Production of live foals via intracytoplasmic injection of lyophilized sperm and sperm extract in the horse

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Abstract

Work with lyophilized sperm helps delineate the factors required for successful fertilization. We investigated the use of lyophilized sperm in equine embryo production. In Experiment 1, sperm DNA fragmentation index was not affected by three freeze/thaw or lyophilization cycles. In Experiment 2, oocytes injected with lyophilized sperm or with sperm from a treatment in which lyophilized sperm were suspended in sperm cytoplasmic extract (SE) yielded blastocyst development rates of 0 and 28% respectively ($P < 0.05$). In Experiment 3, blastocyst development rate was 6–11% after injection of sperm lyophilized from fresh or frozen–thawed semen, suspended in SE. In Experiment 4, sperm lyophilized 3.5 months or 1 week previously, suspended in SE, yielded similar blastocyst rates (6 and 3% respectively). Rates of normal pregnancy after transfer were 7/10 and 5/7 for embryos from control and lyophilized sperm treatments respectively. Three pregnancies from the lyophilized sperm treatments were not terminated, resulting in two healthy foals. Parentage testing determined that one foal originated from the lyophilized sperm; the other was the offspring of the stallion providing the sperm extract. Further testing indicated that two of five additional embryos in the lyophilized sperm treatment originated from the stallion providing the sperm extract. We conclude that both lyophilized stallion sperm and stallion sperm processed by multiple unprotected freeze–thaw cycles (as for sperm extract) can support production of viable foals. To the best of our knowledge, this is the first report on production of live offspring by fertilization with lyophilized sperm in a non-laboratory animal species.

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Introduction

Cryopreservation of semen offers a method to bank genetics or to fulfill a male's genetic potential even if his reproductive life is cut short. Unfortunately, storage of frozen semen necessitates the use of liquid nitrogen containers, which is associated with a number of drawbacks, including need for constant maintenance and the possibility of viral contamination among straws (Tedder *et al.* 1995, Bielanski *et al.* 2000). Shipment of semen frozen in liquid nitrogen is cumbersome, with the potential for loss of sperm quality if the shipment is delayed en route. Unless a large number of straws have been frozen from a stallion, the number of foals that may be obtained using frozen semen via standard insemination is fairly limited.

The development of ICSI as a method of IVF in the horse (Choi *et al.* 2002b, Galli *et al.* 2002) offers many possibilities for the efficient use of semen, as with this technique only one sperm is required to fertilize each oocyte. Thawing, dilution, and refreezing of previously-frozen stallion semen did not affect blastocyst

development after ICSI (Choi *et al.* 2006). ICSI has been used clinically to obtain pregnancies through use of oocytes recovered by follicular puncture in live mares (Colleoni *et al.* 2007) as well as from oocytes recovered from ovaries postmortem (Hinrichs *et al.* 2011).

As the sperm are injected into the oocyte during ICSI, they need not be motile. This allows the possibility of using freeze-dried (lyophilized) sperm for production of embryos. Blastocysts have been produced with lyophilized sperm in cattle (Keskinetepe *et al.* 2002) and pigs (Kwon *et al.* 2004), and live young have been produced in mice (Wakayama & Yanagimachi 1998, Kusakabe *et al.* 2001, Kaneko & Nakagata 2006), rats (Hirabayashi *et al.* 2005, Kaneko *et al.* 2009), and rabbits (Liu *et al.* 2004). Lyophilized sperm would not require liquid nitrogen for storage, and sperm intended for ICSI could be packaged with very few sperm per dose, thus allowing a large number (hundreds of thousands) of aliquots to be prepared from one ejaculate. Importantly, lyophilization is an efficient method for obtaining numerous ICSI doses of sperm from one straw of previously-frozen semen in cases in which only limited

Table 1 DNA fragmentation index (DFI, mean $\pm$ s.d.) of sperm after various processing treatments.

Sperm treatment	DFI			Overall mean
	Number of freeze or lyophilization cycles			
	1	2	3	
Snap-frozen raw	7.56±2.20	8.07±2.27	8.13±2.57	7.9+2.2 ^a
Standard freeze	6.98±1.28	8.65±4.39	7.04±1.97	7.6±2.8 ^{ab}
Fresh-lyophilized	4.72±0.92	5.78±1.75	6.17±1.02	5.6±1.4 ^c
Standard freeze/thaw/lyophilize	5.53±1.85	5.51±1.38	6.34±1.62	5.8±1.6 ^{bc}
Standard freeze/thaw/density gradient/lyophilize	2.33±0.77	2.17±0.74	2.00±0.38	2.2±0.6 ^d

Within the 'Overall mean' column, values with different superscripts differ significantly ($P < 0.05$).

stores of semen are available. However, the effect of previous freezing and thawing on fertilizing capacity of sperm subsequently lyophilized is unknown.

