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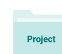

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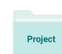

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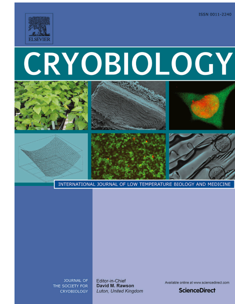

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Magda Teixeira, Loris Commin, Lucie Gavin-Plagne, Pierre Bruyère, Samuel Buff, Thierry Joly

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1 **Title**

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3 Rapid cooling of rabbit embryos in a synthetic medium

4

5 Magda Teixeira[1,*], Loris Commin[1], Lucie Gavin-Plagne[1], Pierre Bruyère[1], Samuel Buff[1], Thierry
6 Joly[1,2]
7 1: Université de Lyon, VetAgro Sup, Interaction cellule environnement, Marcy l'Etoile, France
8 2: Université de Lyon, ISARA-Lyon, Lyon, France
9 [*]corresponding author: magda.guedes-teixeira@vetagro-sup.fr

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11 **Abstract**
12 Embryo cryopreservation media usually contain animal-derived products, such as bovine serum
13 albumin (BSA). These products present two major disadvantages: an undefined variable
14 composition and a risk of pathogen transmission. We aimed to evaluate the effect of replacing BSA
15 in rabbit embryo rapid cooling "freezing" and warming media with a chemically defined medium with
16 no animal-derived products: STEM ALPHA.Cryo3 ("Cryo3").
17 A total of 1540 rabbit morulae were divided into three cryopreservation groups (group 1: BSA,
18 group 2: 20% Cryo3 and group 3: 100% Cryo3) and a fresh controls group. After rapid cooling,
19 embryos were cultured (*in vitro* approach), or transferred into synchronized does (*in vivo*
20 approach). In the *in vitro* approach, post-warm survival rates obtained with 100% Cryo3 (94.9 %)
21 were superior to BSA (90.8%) and 20% Cryo3 (85.6 %). The blastocyst formation rate was similar
22 between BSA, 20% Cryo3 and 100% Cryo3 groups (85.1, 77.9 and 83.3 %, respectively), as was
23 the expansion / hatching rate (63.1, 63.4 and 58.0%, respectively) and embryo mitochondrial
24 activity. In the *in vivo* approach, pregnancy (80.0, 68.0 and 95.2 %, respectively), implantation
25 (40.5, 45.9 and 44.8%, respectively), and live-foetus rates (35.6, 35.5 and 38.1 %, respectively)
26 were similar between the three groups. To conclude, Cryo3 can replace BSA in rabbit embryo rapid
27 cooling "freezing" and warming media.
28
29 **1. Introduction**
30 Over the past few decades, embryo cryopreservation has become crucial to the long-term
31 preservation of genetic material in biobanks. Along with embryo transfer (ET), this technology has
32 contributed to the distribution of genetic materials worldwide, replacing animal exchange [36]. The
33 World Organisation for Animal Health assembled recommendations on risk management
34 procedures concerning embryo collection and processing [74]. Even if these guidelines are the best
35 way to reduce infectious disease transmission, embryo contamination is still of concern to health
36 authorities [36].
37 Animal-derived products, such as bovine serum albumin (BSA) or foetal calf serum, also referred to
38 as foetal bovine serum, are commonly added to animal embryo cryopreservation media
39 [5,49,66,68,77]. Serum-derived product composition is poorly known. Media containing BSA or
40 serum are classified as semi-defined or non-defined, respectively [72]. These products contain
41 growth factors, cell attachment and spreading factors, hormones, carbohydrates, amino acids,
42 proteins (such as albumin), vitamins and various undefined molecules [9,72].
43 Serum-derived products promote embryonic viability and development [4,9,60,69,71,72] and have
44 numerous advantageous properties in cryopreservation media, such as metal chelating activity,
45 oncotic pressure regulation, pH regulation [22] and toxin-scavenging [11]. Additionally, animal sera
46 have surfactant properties, which reduce the surface tension in the media, preventing embryos
47 from floating or sticking to glass and plastic surfaces [22,73], and avoiding the adsorption of some
48 media compounds (as hormones, growth factors and carrier proteins) to the material surfaces [53].
49 Moreover, the addition of serum-derived products to the cryopreservation media seems to protect
50 embryos from possible toxic effects of cryoprotectants during the cryopreservation process [23,55].

