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Freeze-dried spermatozoa: An alternative biobanking option for endangered species

Debora Agata Anzalone[a], Luca Palazzese[a], Domenico Iuso[a,1], Giuseppe Martino[b], Pasqualino Loi[a,*]

[a] Faculty of Veterinary Medicine, University of Teramo, Campus Coste Sant'Agostino, Renato Balzarini Street, 1, 64100 Teramo, Italy
[b] Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Campus Coste Sant'Agostino, Renato Balzarini Street, 1, 64100 Teramo, Italy

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ABSTRACT

In addition to the iconic wild species, such as the pandas and Siberian tigers, an ever-increasing number of domestic species are also threatened with extinction. Biobanking of spermatozoa could preserve genetic heritages of extinct species, and maintain biodiversity of existing species. Because lyophilized spermatozoa retain fertilizing capacity, the aim was to assess whether freeze-dried spermatozoa are an alternative option to save endangered sheep breeds. To achieve this objective, semen was collected from an Italian endangered sheep breed (Pagliarola), and a biobank of cryopreserved and freeze-dried spermatozoa was established, and evaluated using IVF (for frozen spermatozoa) and ICSI procedures (for frozen and freeze-dried spermatozoa). As expected, the fertilizing capacity of cryopreserved Pagliarola's spermatozoa was comparable to commercial semen stocks. To evaluate the activating capability of freeze-dried spermatozoa, 108 MII sheep oocytes were subjected to ICSI, and allocated to two groups: 56 oocytes were activated by incubation with ionomycin (ICSI-FDSa) and 52 were not activated (ICSI-FDSna). Pronuclear formation (2PN) was investigated at 14–16 h after ICSI in fixed presumptive zygotes. Only artificially activated oocytes developed into blastocysts after ICSI. In the present study, freeze-dried ram spermatozoa induced blastocyst development following ICSI at a relatively high proportion, providing evidence that sperm lyophilization is an alternative, low cost storage option for biodiversity preservation of domestic species.

1. Introduction

According to the Second Report on the State of the World's "Animal Genetic Resources for Food and Agriculture" (2000–2014), approximately 99 livestock breeds have become extinct, and about 17% of them are threatened with extinction. Domestic breeds are considered as endangered when there are less than a total of 1000 females and 20 fertile males that are still alive for the breed. In these cases, reproductive programs, such as artificial insemination (AI), *in vitro* fertilization (IVF) and the associated technology, Intra Cytoplasmic Sperm Injection (ICSI), might be an option to increase reproductive rates of the endangered population (Henson, 1992; Comizzoli et al., 2000).

The protocols for sperm cryopreservation are well established in mammalian species including cattle, sheep, mice and humans (Parrish et al., 1995; Salamon and Maxwell, 1995; Storey et al., 1998; O'Connell et al., 2002), but not in wild species where the

* Corresponding author.
E-mail address: ploi@unite.it (P. Loi).
[1] Present address: INSERM, Institute Albert Bonniot, Universitè Grenoble Alpes, 38700 Grenoble, France.

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reproductive physiology has yet to be well characterized (Fickel et al., 2007). The conventional cryopreservation in liquid nitrogen induces a cellular dehydration through a progressive temperature reduction until the temperature of −196 °C is reached. The use of cryoprotectant agents (CPAs) in the procedure is essential to prevent cellular damage due to osmotic stress and membrane shrinkage (Medeiros et al., 2002). These storage conditions are, however, not always available because of the considerable costs and the lack of availability of liquid nitrogen, especially in areas with an arid climate or under developed countries.

The lyophilization of semen represents an innovative and ecological non-cryogenic storage solution (Loi et al., 2013; Kaneko et al., 2014). The freeze-drying process leads to the removal of water, thus, the conservation of specimens in an anhydrous state. Briefly, first the sample needs to be frozen at low temperatures (freezing step); then, by a sublimation process, water is removed passing from a solid to an aeriform state (drying step). The final product in the dry form can be stored at room temperature for long periods of time and product can easily be transported worldwide. Since the breakthrough achieved by Wakayama and Yanagimachi (1998) in producing live offspring using lyophilized mouse spermatozoa for fertilization, viable offspring have been obtained in rabbits, rats and horses by intracytoplasmic sperm injection (ICSI) of lyophilized spermatozoa (Wakayama and Yanagimachi, 1998; Liu et al., 2004; Hirabayashi et al., 2005; Choi et al., 2011).

In the present study, conventional cryopreservation and freeze-drying techniques were used for establishing a genetic bank of male gametes from an endangered Italian sheep breed native of the province of Teramo (Abruzzo, Italy). The breed, name “Pagliarola” (straw-eater) denotes a rustic animal, and was maintained in small flocks by rural families to provide foodstuff, however, there was a decrease in numbers from 350,000 to 25 (21 ewes and four rams) in less than a century. This breed, therefore, serves as a dramatic case of biodiversity loss in a farm animal breed. In addition, technical improvements of ICSI using lyophilized spermatozoa are reported.

2. Materials and methods

2.1. Ethic statement

All experiments were performed in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) in concordance with European Community regulation 86/609 and were approved by CEISA (Inter-Institutional Ethics Committee for Animal Experimentation) Prot. 79/2013/CEISA Prog. 58. The permit n°: CEISA VI, Class 8.1, Prot. 2823. All chemicals were obtained from Sigma Aldrich unless otherwise stated.

