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Boar sperm preservation by freeze-drying

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Abstract. As a consequence of the assisted reproductive technology development, semen dehydration was reconsidered as a possible alternative strategy in animal germplasm long-term preservation. Species with sperm that is more sensitive to low temperature are given special attention. Here, we investigate some aspects related to boar semen freeze-drying protocol, investigating drying periods of 4 and 8 hours and storage temperatures of 4 and 20°C. Freeze-dried Mangalita spermatozoa (after rehydration) were evaluated for membrane integrity (sperm viability, hypo-osmotic swelling test and acrosomal integrity), DNA integrity (DNA fragmentation index) and intracytoplasmic sperm injection (ICSI). Results showed a better resistance of acrosomal membranes compared to plasma membranes. Despite the low percentage of live sperm observed following rehydration, embryos were obtained after ICSI using freeze-dried spermatozoa. In conclusion, freeze-dried spermatozoa could be used for Mangalita germplasm preservation for subsequent embryo production by ICSI.

Key Words: boar spermatozoa, assisted reproductive technology, DNA-fragmentation, ICSI, germplasm preservation.

Introduction. The importance of preserving genetic resources for the next millennium is widely recognized. "Germplasm preservation can provide a cost-effective alternative to the maintenance of breeding colonies for the preservation of rare genotypes and can also provide the means to prevent the loss of rare genotypes" (Hettig et al 2012). Sperm preservation will have a major contribution with great potential applications in agriculture, biotechnology, species conservation and clinical medicine (Yoshida 2000). Long-term preservation of sperm requires the arrest of the cells' metabolism, which can be obtained by freezing them in liquid nitrogen (cryopreservation) or by dehydration (lyophilization). Lyophilization is an alternative method of preservation, earlier proposed for human spermatozoa by Sherman (1954) and for bovine by Bialy & Smith (1957). Lyophilization can be achieved by the conventional heat-drying method (based on evaporation), or by freeze-drying (based on sublimation). Freeze-drying offers advantages over conventional heat-drying by minimizing damage and loss of activity in heat-labile products such as enzymes and hormones (Hochi et al 2011). The main advantages of the freeze-drying method are that spermatozoa can be stored at room temperature in atmospheric pressure, and can be used in males whose spermatozoa are difficult to preserve by freezing. Although freeze-dried spermatozoa after rehydration lose their motility, which is an essential requirement to complete physiological fertilization, the feasibility of this approach is supported by data providing evidence that viable offspring were obtained by intracytoplasmic sperm injection (ICSI) in mice (Wakayama & Yanagimachi 1998), rabbits (Liu et al 2004), rats (Hirabayashi et al 2005), hamsters (Muneto & Horiuchi 2011) and horses (Choi et al 2011). In a recent study, Kaneko & Serikawa (2012) reported successful freeze-drying of sperm using a new and simple method for long-time preservation. Evaluation of long-term preservation exceeding 1 year of freeze-dried sperm is indispensable in the application of this new preservation method for bio-banking. Offspring were obtained from oocytes fertilized by ICSI with rat epididymal sperm treated with diamide before freeze-drying and stored at 4°C for 5 years.

The aim of the present study was to investigate some aspects related to boar semen freeze-drying protocol, including drying period and storage temperature.

Material and Methods

Semen collection and freeze-drying. Semen was collected from Mangalita breed using gloved-hand method and extended with Beltsville Thawing Solution (BTS; Minitüb, Tiefenbach, Germany) at a 1:1 ratio for transportation. The semen with at least 70% motility and 80% morphological integrity was kept for 2 h at room temperature (20°C). Freeze-drying was made by using a modified method described by Kwon et al (2004). According to this, 10 mL diluted semen were centrifuged at 600 x *g* for 10 min and the pellet spermatozoa were washed twice with 10 mL TL (Tyrode lactate) by centrifugation at the same parameters. Each of 150 µL of the final sperm suspension in 10 mL TL was transferred in 2 mL cryovial, which were directly plunged into LN2 for 20 sec. The cryovials were then placed in a precooled (-80°C) box until they were transferred to the freeze-drying system (Christ, Alpha 1-2 LD plus). After 4–8 h under lyophilization, the flask was removed from the system and the cryovials were closed without removing oxygen and stored at refrigerator (4°C) or room temperature (20°C) for 1–6 mo. Freeze-dried sperm samples were rehydrated by adding 150 µL of Milli-Q water and 1 mL of TL 5 min later.

