1 **Freeze-dried sperm: an alternative biobanking option for endangered species.**

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16

17 **Abstract**

18 In addition to the iconic wild species, like pandas and Siberian tigers, an ever-increasing
19 number of domestic species are also threatened with extinction. Biobanking of spermatozoa
20 could preserve genetic heritage, and maintain biodiversity. Given that lyophilized spermatozoa
21 retain fertilizing capacity, our aim was to demonstrate that is possible to use freeze-dried sperm
22 as alternative option to save endangered ram species. To reach the goal, we have collected
23 semen from an Italian endangered sheep breed (Pagliarola), and established a biobank of
24 cryopreserved and freeze-dried spermatozoa, evaluated by IVF (for frozen spermatozoa) and
25 ICSI (for frozen and freeze-dried spermatozoa). As expected, the fertilizing capacity of

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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-FDS*a*) and 52 were un-activated (ICSI-FDS*na*). Pronuclear formation (2PN) was investigated at 14-16 h after ICSI in fixed presumptive zygotes. Only artificially activated oocytes were able to develop to blastocyst after ICSI. In this work, we have demonstrated that freeze-dried ram spermatozoa drive blastocyst development following ICSI in relatively high proportion, enforcing the concept that sperm lyophilization is an alternative, low cost storage option for biodiversity preservation in also in domestic species.

Keywords: freeze-drying, cryopreservation, biobanking, spermatozoa, ICSI, sheep.

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 species have become extinct, and about 17% of them are threatened with extinction. Domestic breeds are considered as endangered when count less than 1000 females and 20 fertile males. In these cases, reproductive programs, such as artificial insemination (AI), in vitro fertilization (IVF) and its variant Intra Cytoplasmic Sperm Injection (ICSI) might be an option to increase reproductive performances of the endangered population (Henson, 1992; Comizzoli et al., 2000).

The protocols for sperm cryopreservation are well established in mammalian species including bovine, sheep, mouse and human (Parrish et al., 1995; Salamon and Maxwell, 1995; Storey et al., 1998; O'Connell et al., 2002), but not in wild species whose reproductive physiology is not well characterized yet (Fickel et al., 2007). The conventional cryopreservation in liquid nitrogen induces a cellular dehydration by a progressive temperature reduction until -196°C.

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51 The use of cryoprotectant agents (CPAs) in the procedure is mandatory to prevent cellular
52 damages due to osmotic stress and membrane shrinkage (Medeiros et al., 2001). However,
53 these storage conditions are not always available because their high cost and the low
54 availability of liquid nitrogen, especially in arid climate or under developed countries.
55 The lyophilization of semen represents an innovative and ecological non-cryogenic storage
56 solution (Loi et al., 2013; Kaneko et al., 2014). The freeze-drying the process leads to the
57 removal of water and implies the conservation of specimens in an anhydrous state. Briefly, first
58 the sample needs to be frozen at low temperatures (freezing step); then, by a sublimation
59 process, water is removed passing from a solid state to aeriform one (drying step). The final
60 product in dry form can be stored at room temperature for long term and can be easily carried
61 worldwide. Since the breakthrough achieved by Wakayama and Yanagimachi in producing live
62 offspring from lyophilized mouse spermatozoa, viable offspring have been obtained in rabbit,
63 rat and horse by intracytoplasmic sperm injection (ICSI) of freeze-dried spermatozoa
64 (Wakayama and Yanagimachi, 1998; Liu et al., 2004; Hirabayashi et al., 2005; Choi et al.,
65 2011).
66 In this work, we set to apply conventional cryopreservation and freeze drying for establishing
67 a genetic bank of male gametes from a truly endangered Italian sheep breed native of the
68 province of Teramo (Abruzzo, Italy). The breed, whose name "Pagliarola" (straw-eater)
69 denotes a rustic animal, was maintained in small flocks by rural family to provide foodstuff
70 and declined in number from 350,000 to 25 (21 ewes and 4 rams) heads in less than a century;
71 indeed, a dramatic cases of biodiversity loss in a farm animal. In addition, we report in here
72 technical improvements of ICSI using dry spermatozoa.
73

74 2. Materials and Methods

75 *2.1. Ethic Statement*

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76 All experiments were performed in accordance with DPR 27/1/1992 (Animal
77 Protection Regulations of Italy) in concordance with European Community regulation 86/609
78 and were approved by CEISA (Inter-Institutional Ethics Committee for Animal
79 Experimentation) Prot. 79/2013/CEISA Prog. 58. The permit n°: CEISA VI, Class 8.1, Prot.
80 2823. All chemicals were obtained from Sigma Aldrich unless otherwise stated.