2.2. Semen collection

Semen was collected from two adult fertile Pagliarola rams using an Artificial Vagina (AV) filled with warm water (40–44 °C) and connected to a 15 ml tube. Immediately after collection, sperm motility was evaluated using a stereomicroscope and sperm concentration was assessed using a Burker chamber. Only ejaculates with sperm concentrations $\geq 1.8 \times 10^9$ spermatozoa/ml and motility ≥70%, were used for the experiments.

2.3. Spermatozoa cryopreservation: media and procedure

Freezing media was prepared in two steps. Initially a basic medium was prepared by dissolving 2.42 g TRIS base, 1.36 g citric acid, 1.00 g fructose, 100.000 IU penicillin G, 0.1 g streptomycin in 67.20 ml of bi-distilled water (ddH_20) at an adjusted pH of 6.7–6.8. Subsequently, the basic medium was divided in two equal volumes (33.60 ml) and preparation of two media continued. Medium A (or 30 °C medium) and Medium B (or 4 °C medium) were prepared by adding 10 ml of egg yolk, 6.40 ml of ddH_20 (for Medium A) and 10 ml of egg yolk, 6.40 ml of glycerol for Medium B. Medium A and B were maintained at 30 and 4 °C, respectively before use. Freezing medium composition is reported in Table 1.

For cryopreservation of spermatozoa, Medium A was initially added to the ejaculate and transferred immediately to a cold room (4 °C) to allow a controlled cooling - from 30 to 4 °C - over 2 h. Subsequently, Medium B was gently added to the suspension, and was stored for 2 h in the cold room. Medium A and Medium B were added in the same volume to dilute the ejaculate to a final concentration of 400×10^6 spermatozoa/ml. Every 30 min the tubes were gently mixed by rotating the plugged flask 180 degrees. Subsequently, 250 µl plastic straws were filled, sealed with polyvinyl alcohol (PVA) and placed on a metallic grid to stabilize for the last 2 h at 4 °C. The straws were then exposed to LN vapors (−80 °C) in a Dewar flask and maintained for 6 min, before being plunged

Table 1
Composition of media for sperm cryopreservation. Medium base was equally added to Medium A and B.

Basic Medium		Medium A (30 °C)		Medium B (4 °C)	
TRIS	2.42 g	Basic Medium	33.60 ml	Basic Medium	33.60 ml
Citric acid	1.36 g	ddH_2O	6.40 ml	Glycerol	6.40 ml
Fructose	1.00 g	Egg yolk	10.00 ml	Egg yolk	10.00 ml
Penicillin G	100.000 IU				
Streptomycin	0.1 g				
ddH_2O	67.20 ml				

into LN and stored in tanks filled with LN until the time of semen use.

2.4. Sperm freeze-drying: media and procedure

Spermatozoa were lyophilized as previously reported (Loi et al., 2008a), using a Freeze-Dry apparatus (SP Scientific-VirTis, 2.0 BenchTop) following the protocol used by Wakayama and Yanagimachi (1998). For the final stage of the process, each ampule was sealed by vacuum and stored at room temperature (RT, 18–23 °C) until use.

To assess membrane integrity, freeze-dried spermatozoa were evaluated by PI staining. Samples were rehydrated by adding 100 µl of bi-distilled water and incubated in 5 µg/ml PI solution for 10 min, in the dark, at room temperature. Subsequently, 10 µl of sample were placed on a microscope slide, mounted with Fluoromount and immediately observed using a confocal microscope (Nikon Eclipse Ti-E).

2.5. Sperm plasma membrane evaluation after lyophilization

To evaluate the integrity of plasma membrane after lyophilization, spermatozoa were first rehydrated by adding 100 µl of ddH_2O, were then incubated 10 min in a 5 µg/ml propidium iodide (PI) solution in PBS, because the PI is a fluorescent dye it can only permeate damaged sperm membranes. A 15 µl drop of semen was then placed on the slide, and a coverslip was placed over the drop of semen which was observed using an epifluorescence microscope (Nikon Eclipse E-600). A minimum of 150 spermatozoa were assessed on each slide.

2.6. Oocyte recovery and in vitro maturation (IVM)

Sheep ovaries were collected from a local slaughterhouse and transferred to the research laboratory within 1–2 h. Oocytes were aspirated using a 21 G needles in the presence of 4-(2- hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered TCM-199 medium (Gibco, Life Technologies, Milan, Italy) and 0.005% (w:v) heparin. Only oocytes having at least 2 or 3 layers of compact cumulus cells were selected for IVM. This process was performed in 4 well-dishes containing 500 µl of IVM medium. The IVM medium was composed of bicarbonate-buffered TCM-199 (Gibco) containing 2 mM glutamine, 0.3 mM sodium pyruvate, 100 µM cysteamine, 10% fetal bovine serum (FBS) (Gibco), 5 µg/ml follicle stimulating hormone (FSH; Ovagen, ICP, Auckland, New Zealand), 5 µg/ml luteinizing hormone (LH) and 1 µg/ml 17β-estradiol. Maturation was completed in a humidified atmosphere at 38.5 °C and 5% CO_2 in air for 24 h, as previously described by Ptak et al. (2002). After IVM, only MII oocytes with an expanded cumulus and normal morphology were selected to be used for ICSI.