Sperm evaluation

Assessment of membrane integrity (sperm viability, hypo-osmotic swelling test and acrosomal integrity). The percentages of viable sperm were determined by eosin-nigrosin staining as described by Dott & Foster (1972). Evaluation was undertaken using a bright-field microscope at 1000x magnification with 200 sperm being examined for each smear. The percentage of HOST positive cells was calculated by placing a 0.1 mL semen sample into 1 mL of HOS (0.0375 mg/mL Natriumcitrat x 2H₂O and 13.5 mg/mL fructose (Roth); 100 mOsm/kg) in a water bath at 37°C for one hour. After incubation a drop of the sperm suspension was placed on the slide and covered with a glass coverslip. The slides were evaluate at phase contrast microscope using 400 x magnification and were counting 200 sperm cells those with any degree of a coiled tail (HOST positive cells) and those with a straight tail (negative HOST cells). The evaluation of acrosomal integrity was made by examining formalin saline fixed samples (2.9 g tri-Natriumcitrat x 2H₂O, 4 mL of 37% formalin and 96 mL of MilliQ water) with a phase-contrast microscopy at 1000 x magnification. At least 200 acrosomes per sample were examined in order to establish acrosomal integrity by normal apical ridge (NAR) and classified using the scoring system reported by Pursel et al (1972).

DNA integrity (DNA fragmentation index). For DNA fragmentation index (DFI) semen was processed according to the instruction of the Sperm-Sus-Halomax[®] kit (ChromaCell SL, Madrid, Spain). For a better examination, spermatozoa from each group were diluted in TL to give a final concentration of 5-10 x 10⁶ spermatozoa per mL. After 5 min in a 90–100°C water bath, vials with agarose were left into a thermostatic water bath at 37°C to equilibrate for 5 min. When the agarose reached 37°C, 25 µL of semen were added to the vial and mixed with a pipette. Then, a drop of the cell suspension was placed on a previously treated and pre-cooled (5°C) slide and it was covered with a glass coverslip at 4°C for 5 min. The coverslip was smoothly removed and the slide was introduced into 10 mL of the lyses solution and maintained for 5 min at room temperature. The slide was then washed in MilliQ water for 5 min, dehydrated in sequential 70 and 100% ethanol baths for 2 min each and air dried. Just before analyzing under bright-field microscopy, the slides were immersed for 5-10 min in staining solution A and B. According to the kit instructions, spermatozoa showing a halo of dispersion equal or wider than the core minor diameter were considered positive for high DFI.

Intracytoplasmic sperm injection (ICSI). Porcine ovaries were collected from pre-pubertal gilts at a slaughterhouse. Contents of follicles of 2–6 mm in diameter were aspirated using a 10 mL syringe with a 18-gauge needle and collected in Petri dishes containing collection

medium (M 199 with HEPES and Earle's salt supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin). Oocytes with a uniform ooplasm and compact cumulus cells were washed twice with collection medium. Groups of 5 oocytes were placed into 30 µL droplet of M 199 maturation medium supplemented with 10 UI/mL Chorulon, 10 UI/mL Folligon, 10% FCS, 1 mg/mL sodium pyruvate and 0.1 mg/mL cysteine. The droplets were covered with mineral oil and incubated at 37.5°C in a 5% CO₂ air for 24 h with an addition of hormones and 20 h more, without hormonal additives. Thereafter, cumulus cells were mechanically removed using a micropipette and the denuded oocytes were washed three times and placed in groups of 5 oocytes in 30 µL droplets of TALP-fert medium (Tyrode's saline solution supplemented with 4 mg/mL BSA, 10 µl/mL sodium lactate, 0.288 µg/mL sodium pyruvate, 100 IU/mL penicillin and 100 µg/mL streptomycin). The mature oocytes were injected with a single spermatozoon, using a Narishige micromanipulation station, equipped with Eppendorf holding pipettes and injection needles, under an inverted contrast heated stage Olympus XI-51 microscope. The mature oocytes were placed with the first polar body at 6 or 12 o'clock, and the injection was made at 3 o'clock. Sperm rehydrated was carried out in a 50 µL drop of 8% Polyvinylpyrrolidone (PVP) in TALP medium. Injected oocytes were placed in 30 µL drops of NCSU 23 medium (medium described by Petters & Wells in 1993) supplemented with 0.504 mg/mL sodium lactate, 0.0363 mg/mL sodium pyruvate, 25 µM β-mercaptoethanol, 0.1 mg/mL cysteine, 4 mg/mL BSA, 75 µg/mL penicillin, 50 µg/mL streptomycin and 50 µg/mL gentamicin. The droplets were covered with sterile mineral oil and incubated at 37.5°C in a 5% CO₂ air. Embryos were observed 48 hours after injection in terms of cleavage rate by using a phase-contrast microscopy.

