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Artificial activation of ovine oocytes is required after ICSI with freeze-dried spermatozoa.

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Background

Cryopreserved mammalian spermatozoa are routinely stored at -196 ºC in Liquid Nitrogen (LN_2). However, storage in LN has some drawbacks: it is hazardous for workers, expensive, requires dry ice for shipments and it is not always reliable.

The **freeze-drying (or lyophilization) technique consists to water removal by sublimation.** As well as many foodstuffs and pharmaceutical products, freeze-drying allows to store the biological samples in a dry state and represents an interesting alternative low-cost strategy of **semen biobanking to save the endangered species.**

Here, we have established a dry sperm biobank from an endangered Italian sheep breed (Pagliarola) and tested its fertility efficiency through ICSI.

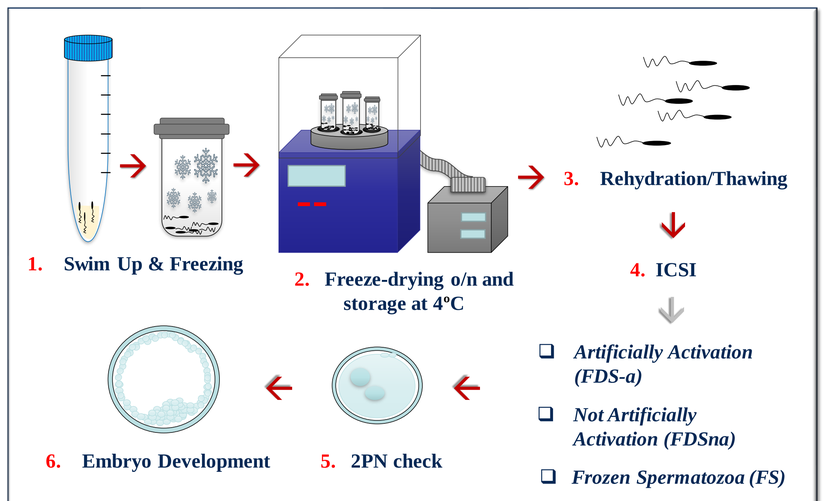

Fig. 1. Experimental Design. ICSI-FDSa: ICSI with freeze-dried spermatozoa, followed by activation in ionomycin 5µM; ICSI-FDSna: ICSI with freeze-dried spermatozoa, not followed by activation; FS: Frozen Spermatozoa; PN: pronuclei.

Material & Methods

Experimental design is illustrated in fig.1.

Semen collection & Freeze-drying

- Ram ejaculate was collected with artificial vagina and the motile spermatozoa were selected by swim-up in TRIS-based medium at 38.5 ºC for 20 min.
- A 100µl aliquot of spermatozoa were frozen in freeze-drying medium (10mM EGTA and 50mM NaCl in 10mM Tris–HCl buffer; pH was adjusted to 8.4) and subsequently lyophilized for 20 hrs. under pressure of 20 mTorr (Freeze-dryer 2.0 BenchTop, SP Scientific-VirTis).
- The glass vials were sealed under vacuum and stored in the dark at 4 ºC for 1-2 months.

Intracytoplasmic Sperm Injection (ICSI)

- Just before ICSI, the freeze-dried spermatozoa were rehydrated by adding 100µl ddH2O.
- To evaluate the fertilizing capability, 108 Mature sheep oocytes were injected with freeze-dried spermatozoa. 56 of them were artificially activated post-ICSI by 5µM ionomycin (ICSI-FDSa), for 5 min; 52 were left non-activated (FDSna). Forty-four oocytes were injected with frozen spermatozoa (ICSI-FS) as control (not artificially activated).
- Pronuclear formation (2PN) and blastocyst development were investigated at 14-16 hours and 7-8 days after ICSI, respectively.

Results

- **All freeze-dried spermatozoa were completely immotile after rehydration and showed damaged membranes** inasmuch as penetrable by Propidium iodide (PI). Only 31% of frozen spermatozoa were reached by PI (fig. 2).