51 Despite the numerous beneficial effects of serum on embryos during and after cryopreservation,
52 negative effects have also been suspected. Ruminant embryos cultured with serum before
53 blastocyst formation may present increased incidence of unusual development, accompanied by
54 "large offspring syndrome": high birth weight, prolonged gestation, frequent dystocia, elevated
55 abortion rates and organ defects [35,76].
56 Sera can be contaminated with pathogenic agents such as bacteria, viruses [20,58], yeast, fungi,
57 and mollicutes such as mycoplasmas [14], or prions [44], even if the risk of prion contamination
58 seems to be low [75]. Although commercial sera are usually declared to be pathogen-free,
59 treatments like heat inactivation and gamma irradiation don't always seem to be efficient [58].
60 The advantages of using synthetic medium, in cryopreservation media are widely recognized as
61 providing more defined, more consistent and more reproducible conditions, in addition to avoiding
62 animal welfare and ethical concerns.
63 Numerous studies have aimed to replace animal products in cryopreservation media with media
64 free of animal-derived products, such as silk protein sericin [24], vegetal peptones [18], HA [25,52],
65 and non-organic macromolecules such as polyvinyl alcohol [21,38,50,62], polyvinylpyrrolidone
66 [21,32,65] and Ficoll [21,32]. Hyaluronic acid (HA) is a glycosaminoglycan that can be synthetized
67 in its pure form [16] and can be found in follicular, oviduct and uterine fluids [37] and its
68 concentration increases in the uterus by the time of implantation [78]. After successfully replacing
69 albumin in embryo culture [17,42], HA became an interesting candidate to replace animal products
70 during cryopreservation.
71 Animal derived sera composition not only changes between batches but is also extremely variable
72 [9]. This variation can occur as a result of physiological and biochemical differences between
73 donors [40], and more generally with gender [2], age [29], diet [41], photoperiod [64] and
74 preparation methods [33]. Regarding embryotrophic properties of BSA, some authors observed
75 considerable variations between suppliers and even between distinct lots from the same supplier
76 [4,27,45].
77
78 STEM ALPHA.Cryo3 (referred to as "Cryo3", Stem-Alpha, Saint-Genis-l'Argentière, France), is a
79 patented serum-free, protein-free and dextran-free medium (manufactured according to good
80 manufacturing practices [cGMP-annex 1] in compliance with 2001/83/EC). CRYO3 is composed of
81 synthetic HA of high molecular weight (> 106 D), glucose, carbohydrates, amino acids, mineral
82 salts, vitamins, fatty acids esters and buffers. This product was originally created for clinical
83 applications, as a serum substituent in somatic and human adult stem cell freezing medium.
84 Bruyère (2013) investigated foetal calf serum thermodynamic properties of three different suppliers
85 and compared them to the synthetic medium Cryo3 (used at 18% v/v). All the solutions presented
86 similar thermodynamic characteristics, but media containing foetal bovine serum presented more
87 variable results, as well as aberrant values, unlike 18% Cryo3 medium, whose results appeared to
88 be more stable.
89 The impossibility of characterizing animal-derived product composition and its variability lead to
90 unpredictable development rates and to experimental results that might not be reproducible.

