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

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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:

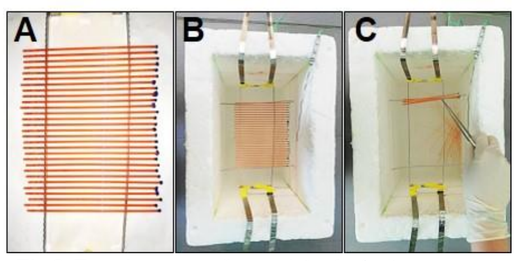


Fig 2. Freezing steps. *See text below for details.*

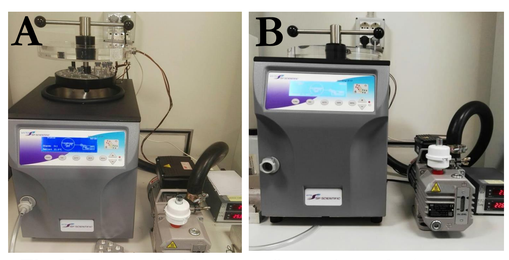


Fig 3. Freeze- drying steps *See text below for details.*

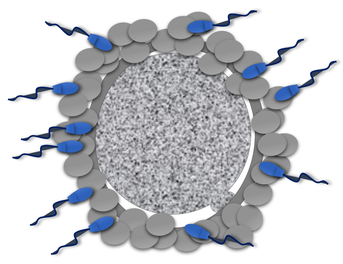
The semen was **diluted** in TRIS based medium containing 20% egg yolk and 6,4% glycerol, cooled in cold room at 0.25°C/min and left for 2 hrs. to equilibrate before been loaded into plastic straws (Fig 2A). Next, straws were exposed to LN2 vapors (-80°C) for 6 min (Fig 2B) then plunged and stored in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

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:

❖ **Thawing**

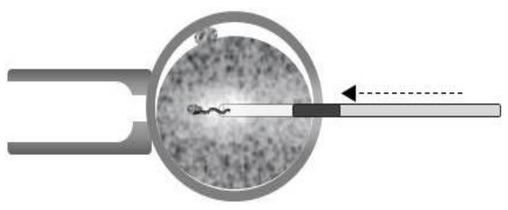
❖ **IVF/ICSI**



- ❑ *IVF with Commercial stock (IVF COMM)*
- ❑ *IVF with Pagliarola semen (IVF PAGL)*

❖ **Rehydration** *(+100µl ddH_2O)*

❖ **ICSI**



- ❑ *Artificially Activated by 5µM ionomycin (FDS-a)*
- ❑ *Not Artificially Activated (FDSna)*
- ❑ *Frozen Spermatozoa (FS)*

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.

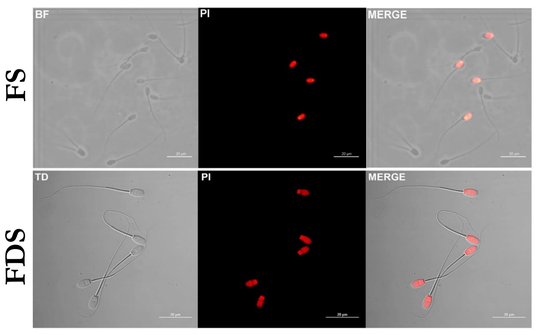


Fig 4. Propidium Iodide (PI) staining. FS: frozen spermatozoa; FDS: freeze-dried spermatozoa; BF: bright field; TD: Transmitted DIC; MERGE: PI + BF/TD. Scale bar=20 µm.

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

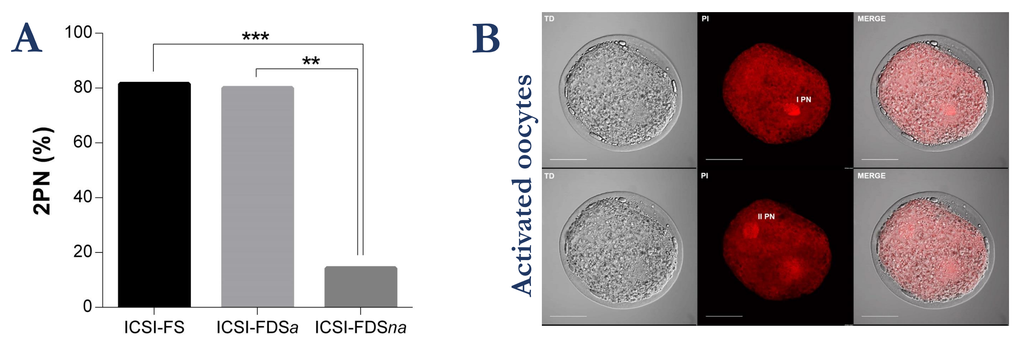


Fig 5. Pronuclear formation. A) ** means $p<0.01$ between ICSI-FDSna and ICSI-FDSa; *** means $p<0.001$ between ICSI-FDSna and ICSI-FS. None difference was observed between ICSI-FDSa and ICSI-FS; B) An activated oocyte showing 2PN (I PN and II PN). All nuclei were counterstained with PI; MERGE means 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)[a]	14/38 (36.8)[b]	12/38 (31.6)[c]
ICSI-FDSna	52	29/45 (64.4)	5/45 (11)	0/45 (0)[d]
ICSI-FDSa	56	24/49 (49)	16/49 (32.7)[b]	5/49 (10.2)[c]

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.

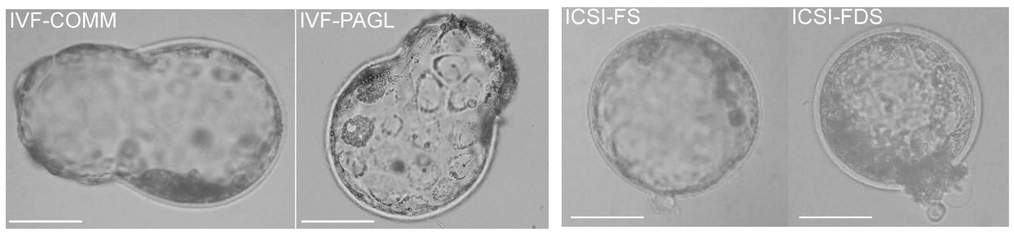


Fig 6. Sheep blastocysts at 8th of culture. Blastocysts were obtained from IVF with frozen Pagliarola (IVF PAGL), IVF with commercial semen (IVF COMM), ICSI with frozen (FS) and freeze-dried spermatozoa (FDS). Scale bar= 100µm

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

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