To our knowledge, no work has been done on ICSI with freeze-dried sperm in the horse. The basic techniques necessary to study this, including ICSI (Choi *et al.* 2004) and *in vitro* embryo culture (Hinrichs *et al.* 2005), are currently in place. Methods for activation of horse oocytes have been investigated; notably, these include injection of cytosolic extract, prepared from sperm via multiple cycles of flash freezing and thawing. Injection of sperm extract is used in our laboratory for activation of equine oocytes after nuclear transfer and is associated with relatively high rates of production of live offspring (Hinrichs *et al.* 2006, 2007, Choi *et al.* 2009b). It is possible that injection of sperm cytosolic extract may not only serve to activate oocytes but also to supply sperm-derived factors, beneficial to embryo development, that are lost from sperm during the lyophilization process. The objective of this study was to evaluate the effect of injection of sperm cytosolic extract in conjunction with injection of lyophilized spermatozoa on the production of blastocysts in the horse and to assess the viability of these blastocysts after transfer to recipient mares.

Results

Experiment 1: effect of lyophilization on DNA fragmentation index

Sperm were evaluated for the DNA fragmentation index (DFI; also known as %COMP or COMP_{at}) as determined by the sperm chromatin structure assay, a flow cytometric analysis of acridine orange-stained samples, after A) snap freezing in the raw (neat) state, B) standard freezing in the presence of cryoprotectants, C) lyophilization, D) standard freezing followed by lyophilization, or E) standard freezing followed by density-gradient centrifugation and then lyophilization. The DFI represents the population of sperm outside the main population and is the most indicative endpoint of the assay in stallions, being correlated with both seasonal pregnancy rate and first-cycle pregnancy rate (Love & Kenney 1998). Lower values for DFI are associated with lower susceptibility to chromatin denaturation and thus

higher sperm quality (Evenson *et al.* 1980, Love & Kenney 1998). Freezing of raw spermatozoa has been reported not to be detrimental to chromatin integrity (Evenson *et al.* 1994, Sailer *et al.* 1995). The values for DFI for these treatments are presented in Table 1. Lyophilization had no significant effect on the DFI. The mean DFI for sperm snap-frozen raw was similar to that for sperm frozen by standard methods but was higher than those for the remainder of treatment groups ($P < 0.05$). The DFI was lower for frozen-thawed sperm passed through a density gradient before lyophilization than for all other treatment groups ($P < 0.05$). Across all treatments, the mean DFI was unaffected ($P > 0.05$) by the number of freeze/thaw or lyophilization cycles (Table 1). There was no treatment-by-cycle interaction.

Experiment 2: effect of sperm extract injection on blastocyst development after ICSI with lyophilized sperm

This experiment was conducted in July and August. For this experiment, 188 compact (Cp) oocytes were cultured, 67 matured (36%), and 1 oocyte lysed after injection. The cleavage and blastocyst development rates for oocytes injected with sperm in four treatment groups (control, control + sperm extract, lyophilized, and lyophilized + sperm extract) are given in Table 2. Control sperm was frozen-thawed sperm, either from the same ejaculate from which the lyophilized sperm was prepared (Stallion 2115, two replicates), or from a separate stallion (Stallion DD, two replicates). Blastocyst

Table 2 Cleavage rates and blastocyst development after ICSI with control (motile, frozen-thawed) or lyophilized sperm, with or without activation by injection of sperm extract at the time of ICSI.

Treatment	Oocytes injected (n)	Cleavage (%)	Blastocysts (%)
Control	18	13 (72) ^a	6 (33) ^a
Control + SE	15	13 (87) ^{a,b}	4 (27) ^a
Lyo	15	5 (33) ^c	0 (0) ^b
Lyo + SE	18	18 (100) ^b	5 (28) ^a

SE, injection of sperm extract at the time of ICSI; Lyo, sperm lyophilized from fresh semen. ^{a,b,c}Within columns, values with different superscripts differ significantly ($P < 0.05$).

development for oocytes injected with lyophilized sperm+sperm extract was not significantly different from that for control or control+sperm extract (28 vs 27–33%; $P>0.1$). No blastocysts developed when oocytes were injected with lyophilized sperm without sperm extract; this rate was significantly lower than those for all other treatments ($P<0.05$).

Experiment 3: effect of previous freezing/thawing on blastocyst development after ICSI with lyophilized sperm

This experiment was conducted in August. For this experiment, of the 319 expanded (Ex) oocytes evaluated after maturation, 195 (61%) were mature. One oocyte lysed after injection, and 15 oocytes were used for another study. Cleavage and blastocyst development rates for five treatments (control, control+sperm extract, lyophilized+sperm extract, frozen-thawed, then lyophilized+sperm extract, and frozen-thawed followed by density-gradient centrifugation, then lyophilized+sperm extract) are presented in Table 3. Control sperm was frozen-thawed sperm either from the same ejaculate from which the lyophilized sperm was prepared (Stallion 2115, three replicates) or from a separate stallion (Stallion DD, two replicates). Blastocyst development in the control treatment (31%) was significantly higher than that for all treatments in which sperm extract was injected (control+sperm extract (12%), $P=0.05$; all other treatments $P<0.05$). Blastocyst development rates in the three lyophilized treatments (6–11%) were not significantly different from that for the control+sperm extract treatment (12%; $P>0.1$).