81

82 *2.2. Semen collection*

83 Semen was collected using an Artificial Vagina (AV), filled with warm water (40 to 44
84 °C) and connected to a 15ml tube from two adults, fertile Pagliarola breed rams. Immediately
85 after collection, sperm motility was evaluated under the stereomicroscope and sperm
86 concentration was assessed by Burker chamber. Only ejaculates with sperm concentration ≥
87 1.8 x 10^9 spermatozoa/ml and motility ≥ 70%, were used for the experiments.

88

89 *2.3. Sperm cryopreservation: media and procedure*

90 Freezing media has been prepared in two steps. First, was made a basic medium by
91 dissolving 2.42g TRIS base, 1.36g citric acid, 1.00g fructose, 100.000 IU penicillin G, 0.1g
92 streptomycin in 67.20 ml of bi-distilled water (ddH_20); pH was adjusted to 6.7-6.8. Then, we
93 divided the basic medium in two equal volumes (33.60 ml) and proceeded with the preparation
94 of two media, referred as Medium A (or 30 °C medium) and Medium B (or 4 °C medium), by
95 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
96 glycerol (for Medium B). Medium A and Medium B have been maintained at 30°C and 4°C,
97 respectively before use. Freezing medium composition is reported in Table 1.
98 For cryopreservation of sperm, first Medium A was carefully added to the ejaculate and
99 transferred immediately at 4 °C (in the cold room) to allow a controlled cooling - from 30 °C
100 to 4 °C - over 2 hours. Afterward, Medium B was gently added to the suspension, and left 2

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101 hours in the cold room. Medium A and Medium B were added in the same volume in order to
102 dilute the ejaculated to a final concentration of 400 x 10^6 spermatozoa/ml. Every 30 minutes
103 the tubes were gently mixed upside down. Next, 250 µl plastic straws were filled, sealed with
104 polyvinyl alcohol (PVA) and placed onto a metallic grid to stabilize for the last 2 hours at 4
105 °C. Finally, the straws were exposed to LN vapors (-80 °C) into a Dewar flask and maintained
106 for six minutes, before being plunged into LN and stored into LN tanks until use.
107

108 *2.4. Sperm freeze-drying: media and procedure*

109 Spermatozoa were lyophilized as previously reported (Loi et al., 2008a), using Freeze-
110 Dry apparatus (SP Scientific-VirTis, Freeze-dryer 2.0 BenchTop), following the protocol used
111 by Wakayama and Yanagimachi (1998). At the end of the process, each ampoule was sealed
112 by vacuum and stored at room temperature (RT, 18–23 °C) until use.
113 To check membrane integrity, freeze-dried spermatozoa were evaluated by PI staining.
114 Samples were rehydrated by adding 100 µl of bi-distilled water and incubated in 5 µg/ml PI
115 solution for 10 minutes, in the dark, at RT. Subsequently, 10 µl of sample were placed on slide,
116 mounted with Fluoromount and immediately observed under confocal microscope (Nikon
117 Eclipse Ti-E).
118

119 *2.5. Sperm plasma membrane evaluation after freeze-drying*

120 To evaluate the integrity of plasma membrane after lyophilization, spermatozoa were first
121 rehydrated by adding 100 µl of ddH_2O, then incubated 10 minutes in a 5 µg/ml propidium
122 iodide (PI) solution in PBS, since PI is a fluorescent dye able to permeate only damaged
123 membranes. A 15 µl drop was the placed on the slide, covered by coverslip and observed under
124 an epifluorescence microscope (Nikon Eclipse E-600). A minimum of 150 spermatozoa were
125 counted.