2.7. In vitro fertilization (IVF)

The fertilizing capability of frozen semen of Pagliarola rams (PAGL) was compared with a commercial stock (COMM) of frozen semen from Sarda rams, using *in vitro* fertilization procedures, as previously described (Ptak et al., 2002). Briefly, semen was fast-thawed in 35 °C water and centrifuged in bicarbonate-buffered synthetic oviductal fluid (SOF-) containing 0.4% BSA (w/v), at 1000 rpm for 5 min. Cumulus-Oocytes-Complexes (COCs) are sticky and difficult to handle after *in vitro* maturation because of the deposition of hyaluronic acid by cumulus cells. To ease the displacement of cumulus cells, COCs were rapidly passed in 300 U/ml hyaluronidase solution initially (dissolved in H199) and subsequently 9 or 10 oocytes were placed in50 µl of IVF medium and covered with mineral oil. Oocytes were subsequently incubated with spermatozoa (5×10^6 sperm/ml) overnight, in a humidified atmosphere at 38.5 °C, 5% CO_2, and 7% O_2. On the next day, oocytes were pipetted in SOF- medium to remove most of the spermatozoa attached to the zona pellucida and cultured as subsequently described.

2.8. Intracytoplasmic sperm injection (ICSI)

2.8.1. Sperm preparation

The ICSI procedure was performed with frozen semen (ICSI-FS) and freeze-dried spermatozoa (ICSI-FDS) as previously described by Anzalone et al. (2016). Briefly, a single straw was fast-thawed by immersion in 35 °C water for a few seconds was opened and transferred using a 1.5 ml Eppendorf pipette and incubated three minutes in a humidified atmosphere at 38.5 °C and 5% CO_2.Subsequently, 5 µl of semen was diluted in 100 µl of IVF medium (SOF- enriched with 20% (v:v) heat-inactivated estrous sheep serum, and 16 µM isoproterenol) buffered with HEPES (referred to as H-IVF medium). This mixture was diluted 1:1 with 12% (w:v) PolyVinylPyrrolidone (PVP) and a 10 µl drop of this mixture was placed on the lid of a Petri dish that was located on a warmed microscope stage covered by mineral oil.

Freeze-dried spermatozoa were rehydrated by adding 100 µl of bi-distilled water, and subsequently a 5 µl aliquot was suspended in 100 µl of H199 medium and processed as described previously for frozen spermatozoa.

2.8.2. ICSI procedure

The intracytoplasmic sperm injection was performed using an inverted microscope (Nikon Eclipse E-800) connected to a micromanipulation system (Narishige NT-88NEN, Tokyo, Japan), using a piezo micropipette manipulating system (PiezoXpert, Eppendorf, Milan, Italy) as previously described (Anzalone et al., 2016). The oocytes were injected in groups of five to avoid

prolonged light exposure and the PVP/sperm drops were refreshed after ten oocytes had been injected.

Oocytes of an aliquot were injected with a freeze-dried sperm that was activated as a result of 5 min of incubation in 5 µM ionomycin dissolved in H199 + 0.4% BSA (ICSI-FDS*a*), while another one non-activated aliquot was placed directly into the culture mixture without further activation (ICSI-FDSna) as previously described.

2.9. Embryo culture

All presumptive zygotes that resulted from oocytes treated with ICSI and IVF procedures, were cultured in numbers of 4 or 5 per drop in 20 µl of SOF- enriched with 2% (v:v) basal medium Eagle essential amino acids (EAA), 1% (v:v) minimum essential medium (MEM) non-essential amino acids (NEAA) (Gibco), 1 mM glutamine, and 8 mg/ml fatty acid-free BSA, covered by mineral oil. The medium was renewed on day 3 of culture [SOF- supplemented with 0.27 mg/ml glucose (SOF^+), 2% EAA, 1% NEAA]; again on day 5 of culture [SOF^+ with 10% of charcoal stripped FBS (cs-FBS), 2% EAA, 1% NEAA]; and again on day 6 (1:1 MEM/M199 enriched with 10% cs-FBS, 2.5 µg/ml gentamicin and 1% sodium pyruvate) until day 7 or 8 of culture. The *in vitro* development was evaluated at 24 h for cleavage (only the 2 cell-stage of embryos was considered to have cleaved) and at day 7 or 8 of culture assessments were made for blastocyst formation.

2.10. Pronuclear staining

To visualize pronuclei (2PN) in ICSI-FS, ICSI-FDS*a* and ICSI-FDS*na*, a total of 27, 30 and 21 presumptive zygotes were fixed in 4% paraformaldehyde (PFA) for 20 min, at 14–16 h after spermatozoa injection. Then, presumptive zygotes were permeabilized with 0.1% Triton X-100, and stained for 5 min with 5 µg/ml PI at room temperature, washed twice in 0.4% PVP (in PBS) and mounted on slides. Images were obtained using a confocal microscope (Nikon Eclipse T*i*-E).

2.11. Statistical analysis

The one-way ANOVA test and Fisher's exact test were used to compare 2PN and *in vitro* embryo developmental stages between the groups. Data were analyzed using PRISM software version 5.0, GraphPad, and the values were considered to be different $P < .05$.

3. Results

3.1. Sperm plasma membrane in freeze-dried spermatozoa

Propidium iodide stained 100% (174/174) of lyophilized-rehydrated spermatozoa, indicating that all freeze-dried spermatozoa were not viable (Fig. 1).