Experiment 4: effect of storage of lyophilized sperm on blastocyst formation after ICSI

This experiment was conducted in November and December. For this experiment, of the 224 Ex oocytes evaluated after maturation, 116 (52%) were mature. Of the 96 Cp oocytes evaluated after maturation, 18 (19%) were mature. One Cp oocyte lysed after injection.

Table 3 Blastocyst development after ICSI with control (motile, frozen-thawed) and lyophilized sperm.

Treatment	Oocytes injected (n)	Cleavage (%)	Blastocysts (%)
Control	36	25 (69)	11 (31) ^a
Control+SE	34	30 (88)	4 (12) ^b
Lyo+SE	35	35 (100)	2 (6) ^b
Frz-Lyo+SE	36	34 (94)	2 (6) ^b
Frz-D-Lyo+SE	38	32 (84)	4 (11) ^b

SE, injection of sperm extract at the time of ICSI; Lyo, sperm lyophilized from fresh semen; Frz-Lyo, sperm lyophilized from frozen-thawed semen; Frz-D-Lyo, sperm lyophilized from frozen-thawed semen passed through a density gradient before lyophilization. ^{a,b}Within columns, values with different superscripts differ significantly ($P\leq 0.05$).

Table 4 Cleavage and blastocyst development after ICSI of oocytes injected with control (motile, frozen-thawed) sperm or with lyophilized sperm of two different storage durations.

Treatment	Oocytes injected (n)	Cleavage (%)	Blastocysts (%)
Control	36	29 (81)	10 (28) ^a
Control+SE	31	28 (90)	8 (26) ^a
Frz-Lyo+SE 1	32	25 (78)	2 (6) ^b
Frz-Lyo+SE 2	34	31 (91)	1 (3) ^b

^{a,b}Within columns, values with different superscripts differ significantly ($P<0.05$).

SE 1, sperm lyophilized from frozen-thawed semen 3.5 months prior to start of experiment and held at 4 °C. SE 2, sperm lyophilized from frozen-thawed semen 1 week prior to start of experiment and held at –20 °C.

There was no difference in blastocyst development rates between oocytes injected with frozen-thawed/lyophilized sperm that had been stored at 4 °C for 3.5 months prior to the start of the experiment and those injected with sperm lyophilized from a frozen-thawed straw from the same ejaculate 1 week prior to the start of the experiment and stored at –20 °C (3–6%, Table 4). Blastocyst development rates for oocytes injected with lyophilized sperm were significantly lower than those for oocytes injected with control sperm, with or without sperm extract (26–28%; $P<0.05$). There was no significant difference in blastocyst formation after injection of lyophilized sperm between Ex and Cp oocytes (2/55, 4 vs 1/11, 9% respectively, $P>0.1$).

Experiment 5: viability of blastocysts in the lyophilized sperm treatments

Pregnancy rates (visualization of an embryonic vesicle on ultrasonographic examination per rectum) and rates of normal pregnancy (fetal heartbeat visualized after day 25) after transfer of ICSI-produced blastocysts to recipient mares are presented in Table 5. There was no difference in pregnancy rate or normal pregnancy rate between embryos produced with control sperm (control and control+sperm extract treatment groups combined), or sperm in the lyophilized treatments (all lyophilized treatment groups combined). The overall pregnancy rate for control sperm was 8/10, with 7/10 (70%) normal pregnancies. The overall pregnancy rate for sperm from the lyophilized treatment was 5/7 (71%), with all the five pregnancies being normal to the heartbeat stage.

The first three pregnancies established from embryos produced in the lyophilized sperm treatment were not terminated. One pregnancy was lost at pasture between 4 and 6 months of gestation. The other two pregnant mares gave birth to healthy foals, a filly and a colt, at 330 and 337 days of gestation respectively (Fig. 1). Parentage testing on the filly showed that Stallion 2115, the stallion that provided the lyophilized sperm, qualified as her sire.

Table 5 Number of blastocysts transferred and resulting pregnancies from embryos produced by ICSI with control (motile, frozen–thawed) or lyophilized sperm.

Treatment	Blastocysts transferred	Pregnancies	Normal pregnancies
Control	5	4	4
Control + SE	5	4	3
Lyophilized + SE*	7	5	5

Normal pregnancies were determined when a fetus with heartbeat was visualized using ultrasonography. *Includes blastocysts from all three lyophilized treatment groups.

Parentage testing on the colt excluded Stallion 2115 and Stallion DD as a sire, but the colt qualified as an offspring of Stallion EG, the stallion from whose semen the sperm extract was produced.

Both foals are healthy and are 5 years old at the time of writing.