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2.6. Oocyte recovery and in vitro maturation (IVM)

Sheep ovaries were collected from local slaughterhouses and transferred to our laboratory within 1–2 hours. Oocytes were aspirated with 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 2–3 layers of compact cumulus cells were selected for IVM, that was performed in 4 wells-dishes containing 500 µl of IVM medium. In vitro maturation medium is composed by bicarbonate-buffered TCM-199 (Gibco) containing 2 mM glutamine, 0.3 mM sodium pyruvate, 100 µM cysteamine, 10% fetal bovine serum (FBS) (Gibco), 5µg/ml follicle stimulating hormone (FSH) (Ovagen, ICP, Auckland, New Zealand), 5 µg/ml luteinizing hormone (LH) and 1 µg/ml β-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 and colleagues (2002). After IVM, only selected MII oocytes with expanded cumulus and normal morphology were used for ICSI.

2.7. In Vitro Fertilization (IVF)

The fertilizing capability of Pagliarola Sheep frozen semen (PAGL) was compared with commercial stock (COMM) from Sarda breed rams, through in vitro fertilization, 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 minutes. Cumulus-Oocytes-Complexes (COCs) are sticky and difficult to hand after in vitro maturation, owing to the deposition of hyaluronic acid by cumulus cells. In order to ease their displacement, COCs were first rapidly passed in 300 U/ml hyaluronidase solution (dissolved in H199) and placed, in number of 9-10/drop, into 50 µl drops of IVF medium

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151 covered by mineral oil and incubated with sperms (5 x 10^6 sperm/ml) overnight, in a humidified
152 atmosphere at 38.5 °C, 5% CO_2, and 7% O_2. The day after, oocytes were pipetted in SOF-
153 medium to remove most of the spermatozoa attached to the zona pellucida and cultured as
154 described below.
155
156 *2.8. Intracytoplasmic sperm injection (ICSI).*
157 *2.8.1. Sperm preparation*
158 We performed ICSI with frozen semen (ICSI-FS) and freeze-dried spermatozoa (ICSI-
159 FDS) as previously described by Anzalone and colleagues (2016). Briefly, a single straw was
160 fast-thawed by immersion in 35 °C water for few seconds, opened into a 1.5 ml Eppendorf and
161 incubated three minutes in a humidified atmosphere at 38.5 °C and 5% CO_2. Next, 5 µl of
162 semen was diluted in 100 µl of IVF medium (SOF- enriched with 20% (v:v) heat-inactivated
163 estrus sheep serum, and 16 µM isoproterenol) buffered with HEPES (referred to as H-IVF
164 medium). The latter was diluted 1:1 with 12% (w:v) PolyVinylPyrrolidone (PVP) and 10 µl
165 drop was placed on the lid of a Petri dish, on a warmed microscope stage covered by mineral
166 oil.
167 Freeze-dried spermatozoa were rehydrated by adding 100 µl of bi-distilled water, then 5 µl
168 aliquot was suspended in 100 µl of H199 medium and processed as described above for frozen
169 spermatozoa.
170 *2.8.2. ICSI procedure*
171 The intracytoplasmic sperm injection was performed under an inverted microscope
172 (Nikon Eclipse E-800) connected to a micromanipulation system (Narishige NT-88NEN,
173 Tokyo, Japan), using a piezo micropipette driving system (PiezoXpert, Eppendorf, Milan,
174 Italy) as previously described (Anzalone et al., 2016). The oocytes were injected in groups of

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175 five to avoid prolonged light exposure and PVP/sperm containing drops were renewed every
176 ten injected oocytes.
177 An aliquot of oocytes injected with freeze dried spermatozoa was *activated* by five minutes
178 incubation in 5 µM ionomycin dissolved in H199 + 0.4% BSA (ICSI-FDS*a*), while another
179 one was *non-activated* and directly placed in culture without further activation (ICSI-FDS*na*)
180 as described above.
181
182 *2.9. Embryo culture*
183 All presumptive zygotes from ICSI and IVF oocytes, were cultured in number of 4-5
184 per drop into 20 µl drops of SOF- enriched with 2% (v:v) basal medium Eagle essential amino
185 acids (EAA), 1% (v:v) minimum essential medium (MEM) non-essential amino acids (NEAA)
186 (Gibco), 1 mM glutamine, and 8 mg/ml fatty acid-free BSA, covered by mineral oil. The
187 medium was renewed on day 3 (SOF- supplemented with 0.27 mg/ml glucose (SOF^{+}), 2%
188 EAA, 1% NEAA), on day 5 (SOF^{+} with 10% of charcoal stripped FBS (cs-FBS), 2% EAA, 1%
189 NEAA), and on day 6 (1:1 MEM/M199 enriched with 10% cs-FBS, 2.5 µg/ml gentamicin and
190 1% sodium pyruvate) until day 7/8. The in vitro development was evaluated at 24 hours by
191 cleavage (only 2 cells-stage embryos were considered as cleaved) and at 7/8th day of culture by
192 blastocyst formation.
193
194 *2.10. Pronuclear staining*
195 To visualize pronuclei (2PN) in ICSI-FS, ICSI-FDS*a* and ICSI-FDS*na*, a total of 27,
196 30 and 21 presumptive zygotes were fixed in 4% paraformaldehyde (PFA) for 20 minutes, from
197 14 to 16 hours after spermatozoa injection. Then, presumptive zygotes were permeabilized with
198 0.1% Triton X-100, and stained for 5 minutes with 5 µg/ml PI at RT, washed twice in 0.4%