3.2. Embryo development

Data for all embryo development outcomes are reported in Table 2.

3.2.1. IVF outcomes

In vitro fertilization with COMM and PAGL frozen semen resulted in very similar embryo development rates in terms of cleavage [50% (43/86) compared with 41% (36/88), COMM compared with PAGL, respectively] and blastocyst rate [29% (25/86) compared with 31.8% (28/88], COMM compared with PAGL, respectively (Table 2, Fig. 2A). Representative images of IVF derived blastocysts

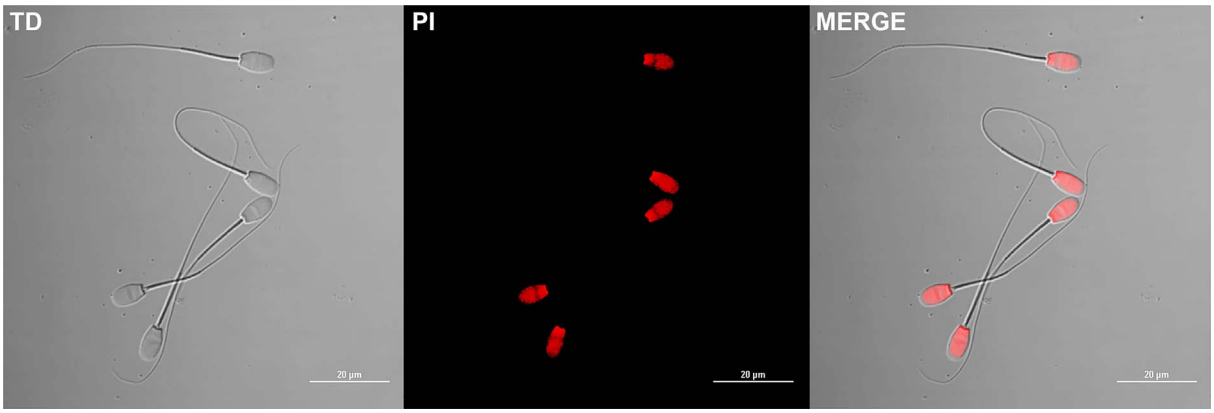

Fig. 1. Sperm plasma membrane integrity after freeze drying. Propidium iodide (PI) stained all sperm heads (nuclei) indicating that all ram freeze-dried spermatozoa were not viable after rehydration. MERGE means PI + TD (Transmitted DIC). Scale bar = 20 µm.

Table 2
Outcomes from IVF and ICSI. *In vitro* fertilization was assessed with commercial (IVF COMM) and Pagliarola (IVF PAGL) frozen semen. ICSI outcomes derived from use of frozen sperm (ICSI-FS) and freeze-dried spermatozoa with and without subsequent oocyte activation (ICSI-FDSa and ICSI-FDSna, respectively).

Groups	No. Oocytes	2PN	Lysed (%)	Fragmented (%)	Non divided (%)	2-Cells (%)	Blastocyst (%)
IVF COMM	93	/	7/93 (7.52)	9/86 (10.5)	34/86 (39.5)	43/86 (50)	25/86 (29)
IVF PAGL	96	/	8/96 (8.3)	12/88 (13.6)	40/88 (45.4)	36/88 (41)	28/88 (31.8)
ICSI-FS	44	22/27 (81.4)*	6/44 (13.6)	4/38 (10.5)	20/38 (52.6)**	14/38 (36.8)***	12/38 (31.5)****
ICSI-FDS*na*	52	3/21 (14.2)	7/52 (13.5)	11/45 (24.4)	29/45 (64.4)	5/45 (11)	0/45 (0)****
ICSI-FDS*a*	56	24/30 (80)*	7/56 (12.5)	9/49 (18.3)	24/49 (49)	16/49 (32.7)***	5/49 (10.2)****

* $P < .05$ ICSI-FDSna compared with ICSI-FDSa and $P < .01$ ICSI-FDSna compared with ICSI-FS.
** $P < .05$ ICSI-FDSna compared with ICSI-FS.
*** $P < .05$ ICSI-FDSna compared with ICSI-FDSa and $P < .01$ ICSI-FDSna compared with ICSI-FS.
**** $P < .05$ ICSI-FDSa compared with ICSI-FDSna and ICSI-FS, and $P < .0001$ ICSI-FDSna compared with ICSI-FS.