Parentage testing of frozen embryos

Seventeen embryos that were not sent for transfer were frozen and then submitted for genetic analysis of 13 microsatellites. In five cases, no DNA was recovered from the vial. Parentage testing of the remaining 12 embryos showed that seven of seven embryos from the control sperm treatments agreed with the appropriate stallion (two of two from replicates in which DD was the control stallion, and five of five in which 2115 was the control stallion). Of the five embryos resulting from the lyophilized sperm treatments, three matched with the stallion used for sperm lyophilization (Stallion 2115) and two matched with the stallion used for preparation of sperm extract (Stallion EG). Examination of sperm extract showed occasional sperm remaining in the extract (Fig. 2). Embryos shown definitively by parentage testing to be a result of injection of lyophilized sperm were produced from three separate lyophilization treatments. These were as follows: one embryo from Experiment 2, in the lyophilized + sperm extract treatment, and three embryos (one of which was validated after the birth of the foal) from Experiment 3, in the lyophilized + sperm extract ($n=1$), frozen–thawed, then lyophilized + sperm extract ($n=1$), and frozen–thawed followed by density-gradient centrifugation, then lyophilized + sperm extract ($n=1$) treatments.

Discussion

In these studies, ICSI with lyophilized stallion sperm in conjunction with injection of sperm extract resulted in the development of blastocysts, normal pregnancies, and a healthy foal. This represents, to the best of our knowledge, the first production of live offspring from lyophilized sperm in a non-laboratory animal species. Blastocysts were produced from lyophilized sperm in

three different treatments, including sperm lyophilized after standard freezing and thawing. Because all the lyophilized sperm used were from one ejaculate of one stallion (2115), it is unknown whether the results obtained can be applied to sperm from other stallions.

On evaluation of the results of parentage testing, a remarkable and unexpected finding was that one of the two foals and at least two of the five additional blastocysts in the lyophilized treatment were produced by injection of sperm from the sperm extract. This extract was prepared by multiple snap-freeze/thaw cycles, followed by centrifugation at 20 000 *g* for 50 min and aspiration of supernatant from the dense sperm pellet. The supernatant was stored at -80°C for over 1.5 years before being used in these experiments. The ability to obtain blastocysts and foals from sperm in this extract, which was produced using a different stallion (EG) from that used for lyophilization, indicates that sperm resistance to processing damage was not specific to Stallion 2115.

Rare sperm (Fig. 2B) were found on examining the extract after the results of the parentage testing were obtained. These sperm are avoided when the extract is aspirated for activation of oocytes after nuclear transfer, likely because they are infrequent and would require manipulation to load into the small diameter (8 μm) injection pipette. In this study, sperm suspensions were mixed with the sperm extract before the mixture was placed on the microscope for sperm injection. In the control + sperm extract treatment, only motile sperm were injected, thus sperm from the sperm extract were avoided because they were immotile. However, in the lyophilized + sperm extract treatments, both the lyophilized sperm and the sperm from the sperm extract were immotile and thus could not be differentiated. Even with the introduced lyophilized sperm, there were few sperm in the droplet, and it was necessary to search the droplet to locate sperm and manipulate them into the pipette. It was only after the results of the parentage testing were known that we realized the possibility that a sperm from the extract could have been injected, instead of a lyophilized sperm.

In future studies, microfiltration of sperm extract, or removal of any sperm from the extract via micromanipulation before adding the subject sperm, would

Figure 1 Foals produced from (A) a lyophilized sperm, 11 days old and (B) a sperm from sperm extract, 16 days old.

Figure 2 Sperm used for ICSI. (A) lyophilized sperm and (B) sperm from sperm extract (two intact sperm and one sperm head). Bar = 10 μ m.

eliminate the possible contribution of sperm from the sperm extract. However, the findings that both lyophilized and snap-frozen/stored sperm can produce live young in the horse should stimulate more work in this area. Unfortunately, the closure of all horse slaughterhouses in the United States in 2007 severely decreased the availability of horse oocytes in this country and has rendered further research in this area problematic for our laboratory.

It is not clear whether the success of these experiments is related to the use of sperm extract for activation, or to the horse as a species. Injection of sperm extract is used in our laboratory for activation of recombined equine oocytes after nuclear transfer, and we have produced cloned foals with relatively high efficiency using this technique (Hinrichs *et al.* 2006, 2007, Choi *et al.* 2009b). Sperm cytoplasmic extract may contain not only the 'sperm factor,' putatively phospholipase C zeta (PLC ζ), which induces calcium oscillations in the oocyte, thus triggering activation (Saunders *et al.* 2002), but may also contain other factors, including mRNAs, that the sperm typically delivers to the oocyte at the time of fertilization (Ostermeier *et al.* 2004). Stallion spermatozoa appeared to be resistant to DNA fragmentation due to lyophilization (Experiment 1). In mice, DNA fragmentation has been shown to be related to the method used for lyophilization and the method of storage, and to be correlated with a lowered ability of lyophilized sperm to yield blastocysts after ICSI (Kawase *et al.* 2009).