Results and Discussion. We used three different tests to analyze membrane integrity after rehydration (Table 1). Plasma membrane integrity was compromised in most cells, as shown by the intra-vital eosin stain and hypo-osmotic swelling test. No statistically significance was observed between experimental trials (drying period or storage temperature). The situation was not the same in the case of acrosomal integrity, where the average value of NAR was very good at freeze-dried spermatozoa. This indicates that the negative influence of freeze-drying method on the acrosomal level is lower. Compared to these data, the average value of acrosomal integrity was 15% lower on the sperm cryopreservation (Zăhan et al 2012).

Table 1

Freeze-dried boar sperm parameters after rehydration

<i>Drying period (h)</i>	<i>Storage temperature (°C)</i>	<i>Live (%)</i>	<i>HOST (%)</i>	<i>NAR (%)</i>	<i>DFI (%)</i>
4	4	4.75	0	97.5	61
	20	8	0.94	84	35.75
8	4	5	2.25	94	63.75
	20	3.25	2	93.5	32
Mean ± SD		5.3 ± 2.0	1.3 ± 1.0	92.3 ± 5.8	48.1 ± 16.6

HOST – hypo-osmotic swelling test, NAR – normal apical ridge, DIF – DNA fragmentation index.

DNA integrity gave surprising results concerning DIF percents, with variations depending on the experimental trial. So, we observed that dehydration period did not influence DNA fragmentation, while the sample storage temperature led to DNA fragmentation (Figure 1) twice as higher at 4°C than at 20°C.

ICSI is a powerful technique in the field of biotechnology of reproduction and provides exciting opportunities to produce pig embryos with sperm that had lost motility. In this study, the cleavage (Figure 2) rate was 16.67%. Our results demonstrate that ICSI leads to successful production of Mangalitsa swine embryos using freeze-dried spermatozoa.

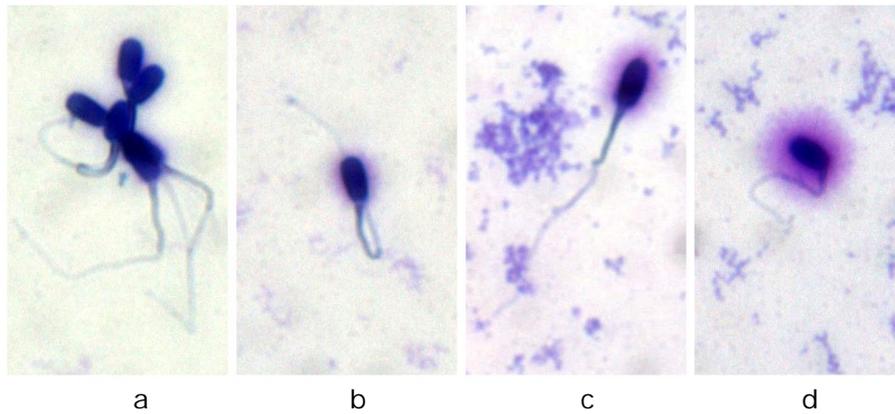


Figure 1. DNA fragmentation analyses of boar sperm under bright field microscopy: sperm without halo or with small halo of chromatin dispersion contains unfragmented DNA (a, b.); sperm with medium size halo or with large halos of chromatin dispersion correspond with sperm heads containing fragmented DNA (c, d.)