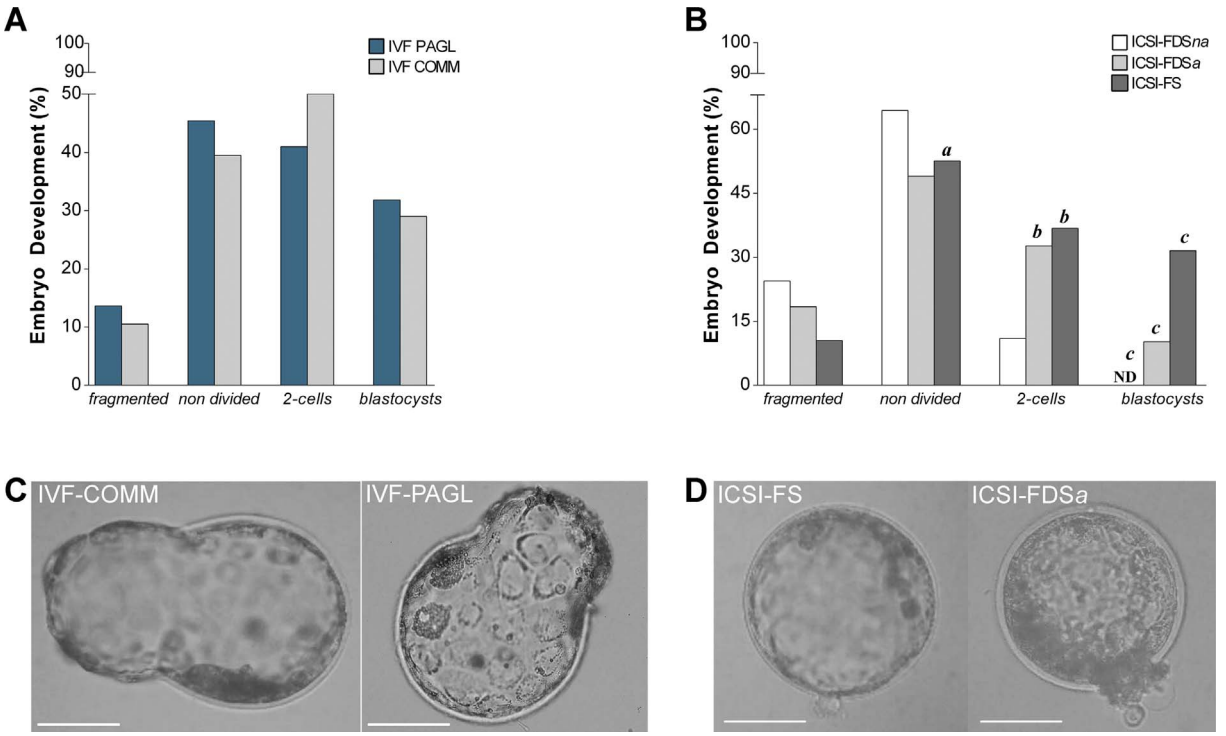

Fig. 2. Embryo development outcomes from IVF and ICSI. A) IVF with Pagliarola's frozen sperm (IVF PAGL) resulted in similar outcomes in terms of 2-cell stage development, non-divided, fragmented and blastocysts, compared to IVF with commercial frozen spermatozoa (IVF COMM). B) Graph shows embryo results from ICSI of frozen (ICSI-FS) and freeze-dried spermatozoa with (ICSI-FDSa) and without (ICSI-FDSna) ionomycin activation. ICSI-FDSna results in less 2-cell stage development at 24 h compared to ICSI-FDSa and to ICSI-FS. Activation by ionomycin after sperm injection (ICSI-FDSa) increased the cleavage rate and led to 10.2% blastocyst development. a = $P < .05$ ICSI-FDSna compared with ICSI-FS; b = $P < .05$ ICSI-FDSna compared with ICSI-FDSa and $P < .01$ ICSI-FDSna compared with ICSI-FS; c = $P < .05$ ICSI-FDSa compared with ICSI-FDSna and ICSI-FS, and $P < .0001$ ICSI-FDSna compared with ICSI-FS. ND means No Development to blastocyst. C, D) Representative photos of blastocysts at the 8th day of culture, obtained from IVF-COMM/IVF-PAGL and from ICSI-FS and ICSI-FDSa, respectively. Scale bar = 100 µm.

are included in Fig. 2C).

3.2.2. ICSI outcomes

The ICSI-FDS*a* derived presumptive zygotes had a greater number of 2PN than the ICSI-FDSna derived presumptive zygotes [80% (24/30) compared with 14.3% (3/21), respectively, $P < .01$] with the ICSI-FDSna derived presumptive zygotes having a lesser number of 2PN than the ICSI-FS derived presumptive zygotes [14.2% (3/21) compared with 81.4% (22/27), respectively, $P < .001$; Table 2]. Representative images of activated and not-activated oocytes are included in Fig. 3A and B, respectively.

The proportion of embryos cleaved at the two-cell stage was less for ICSI-FDSna compared with the ICSI-FDSa and ICSI-FS derived embryos (11% (5/45) compared with 32.7% (16/49) and 36.8% (14/38), respectively, $P < .05$ and $P < .01$). The number of oocytes that was fragmented was similar in all groups [24.4% (11/45), 18.3% (9/49) and 10.5% (4/38) in ICSI-FDSna, ICSI-FDSa and ICSI-FS, respectively]. The percentage of blastocysts produced ranged from 0% in (ICSI-FDSna), to 10.2% (ICSI-FDSa; 5/49); and, as expected, the greatest development was with the ICSI-FS derived blastocysts (31.5%, 12/38; Table 2, Fig. 2B). Representative images