Results of Experiment 2 showed that external activation is required for blastocyst development after ICSI with lyophilized sperm in the horse, as no blastocysts resulted from injection of lyophilized sperm in the absence of sperm factor. This is similar to the situation reported after injection of lyophilized sperm in the rabbit, in which chemical activation treatments, such as calcium ionophore with cycloheximide and 6-dimethylaminopurine, were essential for blastocyst production (Liu *et al.* 2004). In contrast, external activation was not required for the formation of blastocysts after injection of lyophilized sperm in the rat (Kaneko *et al.* 2009) and mouse (Wakayama & Yanagimachi 1998, Kawase *et al.* 2007). Horse oocytes

are resistant to parthenogenetic activation, exhibiting essentially no spontaneous activation (Choi *et al.* 2001); in contrast, in the rat and mouse, the manipulations associated with ICSI may have provided sufficient stimulus for activation. Alternatively, rats and mice may possess sperm-borne oocyte-activating molecules that are resistant to loss during freeze-drying (Wakayama & Yanagimachi 1998, Hirabayashi *et al.* 2005).

Because oocytes were being injected with an activating factor (sperm extract), we performed parentage testing of blastocysts to confirm that blastocysts resulted from fertilization, rather than parthenogenetic activation. All tested blastocysts and foals were products of the sperm from the stallions used and thus were not parthenogenetic. The pregnancies terminated were not recovered for parentage testing; however, normal development to the stage where a heartbeat could be detected (Table 5) indicates that the blastocysts produced in groups injected with sperm extract were unlikely to have resulted from parthenogenetic activation.

The blastocyst rate achieved in the lyophilized sperm treatment appeared to decrease from Experiment 2 to Experiment 3; however, there was a concomitant decrease in blastocyst production in the control + sperm extract treatment between these experiments as well. This may be related to the dose of sperm extract injected. Injection of increasing volumes of sperm extract during activation of nuclear transfer oocytes was associated with a decrease in blastocyst production (Choi *et al.* 2009b). The dose of sperm extract delivered in any given replicate is dependent on characteristics of the pipette used and the column of medium delivered. It is possible that a critical amount of sperm factor must be delivered to the oocyte to support activation without overstimulation and that this amount varies with oocyte type and with the type of sperm used for ICSI, e.g. lyophilized sperm may have lost the majority of their cytosolic active protein (PLC ζ), and thus oocytes injected with lyophilized sperm may require and tolerate a greater amount of sperm factor than do oocytes injected with control sperm, which contain their own sperm factor. In turn, both treatments may respond poorly to an overdose of the sperm factor.

One difference between Experiments 2 and 3 was the use of Cp versus Ex oocytes. In our laboratory, oocytes classified as Cp are identified in lower proportions than Ex and have lower maturation rates, as well as a different required duration of maturation (30 vs 24 h for Ex; Hinrichs *et al.* 2005). However, their blastocyst production after ICSI is equivalent to that for Ex oocytes (Hinrichs *et al.* 2005). Typically, we use only one oocyte type per experiment to minimize variability. In the current work, the first oocyte injection study (Experiment 2) was carried out on Cp oocytes, as they were available from another experiment. After the promising results of Experiment 2, in Experiment 3, Ex oocytes were used because of their greater numbers. Subsequently, to

evaluate whether cumulus type may have been associated with the lower blastocyst rate in Experiment 3, in Experiment 4, both Ex and Cp oocytes were used. In Experiment 4, there was no apparent effect of oocyte type on blastocyst rate using lyophilized sperm (1/11, 9% for Cp versus 2/55, 4% for Ex); however, only a small number of mature Cp oocytes were available for injection. Closure of the horse slaughterhouses in the US has prevented us from pursuing the effect of cumulus type further.

When comparing the data from Experiments 2 and 3, we hypothesized that another possible reason for the lower blastocyst development when using lyophilized sperm in Experiment 3 was a loss of viability in the sperm due to storage. This has been shown in the mouse (Kawase *et al.* 2007). For this reason, we conducted Experiment 4 to compare blastocyst development between the original frozen-thawed/lyophilized sperm, which had now been stored for 3.5 months, and the sperm newly lyophilized from sperm frozen from the same ejaculate. However, these data showed no difference in blastocyst development rates between stored and newly lyophilized sperm; both were low (3–6%; Experiment 4) relative to the findings of Experiment 2.

In conclusion, production of embryos from sperm subjected to lyophilization or to numerous freeze-thaw cycles and storage for over 1.5 years at -80°C suggest that stallion sperm nuclei may be resistant to damage. Use of this sperm in conjunction with activation by injection with sperm extract was associated with production of viable embryos and live foals. Lyophilization of stallion sperm appears to present an alternative to standard freezing for sperm storage; however, further work is needed to define the effect of sperm type and activation stimulus on blastocyst development after ICSI in this species.

Materials and Methods

General methods

Collection and lyophilization of semen

Gel-free semen was collected from three stallions having normal fertility, using an artificial vagina equipped with an in-line micromesh filter. Aliquots of the ejaculates were subjected to each of the five treatments: A) snap freezing in the raw (neat) state, B) standard freezing in the presence of cryoprotectants, C) lyophilization, D) standard freezing followed by lyophilization, or E) standard freezing followed by density-gradient centrifugation and then lyophilization.