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199 PVP (in PBS) and mounted on slides. Images were captured by confocal microscope (Nikon
200 Eclipse T*i*-E).
201
202 *2.11. Statistical analysis*
203 One-way ANOVA test and Fisher's exact test were used to compare 2PN and in vitro
204 embryo developmental stages between groups. Data were analyzed using PRISM software
205 version 5.0, GraphPad, and the results were considered statistically different for $P< 0.05$.
206
207 *2.12. Experimental groups*
208 IVF PAGL: in vitro fertilization with frozen spermatozoa of Pagliarola ram; IVF
209 COMM: in vitro fertilization with frozen spermatozoa from commercial stocks; ICSI-FS:
210 intracytoplasmic sperm injection with Pagliarola frozen spermatozoa; ICSI-FDS:
211 intracytoplasmic sperm injection with Pagliarola freeze-dried spermatozoa; ICSI-FDS*a*:
212 intracytoplasmic sperm injection with Pagliarola freeze-dried spermatozoa, followed by
213 ionomycin activation; ICSI-FDS*na*: intracytoplasmic sperm injection with Pagliarola freeze-
214 dried spermatozoa, not activated by ionomycin.
215
216 **3. Results**
217 *3.1. Sperm plasma membrane in freeze-dried spermatozoa*
218 Propidium iodide stained 100% (174/174) of lyophilized-rehydrated spermatozoa,
219 indicating that all freeze-dried spermatozoa were unviable (Fig. 1).
220
221 *3.2. Embryo development*
222 All embryo development outcomes are reported in Table 2.
223 *3.2.1. IVF outcomes*

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224 In vitro fertilization with COMM and PAGL frozen semen produced very similar
225 embryo development rates in terms of cleavage (50% (43/86) *vs* 41% (36/88), COMM *vs*
226 PAGL respectively) and blastocyst rate (29% (25/86) *vs* 31.8% (28/88), COMM *vs* PAGL
227 respectively (Table 2, Fig. 2A). Representative images of IVF derived blastocysts are reported
228 in Fig. 2C).

229 *3.2.2. ICSI outcomes*

230 ICSI-FDS*a* presumptive zygotes showed a higher number of 2PN than ICSI-FDS*na*
231 (80% (24/30) vs 14.3% (3/21) respectively, $P< 0.01$); the latter ones also displayed a lower
232 number of 2PN than ICSI-FS (14.2% (3/21) vs 81.4% (22/27) respectively, $P< 0.001$) (Table
233 2). Representative images of activated and not-activated oocytes are reported in Fig. 3A and
234 Fig. 3B, respectively.

235 The proportion of embryos cleaved at two-cells stage was significantly lower in ICSI-FDS*na*
236 compared to ICSI-FDS*a* and ICSI-FS (11% (5/45) vs 32.7% (16/49) and 36.8% (14/38)
237 respectively, $P< 0.05$ and $P< 0.01$). The number of oocytes that underwent fragmentation was
238 similar in all groups (24.4% (11/45), 18.3% (9/49) and 10.5% (4/38) in ICSI-FDS*na*, ICSI-
239 FDS*a* and ICSI-FS, respectively). The percentage of blastocyst produced ranged from 0% in
240 (ICSI-FDS*na*), to 10.2% (5/49) in ICSI-FDS*a*; as expected, the highest development was
241 recorded in the ICSI-FS group (31.5%, 12/38) (Table 2, Fig. 2B). Representative images of
242 ICSI derived blastocysts are reported in Fig. 2D).