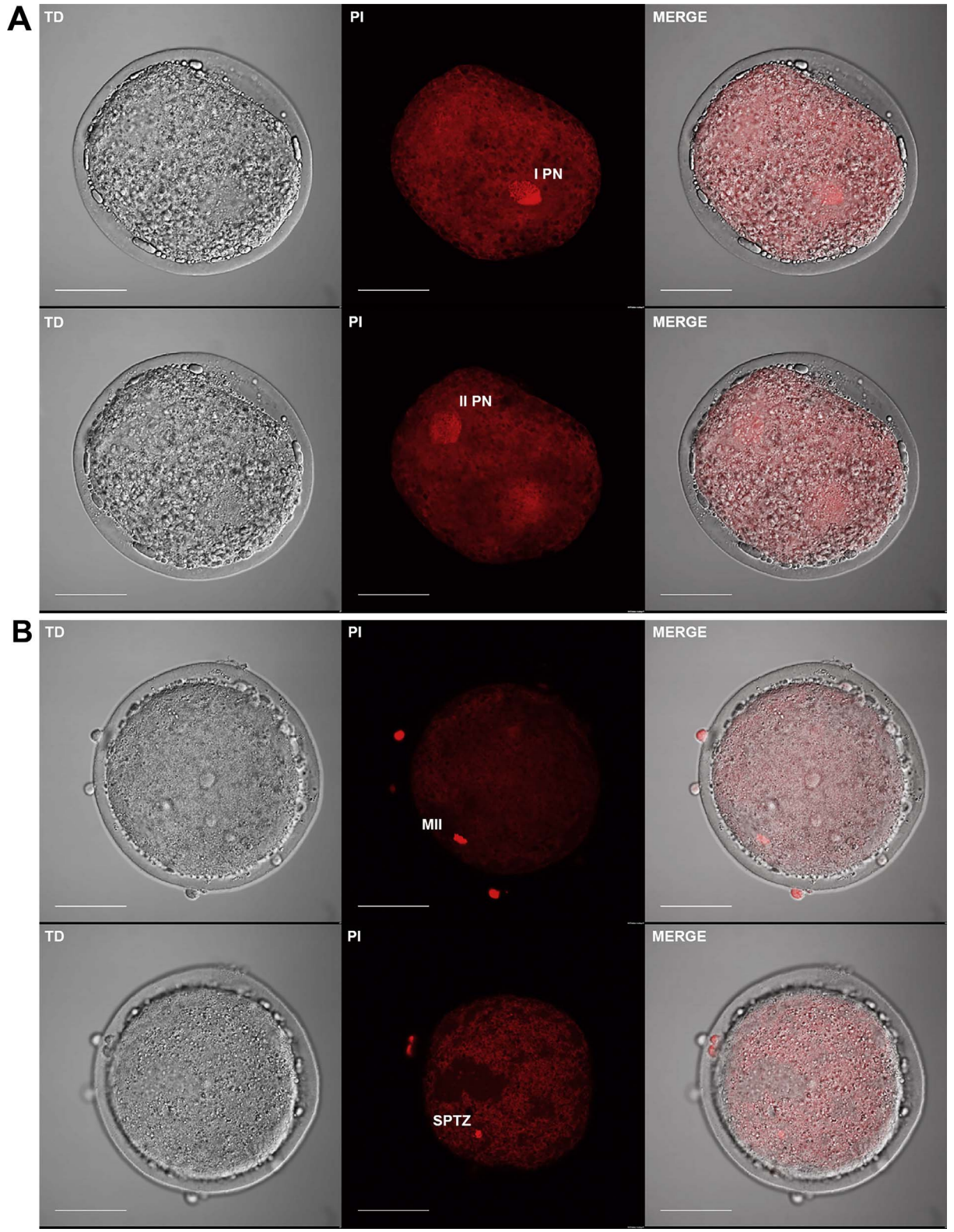

Fig. 3. Pronuclear formation after ICSI. A) Representative image of activated oocytes with two pronuclei (I PN and II PN), on different focal planes (upper and lower line). C) Representative image of non-activated oocytes showing oocytes metaphase (MII) and a non-decondensed sperm head (SPTZ) on different focal planes (upper and lower line). All nuclei were counterstained with propidium iodide (PI); MERGE means PI + TD (Transmitted DIC). Scale bar = 50 μm.

Fig. 4. Pagliarola's semen biobank. Photo shows straws of frozen semen (FS), and glass vials containing freeze-dried spermatozoa (FDS) collected from Pagliarola rams.

of ICSI derived blastocysts are included in Fig. 2D).

3.3. Sperm biobanking

A sperm biobank from *Pagliarola* sheep was established with more than 600 straws of frozen semen stored into a liquid nitrogen tank and with 100 glass vials of freeze-dried spermatozoa, each containing 100×10^6 spermatozoa (Fig. 4).