Snap freezing of raw semen was performed by submerging the samples directly into liquid nitrogen. Freezing by a standard (commercial) method was performed as described previously (Combes *et al.* 2000), with slight modifications. Semen was diluted in a milk-glucose-sucrose centrifugation extender (Blanchard *et al.* 2003) to obtain a sperm concentration of ~ 50 million sperm/ml and then centrifuged at 500 *g* for 10 min in 50-ml conical centrifuge tubes. Following aspiration of the

supernatant, the spermatozoal pellet was resuspended in milk-based freezing extender (Blanchard *et al.* 2003). Semen was packaged in 0.5-ml straws at a sperm concentration of 200 million/ml (100 million sperm/straw), and straws were frozen 3 cm above liquid nitrogen in static vapor for 20 min.

For lyophilization, an aliquot of the fresh ejaculate was extended in skimmed milk glucose extender followed by centrifugation at 500 *g* for 10 min. The sperm pellet was washed once with DMEM/F-12 (Sigma-Aldrich) + 10% fetal bovine serum (FBS; Invitrogen) and resuspended in this medium to 50 000 sperm/ml. Aliquots of 100 μl of the sperm suspension were placed into 2-ml glass lyophilization vials (Wheaton Scientific, Millville, NJ, USA) that were pre-cooled to -80°C . Samples were then transferred to the pre-cooled chamber, a commercial freeze dryer (Advantage; Virtis Industries, Gardiner, NY, USA), and exposed to a 30 h lyophilization cycle with a condenser temperature of -75°C and vacuum of 100 mTorr, with shelf temperatures of -35 , -20 , 0 , and $+20^{\circ}\text{C}$ for 480, 120, 840, and 360 min respectively.

The vials were vacuum-sealed and then removed from the freeze dryer and stored at 4°C . Standard-frozen semen from the same ejaculate was thawed in a waterbath set at 37°C , then washed three times in DMEM/F-12 medium with 10% FBS, and processed for lyophilization as described above. In addition, standard-frozen semen was thawed, then centrifuged through a two-layer density gradient (Equipure, Nidacon International, Gothenburg, Sweden) to select for motile spermatozoa as described previously (Macpherson *et al.* 2002), then washed, and resuspended in DMEM/F-12 + 10% FBS. The sperm were then subjected to lyophilization as described above.

Sperm in the above five treatment groups were exposed to one, two, or three freeze/thaw or lyophilization/resuspension cycles and then processed for determination of DFI.

Flow cytometric analysis of DFI

Analysis of DFI was conducted as described previously (Evenson *et al.* 1980, Love & Kenney 1998) with modifications. Briefly, a 2–7 μl aliquot of thawed or reconstituted semen (aliquot size was adjusted to obtain a flow rate of 100–200 cells/s) was diluted to 200 μl in a buffer solution (1 mM disodium EDTA, 10 mM Tris-HCl, and 150 mM NaCl, pH 7.4). This was mixed with 400 μl acid detergent solution (150 mM NaCl, 4.0% 2 M HCl, and 0.1% Triton-X). After 30 s, 1.2 ml acridine orange solution (37 mM citric acid monohydrate, 126 mM Na_2HPO_4 , 150 mM NaCl, 1 mM disodium EDTA, and 4 $\mu\text{g/ml}$ acridine orange, pH 6.0) was added. The sample was placed in the flow cytometer and allowed to pass through the tubing for 2 min prior to counting of the cells. A total of 5000 events were evaluated for each sample. The flow cytometer was adjusted using sperm from a fertile control stallion such that the mean green fluorescence was set at 500 channels (FI-1 @ 500) and mean red fluorescence at 150 channels (FI-3 @ 150). The ratio of red/(red+green) fluorescence, i.e. single-stranded/total, was measured for each spermatozoan. The DFI (%COMP) was calculated as the percentage of sperm outside the main population, using WinList Software (Verity Software House, Topsham, ME, USA).

Preparation of sperm extract

Stallion sperm cytosolic extract was prepared from sperm from one stallion (Stallion EG) as described previously (Choi *et al.* 2002a) but using Chatot–Ziomek–Bavister (CZB) medium (Chatot *et al.* 1989) modified for use with sperm (Sp-CZB; Choi *et al.* 2003) for sperm washing. Briefly, procedures as reported for the mouse (Perry *et al.* 1999) were used with modifications. Ejaculated stallion sperm were centrifuged at 900 *g* for 10 min to remove seminal plasma. The pellet was then suspended in Sp-CZB containing 5 mg/ml BSA and centrifuged at 900 *g* for 10 min. The resulting pellet was resuspended in nuclear isolation medium (NIM: 125 mM KCl, 2.6 mM NaCl, 7.8 mM Na₂HPO₄, 1.4 mM KH₂PO₄, and 3.0 mM EDTA disodium salt; pH 7.45; Kuretake *et al.* 1996) and centrifuged to remove Sp-CZB. The pellet was then resuspended to the same volume with NIM containing 1 mM dithiothreitol, 100 μ M leupeptin, 100 μ M antipain, and 100 μ g/ml soybean trypsin inhibitor. The suspension was subjected to four cycles of freezing (5 min/cycle) in liquid N₂ and thawing (5 min/cycle at 15 °C) and then sperm were pelleted at 20 000 *g* for 50 min at 2 °C. The resultant supernatant was carefully removed, aliquoted, and kept at –80 °C until used.

