Noguchi, J., Shimada, A., Soloy, E. & Kaneko, H. (1998). Cryopreservation and ensuing in vitro fertilization ability of boar spermatozoa from epididymides stored at 4 ℃. *Theriogenology* 50, 615-623.

Kikuchi, K., Kashiwazaki, N., Noguchi, J., Shimada, A., Takahashi, R., Hirabayashi, M., Shino, M., Ueda, M. & Kaneko, H. (1999). Developmental competence, after transfer to reciepients, of porcine matured, fertilized, and cultured in vitro. *Biol. Reprod.* 60, 336-340.

Kure-bayashi, S., Miyake, M., Okabe, K. & Kato, S. (2000). Successful implantation of in vitro matured, electro activated oocytes in the pig. *Theriogenology* 53, 1105-1119.

Kusakabe, H., Szczygiel, M.A., Whittingham, D.G. & Yanagimachi, R. (2001). Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa. *Proc. Natl. Acad. Sci. U.S.A.* 98, 13501-13506.

Kwon, IK., Park, K.E. & Niwa, K. (2004). Activation, pronuclear formation, and development in vitro of pig oocytes following intracytoplasmic injection of freeze-dried spermatozoa. *Biol. Reprod.* 71, 1430-1436.

Liu, J.L., Kusakabe, H., Chang, C.C., Suzuki, H., Schmidt, D.W., Julian, M., Pfeffer, R., Bormann, C.L., Tian, X.C., Yanagimachi, R. & Yang, X. (2004). Freeze-dried sperm fertilization leads to full-term development in rabbits. *Biol. Reprod.* 70, 1776-1781.

Lopes, S., Sun, J.G., Jurisicova, A., Meriano, J. & Casper, R.F. (1998). Sperm deoxyribonucleic acid fragmentation is increased in poor quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil. Steril.* 69, 528-532.

Nakai, M., Kashiwazaki, N., Takizawa, A., Hayashi, Y., Nakatsukasa, E., Fuchimoto, D., Noguchi, J., Kaneko, H., Shino, M. & Kikuchi, K. (2003). Viable piglets generated from porcine oocytes matured in vitro and fertilized by intracytoplasmic sperm head injection. *Biol. Reprod.* 68, 1003-1008.

Nakai, M., Kashiwazaki, N., Takizawa, A., Maedomari, N., Ozawa, M., Noguchi, J., Kaneko, H., Shino, M. & Kikuchi, K. (2006). Morphological changes in boar sperm nuclei with reduced disulfide bonds in electrostimulated porcine oocytes. *Reproduction* 131, 603-611.

Petters, R.M. & Wells, K.D. (1993). Cultured of pig embryos. *J. Reprod. Fertil. Suppl.* 48, 61-73.

Pursel, V.G., & Johnson, L.A. (1975). Freezing of boar spermatozoa: fertilizing capacity with cocncentrated semen and a new thawing procedure. *J. Anim. Sci.* 40, 99-102.

Rubin, H. (1975). Central role for magnesium in coordinate control of metabolism and growth in animal cells. *Proc. Nat. Acad. Sci. U.S.A.* 72, 3551-3555.

Salamon, G.W. & Lightfoot, R.J. (1967). Fertilization and embryonic loss in sheep after insemination with deep frozen semen. *Nature* 216, 194-195.

Sanui, H. & Pace, N. (1967). Effect of ATP, EDTA and EGTA on the simultaneous binding of Na, K, Mg and Ca

by rat liver microsomes. *J. Cell. Physiol.* 69, 11-20.

Snedecor, G.W. & Cochran, W.G. (1989). Statistical Methods, 8th ed. pp.273-296. Ames: The Iowa State University Press.

Sotolongo, B., Huange, T.T.F., Isenberger, E. & Ward, W.S. (2005). An endogenous nuclease in hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments. *J. Androl.* 26, 272-280.

Stewart, D.L. (1951). Storage of bull spermatozoa at low temperatures. *Vet. Rec.* 63, 65-66.

Sun, J.G., Jurisicova, A. & Casper, R.F. (1997). Detection of deoxyribonucleic acid fragmentation in human sperm: crrelation with fertilization in vitro. *Biol. Reprod.* 56, 602-607.

Suzuki, K., Asano, A., Eriksson, B., Niwa, K., Nagai, T. & Rodriguez-Martinez, H. (2002) Capacitation atatus and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. *Int. J. Androl.* 25, 84-93.

Talbot, T. & Chacon, R.S. (1981). A triple-stain technique for evaluating normal acrosome reactions of human sperm. *J. Exp. Zool.* 215, 201-208.

Twigg, J.P., Irvine, D.S. & Aitken, R.J. (1998). Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Hum. Reprod.* 13, 1864-1871.

Wakayama, T. & Yanagimachi, R. (1998). Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nat. Biotechno.* 16, 639-641.

Weitze, K.F. (1991). Long-term storage of extended boar semen. *Reprod. Domes. Anim. Suppl.* 1, 231-253.

Ward, M.A., Kaneko, T., Kusakabe, H., Biggers, J.D., Whittingham, D.G.. & Yanagimachi, R. (2003). Long-term preservation of mouse spermatozoa after freeze-drying and freezing without cryoprotection. *Bol. Reprod.* 69, 2100-2108.

Westendorf, P., Richter, L. & Treu, H. (1975). Deep freezing of boar semen: laboratory findings and insemination results with the Hulsenberger Pailleten technique. *Dtsch. Tierarztl. Wschr.* 82, 261-300.