4. Discussion

In the present study, the use of lyophilization was demonstrated to be a viable method for preserving ram spermatozoa from an endangered domestic sheep breed, Pagliarola. Considering the lack of viability of re-hydrated spermatozoa, the ICSI procedure was used to assess the fertilization capacity of these spermatozoa similar to the procedures previously used for these assessments (Anzalone et al., 2016). Previously it was reported that fresh ram spermatozoa activate the oocyte after ICSI, without the need of further chemical activation (*i.e.*, by ionomycin; Anzalone et al., 2016). It is assumed, similar to what occurs in other species, that the Sperm-Oocyte-Activating-Factor (SOAF) localized in the sperm plasma membrane induces oocyte activation eliciting a Ca^{2+} release from ooplasmic stores (Saunders et al., 2002; Kashir et al., 2010; Amdani et al., 2015). Spermatozoa with damaged membranes might, however, lose the capacity to activate the oocyte after ICSI, due to the loss of sperm activation factor(s) (Yanagimachi, 2005), rendering artificial activation a compulsory step for use of ICSI (Tesarik and Sousa, 1995; Yanagida et al., 1999; Zhang et al., 1999; Eldar-Geva et al., 2003). Accordingly, it has been observed that pronuclear formation after ICSI occurs with use of freeze-dried spermatozoa and pronuclear formation is markedly reduced when non-activated oocytes (ICSI-FDSna) are used. With artificially activated oocytes (ICSI-FDSa), pronuclear formation was increased to the same extent as the control group (ICSI-FS). Accordingly, cleavage to 2 cell-stage embryos, as well as blastocyst development rates increased with use of ICSI-FDSa. With the exception of the mouse model, where use of ICSI with dry spermatozoa resulted in very high developmental rates (Wakayama and Yanagimachi, 1998; Kusakabe et al., 2008), blastocysts rate ranging from 10% to 12% occurs in large animals (horses, cattle and pigs) (Keskintepe et al., 2002; Kwon et al., 2004; Choi et al., 2011) with a greater rate of about 24% in rabbits (Liu et al., 2004). Recently, it has been reported for the first time the production of blastocysts from sheep oocytes following ICSI with freeze-dried spermatozoa (Olaciregui et al., 2017). Although the developmental rates were greater in this previous compared with the present study (25% of embryos reaching blastocyst stage), the previously reported study had some shortcomings, starting with the difficulty of identification of normal blastocyst stage embryos in the photos provided, and furthermore and more importantly, the lack of a proper control excluding parthenogenetic development after use of spermatozoa for injecting oocytes. Efforts in the present study were aimed at the development of a robust and repeatable protocol to produce acceptable quality blastocysts. In this regard, activation of oocytes was found to be necessary to induce embryonic development following injection of lyophilized spermatozoa. While activation rates were improved in the ICSI-FDSa group, being comparable to the ICSI-FS group, the disappointing aspect of the present results was the low cleavage frequency in both groups (32.7% and 36.8%, respectively). This finding is not consistent with the resulting high proportion of pronuclei detected in both groups (about 80%), and clearly indicates that other factors, rather than activation, are responsible for embryonic development problems with use of sheep ICSI procedures. Further studies focusing on important processes such as centriole dynamics, or S phase entry/exit will provide helpful insights as to the defective steps that hamper development with use of sheep ICSI.

Besides the current limits of ICSI in sheep, the development to blastocysts after oocytes are fertilized by lyophilized spermatozoa is about a third of what occurs when using frozen spermatozoa, indicating that the conservation of spermatozoa in the anhydrous state needs further development for these procedures to be optimal. The current lyophilization procedures applied to spermatozoa are essentially similar to those used for foodstuff or pharmaceutical product preservation. Lyophilization technologies for spermatozoa

are, therefore, in the early stages of development, and there is ample opportunity for improving the use of this technology for this purpose. These advancements could be in development of enhanced media for use of this technology, to other procedural changes such as changes with freezing temperatures and use of vacuum conditions. The findings to the present provide for cautious optimism for development of effective lyophilization technologies for sperm storage (Wakayama and Yanagimachi, 1998; Kusakabe et al., 2001; Hirabayashi et al., 2005; Kusakabe et al., 2008; Loi et al., 2008a, 2008b; Gianaroli et al., 2012; Iuso et al., 2013) and indicates lyophilization might be effective for replacing the traditional cryopreservation storage methods for spermatozoa. To conclude, in the present study a sperm bank was established for a seriously endangered sheep breed. Furthermore, lyophilized, as well as cryopreserved spermatozoa have the capacity to induce development to blastocyst stage embryos with use of ICSI in acceptable proportions after storage of the spermatozoa at room temperatures. This finding supports the development of low costs, on-the-shelf genetic biobanks for domestic as well as endangered species.

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Conflict of interest

The authors declare that there is no conflict of interest in publishing this work.