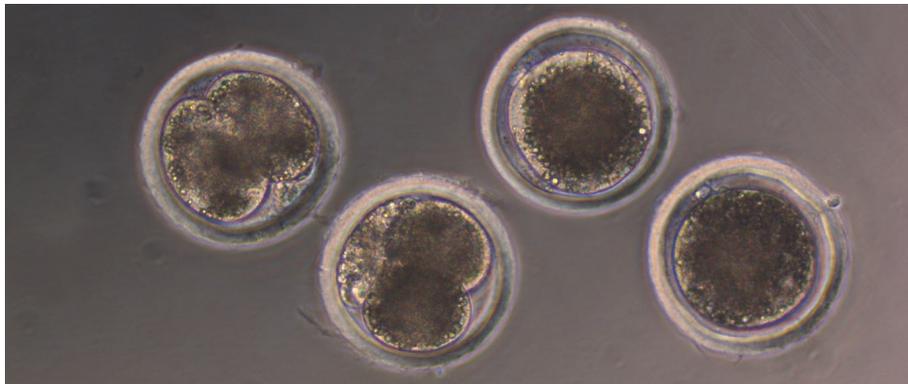


Figure 2. Swine embryo cleavage after ICSI with freeze-dried spermatozoa.

Chromosomal integrity and participation in embryonic development of boar freeze-dried spermatozoa rehydration can be maintained by adding EDTA, EGTA (Nakai et al 2007) or trehalose (Meng et al 2010) to freeze-drying solution. Based on the ultrastructure observation, main cryogenic damage in freeze-dried boar spermatozoa was swelling, damage or rupture in the sperm acrosome, neck and tail (Meng et al 2010).

One of the most important factors that influence the success of ICSI technique in swine is the origin of sperm with differences between fresh or frozen sperm. This is probably due to the fact that the freezing process may lead to DNA or centriole degradation making the male pronucleus impossible to form (Garcia-Rosello et al 2009). Using freeze-dried sperm for injection gave good results in forming the male pronucleus and oocyte activation, but cleavage and embryo developing rates towards morula and blastula stages is lower than we expected. Injection of frozen-thawed boar head spermatozoa leads to improvement of cleavage rate after ICSI (Lee & Yang 2004). However, activation and development of oocytes produced with freeze-dried boar spermatozoa were largely impaired compared with those produced with fresh spermatozoa (Kwon et al 2004). Injection of sperm head only in the case of freeze-dried spermatozoa can lead to accelerate oocytes activation and female pronucleus formation. In spite of these, the incidence of activation, pronucleus forming and early oocyte development were significantly lower in the case of freeze-dried sperm head injection than in the case of freshly collected whole sperm injection, with an intact membrane. As suggested in studies on rabbits (Liu et al 2004), the freeze-dried process may induce chemical modifications on sperm membrane making their development more difficult, compared with freshly collected spermatozoa.

In the case of sperm preserving by freeze-drying, although there are different systems for that, protocols say that sperm must be subjected to freeze-drying for about 4 hours at mice and rabbits (Ward et al 2003), and 12-18 hours at mice and bovine (Keskintepe et al 2002). Other results show that the incidence of oocyte activation, pronucleus forming and cleavage rate were significantly reduced in the case of 24 hour freeze-dried sperm. The most probable reason for this depreciation is that the samples had been dehydrated for too long. When humidity decreases under a certain level, molecular motility that decreased during dehydration starts to increase. Prolongation of the freezing-drying period for boar spermatozoa from 4 to 24 h gradually reduced the ability of spermatozoa to participate to *in vitro* embryo development, particularly when the freeze-dried spermatozoa were stored at 25°C (Kwon et al 2004).

In the case of mouse freeze-dried spermatozoa, Wakayama & Yanagimachi (1998) reported that the ability to activate the oocytes or to participate into *in vitro* or *in vivo* embryo development was not affected by storage neither at 4°C nor at 25°C for up to 3 months. However, Kwon et al (2004) observed that the ability of boar freeze-dried spermatozoa to participate into *in vitro* embryo development was more hampered after one month storage at 25°C than 4°C and that porcine oocytes injected with freeze-dried spermatozoa stored for 3 months at 4°C did not develop *in vitro*.

Conclusions. The process of freeze-drying boar spermatozoa deeply damages cell membrane, but it does not affect acrosome integrity. Depending on the storage temperature, DNA integrity was mostly affected on refrigeration than on room temperature. However, Mangalitsa spermatozoa can be freeze-dried and then these spermatozoa can be used by ICSI in order to obtain embryos. Further experiments are needed to improve boar spermatozoa freeze-drying process until the first piglets will be born.

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