91 Consequently, all serum-derived products seem to be unsuitable when the goal of a study is to
92 obtain defined media and standardized cryopreservation methods.
93
94 Bruyère observed that Cryo3 can successfully replace animal products in rabbit embryo and bovine
95 embryo slow-cooling “freezing” media [7,8].
96 Rapid-cooling “freezing” procedures comprise the use of higher solute concentrations than slow-
97 cooling “freezing”. These solutions, combined with a rapid cooling technique (such as direct
98 plunging in liquid nitrogen), allow the formation of an amorphous state during cooling, avoiding the
99 danger of ice crystal formation that occurs during slow-cooling “freezing”. However, unlike
100 vitrification media, the formation of ice crystals during rapid-cooling “freezing” procedures is
101 possible, especially during warming, if (i) warming rates are not quick enough [70], (ii) insufficient
102 high total solute concentration or (iii) exposure to cryopreservation solution was too brief.
103
104 The aim of our study was to evaluate the effect of replacing BSA with Cryo3 in rapid cooling
105 “freezing” and warming solutions on the *in vitro* and *in vivo* development of rabbit morulae.
106
107 **2. Materials and methods**
108 The Ethical and Animal Welfare Committee of VetAgro Sup approved this study (Permit Number:
109 05/26). All animals were handled according to the EU Directive 2010/63/EU for animal experiment
110 guidelines. Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich (Saint
111 Quentin Fallavier, France).
112
113 **2.1 Embryo production and recovery**
114 A total of 62 rabbit New Zealand does (SARL HYCOLE, Marcoing, France) were housed in groups
115 of five and fed a commercial diet. Does received five doses of a pFSH:LH (ratio 5:1, 31.5 µg total,
116 Stimufol, Reprobiol, Belgium) preparation (administered twice-daily, subcutaneously). Eight hours
117 after the last injection, does were inseminated with sperm from multiple males (pooled ejaculates),
118 and an intramuscular injection of buserelin (2.0 µg Receptal, MSD Animal Health, Beaucouzé,
119 France) was administered.
120 Rabbit does were euthanized 65 to 68 h after the buserelin administration by cervical dislocation.
121 The oviducts and uteri were flushed using Euroflush (IMV Technologies, L’Aigle, France) at room
122 temperature. Embryos were recovered at the morula stage and classified according to the
123 International Embryo Transfer Society (IETS) manual, [6][5][48]Bó and Mapletoft 2013)
124 and quality 1 embryos [6] were pooled. Embryos (n = 1540) were randomly divided into three
125 cryopreservation groups and two control groups.
126 A group of embryos (n = 40) was cultured without cryopreservation (*in vitro* fresh control), and a
127 group of embryos (n = 59) was transferred without cryopreservation (*in vivo* fresh control).
128
129 **2.2 Embryo rapid cooling**
130 Unless specified otherwise, all percentages are expressed as volume/volume.

