

## Freeze-dried sperm: an alternative biobanking solution for endangered farm species

Debora Agata Anzalone<sup>1</sup>, Luca Palazzese<sup>1</sup>, Domenico Iuso<sup>2</sup> and Pasqualino Loi<sup>1</sup>.

<sup>1</sup>University of Teramo, Faculty of Veterinary Medicine, Teramo, Italy;

<sup>2</sup>current affiliation: INSERM, Institute Albert Bonniot, University Grenoble Alpes, Grenoble, France.

### BACKGROUND

An ever-increasing number of domestic species are threatened with extinction. **Biobanking of spermatozoa** could represent a feasible and efficient way for preserving genetic heritage and to maintain biodiversity. Given the published evidence that freeze-dried (or lyophilized) spermatozoa retain their fertilizing capacity, we created a **Biobank of cryopreserved and freeze-dried spermatozoa from an Italian endangered sheep breed (Pagliarola Sheep, Fig 1).**



Fig 1. Pagliarola's Ram (Abruzzo, Italy).

### MATERIAL & METHODS

Semen from Pagliarola rams was collected by an artificial vagina and immediately assessed for motility. Then frozen and lyophilized as follows:

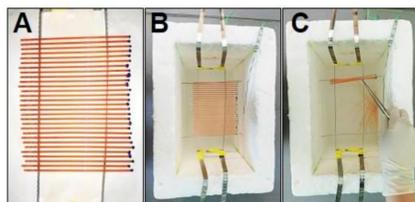


Fig 2. Freezing steps. See text below for details.

The semen was **diluted** in TRIS based medium containing 20% egg yolk and 6,4% glycerol, cooled in cold room at 0.25°C/min and left for 2 hrs. to equilibrate before been loaded into plastic straws (Fig 2A). Next, straws were exposed to LN2 vapors (-80°C) for 6 min (Fig 2B) then plunged and stored in tanks until use. (Fig 2C)

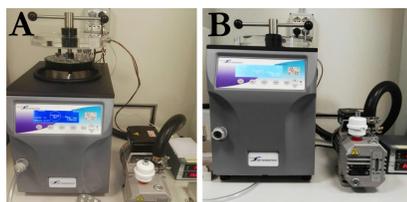
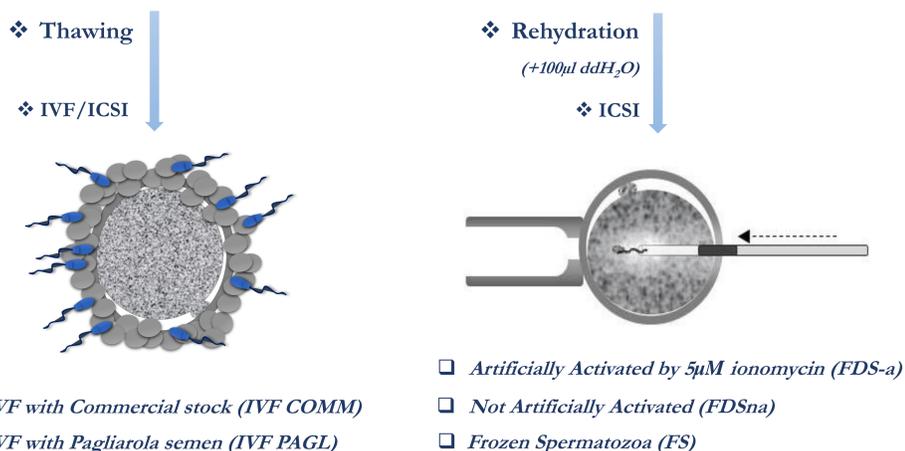


Fig 3. Freeze-drying steps See text below for details.

Spermatozoa were selected by swim up in TRIS based medium at 38.5 °C for 20 min. A 100µl aliquot was frozen in freeze-drying medium (10mM EGTA and 50mM NaCl in 10mM Tris-HCl buffer; pH adjusted to 8.4) and **lyophilized** for 20 hrs. at 20 mTorr (Fig 3A). The glass vials were sealed under vacuum (Fig 3B) and stored at 4 °C until use.

After thawing or rehydration, the fertilizing capability of cryopreserved and lyophilized semen was evaluated by **in vitro fertilization (IVF)** and **IntraCytoplasmic Sperm injection (ICSI)** as follows:



At 14-16h after ICSI, presumptive zygotes were evaluated by pronuclear formation (2PN). Embryo development was evaluated by cleavage and blastocyst rate at 24 h and 7-8 days respectively, after IVF/ICSI.

*Chi-square* test was used to compare 2PN and embryo development. Statistical significance was considered when  $p < 0.05$  (Data analyzed by PRISM 5.0; GraphPad).

### RESULTS

All freeze-dried spermatozoa were immotile after rehydration and showed damaged membranes after staining with Propidium Iodide (PI). Only 31% of frozen spermatozoa were reached by PI.

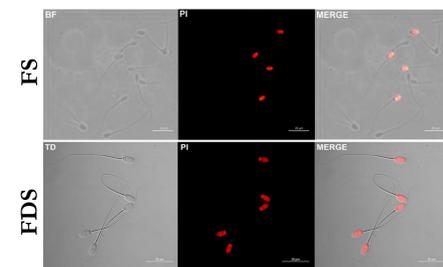


Fig 4. Propidium Iodide (PI) staining. FS: frozen spermatozoa; FDS: freeze-dried spermatozoa; BF: bright field; TD: Transmitted DIC; MERGE: PI + BF/TD. Scale bar=20 µm.

