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 °C in Liquid Nitrogen (LN2). However, storage in LN2 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.

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 °C 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 °C 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).
- 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).
- 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-FDSna vs ICSI-FDSa and ICSI-FS; p<0.001 ICSI-FDSna vs ICSI-FS) (fig. 4).

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
This work was supported by “DRYNET”- H2020 MSCA-RISE 2016, GA No. 734434 and by “ERAoFART”- H2020 Research and Innovation Program Twinning action 2015, GA No. 698165 TWNN-2015. The authors thank Dr. Luca Valbonetti, University of Teramo, Faculty of Veterinary Medicine, for the kind help in confocal microscopy analysis.