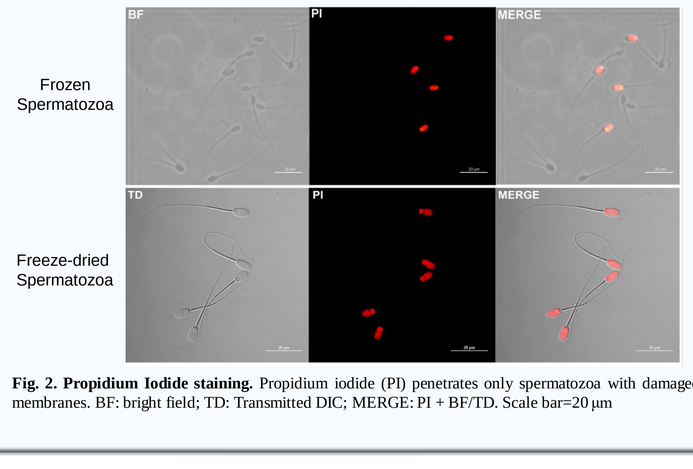

Fig. 2. Propidium Iodide staining. Propidium iodide (PI) penetrates only spermatozoa with damaged membranes. BF: bright field; TD: Transmitted DIC; MERGE: PI + BF/TD. Scale bar=20 µm

- **Two PN were found in 83.3% of ICSI-FDSa, 81.4% of ICSI-FS while only in 14.3% of ICSI-FDSna** ($p<0.05$ ICSI-FDSna vs ICSI-FDSa; $p<0.01$ ICSI-FDSna vs ICSI-FS) (fig. 3).

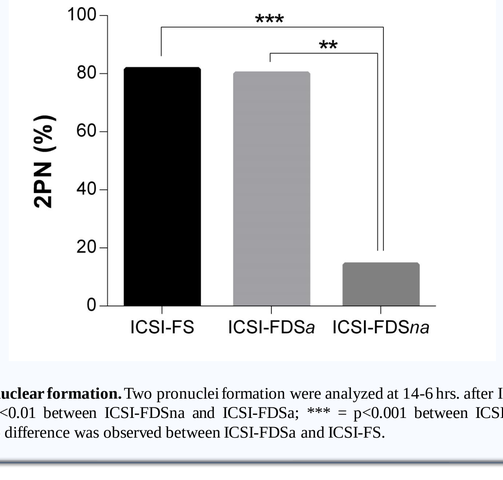

Fig. 3. Pronuclear formation. Two pronuclei formation were analyzed at 14-6 hrs. after ICSI. ** means $p<0.01$ between ICSI-FDSna and ICSI-FDSa; *** = $p<0.001$ between ICSI-FDSna and ICSI-FS. No difference was observed between ICSI-FDSa and ICSI-FS.

- **The ICSI by freeze-dried spermatozoa yielded blastocysts only following artificial activation** (ICSI-FDSa: 10.2%; ICSI-FS: 31%; ICSI-FDSna: 0%; $p<0.05$ ICSI-FDSa vs ICSI-FDSna and ICSI-FS; $p<0.0001$ ICSI-FDSna vs ICSI-FS) (fig. 4)

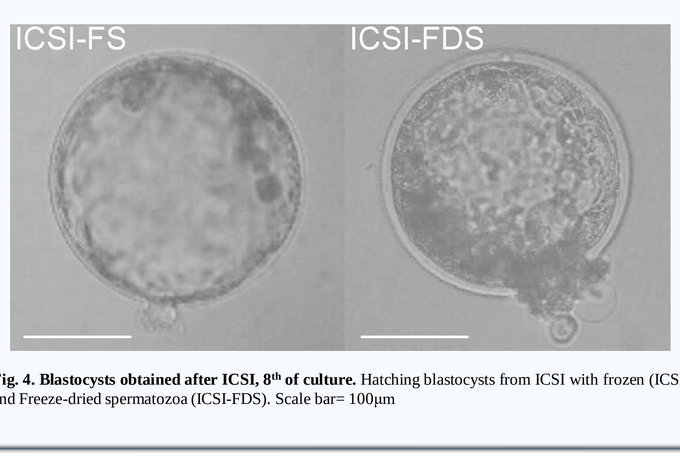

Fig. 4. Blastocysts obtained after ICSI, 8th of culture. Hatching blastocysts from ICSI with frozen (ICSI-FS) and Freeze-dried spermatozoa (ICSI-FDS). Scale bar= 100µm

Conclusions

Freeze-dried spermatozoa have lost the capacity to trigger oocyte activation but maintained their nuclear viability, whose developmental potential was fully released following artificial activation. Our results support the evidence that freeze-drying might be an effective approach of spermatozoa storage to save endangered species.

Acknowledgments

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