Two pronuclei (2PN) were observed in 14.3%, 80% and 81.4% of ICSI-FDSna, ICSI-FDSa and ICSI-FS, respectively.

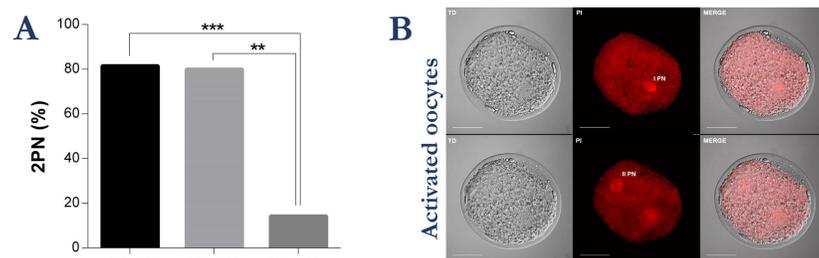


Fig 5. Pronuclear formation. A) \*\* means  $p < 0.01$  between ICSI-FDSna and ICSI-FDSa; \*\*\* means  $p < 0.001$  between ICSI-FDSna and ICSI-FS. None difference was observed between ICSI-FDSa and ICSI-FS; B) An activated oocyte showing 2PN (I PN and II PN). All nuclei were counterstained with PI; MERGE means PI + TD (Transmitted DIC). Scale bar=50 µm.

**Freeze-dried spermatozoa yielded blastocysts only following artificial activation.** FS and FDS produced 31,8% and 10,2% of good quality blastocysts, respectively (Fig 6).

Groups	Oocytes	Non divided at 24h (%)	2-Cells at 24h (%)	Blastocyst at 7-8 days(%)
IVF COMM	93	34/86 (39.5)	43/86 (50)	25/86 (29)
IVF PAGL	96	40/88 (45.4)	36/88 (41)	28/88 (31.8)
ICSI-FS	44	20/38 (52.6) <sup>a</sup>	14/38 (36.8) <sup>b</sup>	12/38 (31.6) <sup>c</sup>
ICSI-FDSna	52	29/45 (64.4)	5/45 (11)	0/45 (0) <sup>d</sup>
ICSI-FDSa	56	24/49 (49)	16/49 (32.7) <sup>b</sup>	5/49 (10.2) <sup>c</sup>

Table 1. Outcomes from IVF and ICSI. a =  $p < 0.05$  ICSI-FDSna vs ICSI-FS; b =  $p < 0.05$  ICSI-FDSna vs ICSI-FDSa and  $p < 0.01$  ICSI-FDSna vs ICSI-FS; c =  $p < 0.05$  ICSI-FDSa vs ICSI-FDSna and ICSI-FS, and  $p < 0.0001$  ICSI-FDSna vs ICSI-FS.



Fig 6. Sheep blastocysts at 8<sup>th</sup> of culture. Blastocysts were obtained from IVF with frozen Pagliarola (IVF PAGL), IVF with commercial semen (IVF COMM), ICSI with frozen (FS) and freeze-dried spermatozoa (FDS). Scale bar= 100µm

**Sperm Biobank from Pagliarola sheep has been established.**

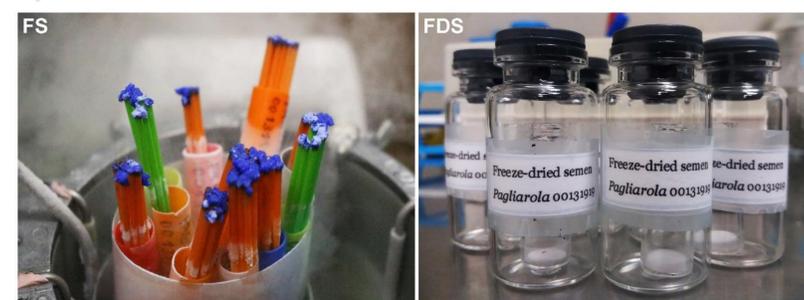


Fig 7. Pagliarola Sperm Biobank. Frozen and freeze-dried semen (FS and FDS respectively) stored in LN2 tanks and at 4°C, respectively.

### CONCLUSIONS

The fertilizing capacity of cryopreserved Pagliarola's spermatozoa was comparable to commercial semen stocks. Although the developmental potential of embryos derived from lyophilized spermatozoa was significantly lower than cryopreserved ones, **sperm lyophilization may be an alternative, low cost storage option to save biodiversity in domestic species.**

### ACKNOWLEDGMENTS

This work was supported by: MIUR/CNR and Program FIRB "GenHome", GA B81 J12002520001; by "DRYNET"- H2020 MSCA-RISE 2016, GA No. 734434 and by "ERAofART" - H2020 Research and Innovation Program Twinning action 2015, GA No. 698165 TWINN-2015. The authors are also participating in COST Action CA16119. The authors thank Dr. Luca Valbonetti, University of Teramo, Faculty of Veterinary Medicine, for the kind help in confocal microscopy analysis.