243

244 *3.3. Sperm biobanking*

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

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249 **4. Discussion**

250 In this work, we have demonstrated that lyophilization might be conveniently used for
251 preserving spermatozoa from a heavily endangered domestic sheep breed, *Pagliarola*. Given
252 the lack of viability of re-hydrated spermatozoa, we had to resort to ICSI to test their
253 fertilization capacity, according to our previously published report (Anzalone et al., 2016). In
254 that paper, we have shown that fresh ram spermatozoa activate the oocyte after ICSI, without
255 the need of further chemical activation (i.e. by ionomycin) (Anzalone et al., 2016). It was
256 assumed, in analogy with other species, that the Sperm-Oocyte-Activating-Factor (SOAF)
257 localized in the sperm plasma membrane, induces oocyte activation, eliciting a Ca^{2+} release
258 from ooplasmic stores (Saunders et al., 2002; Kashir et al., 2010; Amdani et al., 2015). On the
259 contrary, spermatozoa with damaged membranes might lose the ability to activate the oocyte
260 after ICSI, due to the loss of sperm activation factor(s) (Yanagimachi, 2005), rendering
261 artificial activation a compulsory step (Tesarik and Sousa, 1995; Yanagida et al., 1999; Zhang
262 et al., 1999; Eldar-Geva et al., 2003). Accordingly, we have observed that pronuclear formation
263 after ICSI with freeze-dried spermatozoa dramatically decreased in non-activated oocytes
264 (ICSI-FDS*na*); while in artificially activated ones (ICSI-FDS*a*) the pronuclear formation raised
265 to the same level of control (ICSI-FS). Accordingly, cleavage to 2 cells-stage embryos, as well
266 as blastocysts development rates increased in ICSI-FDS*a*. With the exception of the murine
267 model, where ICSI with dry spermatozoa produced very high developmental rates (Wakayama
268 and Yanagimachi, 1998; Kusakabe et al., 2008), blastocysts rate ranges around 10-12% in large
269 animals (horse, bovine and pig) (Keskintepe et al., 2002; Kwon et al., 2004; Choi et al., 2011)
270 peaking to 24% in rabbit (Liu et al., 2004). Recently, it has been reported for the first time the
271 production of blastocysts from sheep oocytes following ICSI with freeze-dried spermatozoa
272 (Olaciregui et al., 2017). Although the authors report a higher developmental rate comparing
273 to ours (25% of embryos reaching blastocyst stage), the paper presents serious shortcomings,

274 starting with the difficulty to identify normal blastocyst stage embryos in the photos provided,
275 and uppermost, for the lack of proper control excluding parthenogenetic development of the
276 spermatozoa injected oocytes. Our efforts aimed at the development of a robust and repeatable
277 protocol to produce good quality blastocysts. To this extent, we have found that activation of
278 oocytes is mandatory to start embryonic development following injection of dry spermatozoa.
279 While we managed to improve activation rates in ICSI-FDSa, making it comparable to ICSI-
280 FS, the disappointing note is the low cleavage frequency in both groups (32.7% and 36.8%
281 respectively). This finding clash with the high proportion of pronuclei found in both groups
282 (about 80%), and clearly indicates that other factors, rather than activation, are responsible for
283 development restrains in sheep ICSI. Further studies focusing on crucial events like centriole
284 dynamics, or S phase entry/exit will provide helpful clues to remove the defective steps that
285 hamper development in sheep ICSI.