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References

Amdani, S.N., Yeste, M., Jones, C., Coward, K., 2015. Sperm factors and oocyte activation: current controversies and considerations. Biol. Reprod. 93, 50 Review.
Anzalone, D.A., Iuso, D., Czernik, M., Ptak, G., Loi, P., 2016. Plasma membrane and acrosome loss before ICSI is required for sheep embryonic development. J. Assist. Reprod. Genet. 33, 757–763.
Choi, Y.H., Varner, D.D., Love, C.C., Hartman, D.L., Hinrichs, K., 2011. Production of live foals via intracytoplasmic injection of lyophilized sperm and sperm extract in the horse. Reproduction 142, 529–538.
Comizzoli, P., Mermillod, P., Mauget, R., 2000. Reproductive biotechnologies for endangered mammalian species. Reprod. Nutr. Dev. 40, 493–504.
Eldar-Geva, T., Brooks, B., Margalioth, E.J., Zylber-Haran, E., Gal, M., Silber, S.J., 2003. Successful pregnancy and delivery after calcium ionophore oocyte activation in a normozoospermic patient with previous repeated failed fertilization after intracytoplasmic sperm injection. Fertil. Steril. 79, 1656–1658.
Fickel, J., Wagener, A., Ludwig, A., 2007. Semen cryopreservation and the conservation of endangered species. Eur. J. Wildl. Res. 53, 81–89.
Gianaroli, L., Magli, M.C., Stanghellini, I., Crippa, A., Crivello, A.M., Pescatori, E.S., Ferraretti, A.P., 2012. DNA integrity is maintained after freeze-drying of human spermatozoa. Fertil. Steril. 97, 1067–1073.
Henson, E.L., 1992. In situ conservation of livestock and poultry. FAO Anim. Health Prod. 99, 112.
Hirabayashi, M., Kato, M., Ito, J., Hochi, S., 2005. Viable rat offspring derived from oocytes intracytoplasmically injected with freeze-dried sperm heads. Zygote 13, 79–85.
Iuso, D., Czernik, M., Di Egidio, F., Sampino, S., Zacchini, F., Bochenek, M., Smorag, Z., Modlinski, J.A., Ptak, G., Loi, P., 2013. Genomic stability of lyophilized sheep somatic cells before and after nuclear transfer. PLoS One 8, e51317.
Kaneko, T., Ito, H., Sakamoto, H., Onuma, M., Inoue-Murayama, M., 2014. Sperm preservation by freeze-drying for the conservation of wild animals. PLoS One 9, e113381.
Kashir, J., Heindryckx, B., Jones, C., De Sutter, P., Parrington, J., Coward, K., 2010. Oocyte activation, phospholipase C zeta and human infertility. Hum. Reprod. Update 16, 690–703.
Keskintepe, L., Pacholczyk, G., Machnicka, A., Norris, K., Curuk, M.A., Khan, I., Brackett, B.G., 2002. Bovine blastocyst development from oocytes injected with freeze-dried spermatozoa. Biol. Reprod. 67, 409–415.
Kusakabe, H., Szczygiel, M.A., Whittingham, D.G., Yanagimachi, R., 2001. Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa. PNAS 98, 13501–13506.
Kusakabe, H., Yanagimachi, R., Kamiguchi, Y., 2008. Mouse and human spermatozoa can be freeze-dried. Biol. Reprod. 67, 233–239.
Kwon, I.K., 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., 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, 79–85.
Loi, P., Matzukawa, K., Ptak, G., Natan, Y., Fulka Jr., J., Arav, A., 2008a. Nuclear transfer of freeze-dried somatic cells into enucleated sheep oocytes. Reprod. Domest. Anim. 43, 417–422.
Loi, P., Matsukawa, K., Ptak, G., Clinton, M., Fulka Jr., J., Nathan, Y., Arav, A., 2008b. Freeze-dried somatic cells direct embryonic development after nuclear transfer. PLoS One 3, e2978.
Loi, P., Iuso, D., Czernik, M., Zacchini, F., Ptak, G., 2013. Towards storage of cells and gametes in dry form. Trends Biotechnol. 31, 688–695.
Medeiros, C.M., Forell, F., Oliveira, A.T., Rodrigues, J.L., 2002. Current status of sperm cryopreservation: why isn't it better? Theriogenology 57, 327–344 Review.
O'Connell, M., McClure, N., Lewis, S.E.M., 2002. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum. Reprod. 17, 704–709.
Olaciregui, M., Luño, V., Domingo, P., González, N., Gil, L., 2017. In vitro developmental ability of ovine oocytes following intracytoplasmic injection with freeze-dried spermatozoa. Sci. Rep. 7, 1096.
Parrish, J.J., Krogenaes, A., Susko-Parrish, J.L., 1995. Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. Theriogenology 44, 859–869.
Ptak, G., Clinton, M., Tischner, M., Barboni, B., Mattioli, M., Loi, P., 2002. Improving delivery and offspring viability of in vitro produced and cloned sheep embryos. Biol. Reprod. 67, 1719–1725.

Salamon, S., Maxwell, W.M.C., 1995. Frozen storage of ram semen. I. Processing, freezing, thawing and fertility after cervical insemination. Anim. Reprod. Sci. 37, 185–249.
Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K., Lai, F.A., 2002. PLC zeta: a sperm-specific trigger of Ca2+ oscillations in eggs and embryo development. Development 129, 3533–3544.
Storey, B.T., Noiles, E.E., Thompson, K.A., 1998. Comparison of glycerol, other polyols, trehalose, and raffinose to provide a defined cryoprotectant medium for mouse sperm cryopreservation. Cryobiology 37, 46–58.
Tesarik, J., Sousa, M., 1995. More than 90% fertilization rates after intracytoplasmic sperm injection and artificial induction of oocyte activation with calcium ionophore. Fertil. Steril. 63, 343–349.
Wakayama, T., Yanagimachi, R., 1998. Development of normal mice from oocytes injected with freeze-dried spermatozoa. Nat. Biotechnol. 16, 639–641.
Yanagida, K., Katayose, H., Yazawa, H., Kimura, Y., Sato, A., Yanagimachi, H., Yanagimachi, R., 1999. Successful fertilization and pregnancy following ICSI and electrical oocyte activation. Hum. Reprod. 14, 1307–1311.
Yanagimachi, R., 2005. Intracytoplasmic injection of spermatozoa and spermatogenic cells: its biology and applications in humans and animals. Reprod. Biomed. Online 10, 247–288.
Zhang, J., Wang, C.W., Blaszcyzk, A., Grifo, J.A., Ozil, J., Haberman, E., Adler, A., Krey, L.C., 1999. Electrical activation and in vitro development of human oocytes that fail to fertilize after intracytoplasmic sperm injection. Fertil. Steril. 72, 509–512.