Collection and maturation of oocytes

Ovaries were obtained from one of the two horse abattoirs located at 3–4 h drive from the laboratory. Oocytes were collected from the ovaries by follicular scraping and were classified as having a Cp or Ex cumulus, or as degenerating, as described previously (Hinrichs *et al.* 1993, 2005).

Oocytes were cultured in TCM199 (Invitrogen) with 5 mU/ml FSH (Sioux Biochemicals, Inc., Sioux Center, IA, USA), 10% FBS, and 25 μ g/ml gentamicin (Invitrogen) at a ratio of 10 μ l/oocyte at 38.2 °C in a humidified atmosphere of 5% CO₂ in air. Oocytes classified as Ex were matured for 24 h and those classified as Cp were matured for 30 h.

ICSI

Not all treatments could be performed concurrently on some oocyte collection days, depending on the number of oocytes recovered, especially for Cp oocytes. A minimum of three and maximum of five replicates were performed for each treatment. Additional replicates (above 3) were performed to equalize the number of oocytes injected in each treatment.

The ICSI procedure was conducted as described previously (Choi *et al.* 2002b). Briefly, after maturation, oocytes were denuded of cumulus by pipetting in 0.05% hyaluronidase and those with a polar body were used for ICSI. For ICSI with frozen–thawed (control) sperm, a swim-up procedure was used (Choi *et al.* 2004). Briefly, semen was thawed at 37 °C for 30 s and 200 μ l were layered under 1 ml Sp-CZB for swim-up. After a 20 min incubation at 38.2 °C, the top 0.6 ml of the medium were collected and washed by centrifugation at 327 *g* for 3 min, resuspension in Sp-CZB, and re-centrifugation. The supernatant was removed and the sperm pellet was resuspended in the remaining medium. For use in ICSI, 1 μ l of this sperm suspension was placed on the surface of a 3- μ l droplet of Sp-CZB containing 10% polyvinylpyrrolidone (PVP),

and motile sperm were identified after swimming down into the droplet.

Lyophilized sperm were reconstituted by adding 100 μ l embryo-grade water to each ampoule. The reconstituted sperm were pipetted into an Eppendorf vial and centrifuged at 327 *g* for 3 min. The supernatant was removed and the sperm were resuspended in Sp-CZB, then centrifuged again, and the supernatant was then removed. The sperm pellet was then resuspended in the fluid remaining in the vial. For use in ICSI, 1 μ l of this sperm suspension was mixed with 3 μ l Sp-CZB containing 10% PVP. Sperm injection was carried out in a separate 50- μ l drop of CZB-M containing 10% FBS. The sperm were injected into the oocyte cytoplasm using the Piezo drill.

In treatments using sperm extract, a sperm extract/PVP solution was prepared as for use in nuclear transfer (Choi *et al.* 2002a) by thawing aliquots of sperm extract and mixing these 1:1 with NIM medium containing 20% PVP. For sperm injection, 1 μ l sperm suspension was layered on (control sperm) or mixed with (lyophilized sperm) 3 μ l sperm extract/PVP solution rather than with Sp-CZB/PVP.

Embryo culture was performed in microdroplets of DMEM/F-12 + 10% FBS containing 25 μ g/ml gentamicin under light white mineral oil at a ratio of 1 μ l medium/embryo, in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 38.2 °C. The medium was changed at 3 and 5 days.

Parentage testing

The blastocysts that were not transferred to recipient mares (see Experiment 5) were submitted for parentage testing to confirm their fertilization status. To increase the number of cells available for DNA recovery, the zonae pellucidae were removed from the embryos by placing the embryo into 3 ml acid Tyrode's solution (pH 2, 137 mM NaCl, 2.7 mM KCl, 1.4 mM CaCl₂·2H₂O, 0.5 mM MgCl₂·6H₂O, 0.3 mM Na₂HPO₄·H₂O, 5.5 mM glucose, and 4 mg/ml PVP) and monitoring the embryo using a dissection microscope. When the zona pellucida started to dissolve, the embryo was removed from the Tyrode's solution and washed thrice in DMEM/F-12 with 20% FBS, during which the zona was shed. The embryo was then placed back into 0.5 ml DMEM/F-12 with 20% FBS in a well of a 4-well multidish for 3–4 days.

This allowed the blastocysts to expand and increase in size (Choi *et al.* 2009a). The blastocysts were loaded into 0.6 or 1.7-ml tubes in PBS and were plunged into liquid nitrogen for later analysis.