286 Besides the current limits of ICSI in sheep, the development to blastocysts of oocytes
287 fertilized by dry spermatozoa is still a third of the frozen control ones, indicating that the
288 conservation of spermatozoa in anhydrous state needs further developments. It has to be
289 pointed out however, that the current lyophilization procedures applied to spermatozoa are
290 essentially similar to those used to foodstuff or pharmaceutical products. In other words, drying
291 spermatozoa is in its infancy, and there is ample margin for improving, starting from the
292 development of media, to radical chances in all the parameters, like freezing temperatures and
293 the vacuum conditions. The finding so far achieved using the current, empirical state of art
294 induces to a cautious optimism (Wakayama and Yanagimachi, 1998; Kusakabe et al., 2001;
295 Hirabayashi et al., 2005; Kusakabe et al., 2008; Loi et al., 2008a, 2008b; Gianaroli et al., 2012;
296 Iuso et al., 2013) and indicates that lyophilization might constitute in the medium run a valid
297 method for replacing the traditional cryopreservation storage. To conclude, in this work we
298 have established a sperm bank from a seriously endangered sheep breed, and have

299 demonstrated that the dry spermatozoa, as well as cryopreserved ones, are able to develop to
300 blastocyst stage embryos upon ICSI in acceptable proportion after storage at room temperature.
301 This finding supports the development of low cost, on the shelf genetic biobanks from domestic
302 and also wild endangered species.

303

304 **Conflict of interest**

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

306

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310

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Figure captions

Fig. 1. Sperm plasma integrity after freeze drying. Propidium iodide (PI) stained all sperm heads (nuclei) indicating that all ram freeze-dried spermatozoa were unviable after rehydration. MERGE means PI + TD (Transmitted DIC). Scale bar = 20 µm.

Fig. 2. Embryo development outcomes from IVF and ICSI. A) IVF with Pagliarola's frozen sperm (IVF PAGL) produced similar outcomes in terms of 2-cells stages, non-divided, fragmented and blastocysts, compared to IVF with commercial frozen sperm (IVF COMM). B) Graph shows embryo results from ICSI of frozen (ICSI-FS) and freeze-dried spermatozoa with (ICSI-FDS*a*) and without (ICSI-FDS*na*) ionomycin activation. ICSI-FDS*na* results in less 2-cells stage at 24 hours compared to ICSI-FDS*a* and to ICSI-FS. Activation by ionomycin after sperm injection (ICSI-FDSa) increased the cleavage rate and lead to 10.2% blastocyst development. a = P<0.05 ICSI-FDS*na* vs ICSI-FS; b = P<0.05 ICSI-FDS*na* vs ICSI-FDS*a* and P<0.01 ICSI-FDS*na* vs ICSI-FS; c = P<0.05 ICSI-FDSa vs ICSI-FDS*na* and ICSI-FS, and P<0.0001 ICSI-FDS*na* vs ICSI-FS. ND means No Development to blastocyst. C, D) Representative photos of blastocysts at 8th day of culture, obtained from IVF-COMM/IVF-PAGL and from ICSI-FS and ICSI-FDS*a*, respectively. Scale bar = 100µm.

Fig. 3. Pronuclear formation after ICSI. A) Representative image of activated oocytes showing 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.

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427 **Fig. 4.** Pagliarola's semen biobank. Photo shows straws of frozen semen (FS), and glass vials

428 containing freeze-dried spermatozoa (FDS) collected from Pagliarola rams.

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Table 1

Composition of media for sperm cryopreservation. Medium base was equally added at 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				

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)[a]	6/44 (13.6)	4/38 (10.5)	20/38 (52.6)[b]	14/38 (36.8)[c]	12/38 (31.5)[d]
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)[d]
ICSI-FDS*a*	56	24/30 (80)[a]	7/56 (12.5)	9/49 (18.3)	24/49 (49)	16/49 (32.7)[c]	5/49 (10.2)[d]

Table 2. Outcomes from IVF and ICSI. In vitro fertilization was assessed with commercial (IVF COMM) and Pagliarola's (IVF PAGL) frozen semen. ICSI outcome derived from frozen sperms (ICSI-FS) and freeze-dried spermatozoa with and without subsequent oocyte activation (ICSI-FDS*a* and ICSI-FDS*na*, respectively). a = $P<0.05$ ICSI-FDS*na* vs ICSI-FDS*a* and $P<0.01$ ICSI-FDS*na* vs ICSI-FS; b = $P<0.05$ ICSI-FDS*na* vs ICSI-FS; c = $P<0.05$ ICSI-FDS*na* vs ICSI-FDS*a* and $P<0.01$ ICSI-FDS*na* vs ICSI-FS; d = $P<0.05$ ICSI-FDS*a* vs ICSI-FDS*na* and ICSI-FS, and $P<0.0001$ ICSI-FDS*na* vs ICSI-FS.

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