For analysis, embryos were shipped to an outside laboratory (Veterinary Genetics Laboratory, University of California, Davis, CA, USA). Parentage testing was performed based on genetic typing of the 13 microsatellite markers comprising the standard parentage panel of the International Society of Animal Genetics, using STRand fragment analysis (Toonen & Hughes 2001) with modifications (<http://www.vgl.ucdavis.edu/STRand>, Veterinary Genetics Laboratory).

Statistical analysis

Flow cytometric data were analyzed by a two-way ANOVA procedure, with Tukey's studentized range test used for mean

separation when F-ratios were different ($P < 0.05$). The proportions of injected oocytes cleaving and injected oocytes developing to blastocyst was compared among groups by Fisher's exact test.

Experiment 1: effect of lyophilization on DFI

Semen from ejaculates from three stallions was processed using the five methods detailed above and analyzed in duplicate for DFI after 1, 2, or 3 freeze/thaw or lyophilization/reconstitution cycles.

Experiment 2: effect of sperm extract injection on blastocyst development after ICSI with lyophilized sperm

Oocytes classified as Cp were used for this experiment. Semen from one ejaculate from one stallion (Stallion 2115) was used for all sperm injection in this and the following experiments, unless otherwise indicated. Oocytes in metaphase II were allocated to one of three treatments: 1) control, injection with motile frozen-thawed sperm; 2) control + sperm extract; 3) lyophilized, injection with rehydrated lyophilized sperm; or 4) lyophilized + sperm extract. Of the four replicates using motile frozen-thawed (control) sperm, two were performed with sperm from Stallion 2115 and two were performed with sperm from a different stallion (Stallion DD). Injected oocytes were placed in culture and were evaluated for blastocyst development at 7 and 8 days. Blastocysts were either transferred to recipient mares (see Experiment 5) or, if no recipient mares were available or sufficient embryos in that treatment had been transferred, were frozen for later parentage testing.

Experiment 3: effect of previous freezing/thawing on blastocyst development after ICSI with lyophilized sperm

Oocytes classified as Ex were used for this experiment. As results of Experiment 2 showed that no blastocyst development was obtained from oocytes injected with lyophilized sperm without sperm extract, all lyophilized treatments in this experiment were accompanied by injection of sperm extract.

After maturation, oocytes in metaphase II were allocated to one of the five treatments: 1) control, injected with motile sperm from frozen-thawed semen; 2) control + sperm extract; 3) lyophilized + sperm extract; 4) lyophilized sperm prepared from frozen-thawed semen + sperm extract; or 5) lyophilized sperm prepared from frozen-thawed semen that had been passed through a density gradient + sperm extract. Of the five replicates using motile frozen-thawed (control) sperm, three were performed with sperm from Stallion 2115 and two were performed with sperm from Stallion DD. Oocyte recovery, maturation, ICSI, embryo culture, blastocyst assessment, and disposition were performed as described in Experiment 2.

Experiment 4: effect of storage of lyophilized sperm on blastocyst formation after ICSI

An additional straw of standard-frozen semen from the original ejaculate from Stallion 2115 was thawed and lyophilized as described above, and the lyophilized sperm were stored at -20°C for the duration of this experiment. The experiment started within 1 week of this second lyophilization procedure; sperm from the original lyophilization procedure had been stored for 3.5 months at the time the experiment began. Both Ex and Cp oocytes were used. Blastocyst development rate was compared among control (motile, frozen-thawed sperm from Stallion 2115); control + sperm extract; sperm from the original frozen-thawed lyophilization; or sperm from the second frozen-thawed lyophilization. All other procedures were performed as in Experiment 2.

Experiment 5: viability of blastocysts produced by injection with lyophilized sperm

When recipient mares were available, blastocysts resulting from Experiments 2, 3, and 4 were transferred to determine their developmental competence. Overall, ten blastocysts produced by ICSI with control sperm (five with injection of sperm extract and five without injection of sperm extract) and seven blastocysts produced by ICSI in the lyophilized sperm treatment (four from fresh semen and three from frozen semen) were transferred. For transferring to recipient mares, blastocysts were loaded in 1.1 ml DMEM/F-12 with 10% FBS into glass vials, wrapped in 120 ml ballast at 38.2°C , placed in a shipping container (Equitainer, Hamilton Research, Inc., South Hamilton, MA, USA) in which the coolant cans had been warmed to 38.2°C , and shipped 4–6 h before transferring to recipient mares. Transcervical transfer (one blastocyst per mare) was performed using recipients that had ovulated from the same day that ICSI was performed to 4 days afterward. Pregnancy status was determined by transrectal ultrasonography starting 5 days after embryo transfer. The first three pregnancies established in the lyophilized sperm treatment were allowed to continue; all others were terminated by administration of prostaglandin $\text{F}_{2\alpha}$, 10 mg i.m., after either a fetus with heartbeat was visualized within the conceptus (expected between 25 and 28 days) on transrectal ultrasonography or after 32 days gestation, if no fetal heartbeat was seen.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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