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131 Prior to rapid cooling, embryos (n = 1441) were randomly divided into three cryopreservation
132 groups. All media contained the same cryoprotectant composition and the following base media:
133 group 1 cryopreservation medium: IMV Embryo holding medium (IMV Technologies, L'Aigle,
134 France), containing 0.4 % (w/v) BSA (n = 543); group 2 cryopreservation medium: D-PBS
135 supplemented with 20% of Cryo3 (n = 423); group 3 cryopreservation medium: 100% Cryo3
136 medium (n = 475). Embryos were transferred into a first equilibration solution composed of 5 %
137 Me_2SO and 5 % ethylene glycol (EG) (5 min), and a second equilibration solution composed of
138 10% Me_2SO and 10% EG (2 to 3 min). Embryos were then exposed to the cryopreservation
139 solution of the correspondent group (30 sec) containing 20% Me_2SO (approx. 2.8 M) and 20% EG
140 (approx. 3.6 M), before being loaded to a Fibreplug (CVM kit, Cryologic) and cryopreserved by
141 solid surface vitrification (three to four embryos per Fibreplug). Warming was performed by
142 immersing the end of the Fibreplug directly into a thawing solution (0.5 M sucrose in group 1,
143 group 2, or group 3 base medium, respectively) at 38.5 ℃ for 5 min, followed by three successive
144 dilution baths (0.3 M, 0.1 M and 0.0 M sucrose).
145
146 **2.3 *In vitro* embryo culture and morphology assessment**
147 Embryos (n = 40) from the *in vitro in vitro* fresh control group were cultured (38.5 ℃, 5 % CO_2) to
148 the expanded blastocyst stage in Medium 199 (without glutamate) supplemented with 10% foetal
149 calf serum and antibiotics (67 UI/mL penicillin and 67 µg/mL streptomycin, Dutscher, Brumath,
150 France). *In vitro* development was assessed after 24 h and 48 h of culture and classified according
151 to their development stage as morula, blastocyst, expanded and hatching embryos. Slightly
152 expanded blastocysts with herniation of embryonic cells (Figure 1) were included in the expanded /
153 hatching embryo group.
154
155 **2.4 *In vivo* embryo transfer**
156 Fresh embryos (n = 59) and warmed vitrified (total = 905; group 1 n = 358, group 2 n = 270,
157 group 3 n = 277) embryos were transferred to synchronized New Zealand recipient does (n = 84),
158 according to the protocol described by Salvetti [61]. Briefly, recipient does were synchronized with
159 a buserelin injection (0.8 µg, intramuscular, Receptal), 50 to 60 h before transfer. After
160 anaesthesia, a midventral laparotomy was performed, and 4 to 7 embryos (mean = 5.4) were
161 transferred to each uterine horn. Pregnancy diagnosis was realized by palpation 20 days after
162 embryo transfer.
163
164 **2.5 Mitochondrial activity assessment with JC-1**
165 The cationic dye JC-1 (5,5'6,6'-tetrachloro-1,1,3,3'tetraethylbenzimidazolycarbocyanine iodide;
166 Thermofisher Scientific, Illkirch, France) exhibits different fluorescent properties, based on its
167 accumulation within mitochondria. J-aggregates accumulate in mitochondria with high
168 mitochondrial membrane potential (MMP), showing red fluorescence, while J monomers
169 accumulate in low MMP mitochondria, presenting green fluorescence [54]. Consequently, embryos
170 with more active mitochondria exhibit higher red to green ratios than less active or inactive

171 embryos. At the end of embryo culture, living embryos (n = 89) at the expanded / hatching stage
172 from the three cryopreservation groups were subjected to a pretreatment of pronase (a protease,
173 from *Streptomyces griseus*, 5 mg/mL) in Dulbecco's Phosphate-Buffered Saline medium (D-PBS)
174 supplemented with D-glucose (5.56 mM), sodium pyruvate (0.33 mM) and bovine serum albumin (3
175 mg/mL), at 38.5 ℃, until the mucin coat began to d issolve. Embryos were then washed in six drops
176 of modified D-PBS. Embryos were incubated with JC-1 for 75 min (1.5 µM, 38.5 ℃, 5 % CO2) and
177 observed using an Olympus IX71 epifluorescence microscope, with an excitation wavelength of
178 488 nm. JC-1 aggregates were detected with a red filter (590 nm wavelength), whereas JC-1
179 monomers were detected with a green filter (530 nm wavelength). To evaluate embryo
180 mitochondrial activity, the staining intensity (by pixel) was measured, from both channels, in two
181 randomly defined areas on each embryo, using the Fiji package [63] of ImageJ software (National
182 Institute of Health, Bethesda, Maryland, USA), and the red to green ratio was quantified.
183 An MMP disruptor (CCCP, carbonyl cyanide 3-chlorophenylhydrazone; Thermofisher scientific,
184 Illkirch, France) was used as a control to confirm that directional changes in the dye signal were
185 correctly interpreted.
186
187 **2.6 Statistical analysis**
188 *In vitro* and *in vivo* development rates were analysed with the chi-square test, whereas JC-1
189 red/green ratios were analysed by one-way analysis of variance. All tests were performed with R-
190 Studio software [57]. Groups were considered significantly different at $p < 0.05$.
191
192 **3. Results**
193 ***In vitro* and *in vivo* embryo development after cryopreservation**
194 The *in vitro* blastocyst formation and expansion/hatching rates and *in vivo* development rates
195 (pregnancy rate, implantation live-birth rates) after rapid cooling with media containing animal
196 products or chemically defined products (group 2 and group 3) are summarized in Table 1. *In vitro*
197 fresh control embryos expressed significantly superior blastocyst and expansion / hatching rates.
198 The group 3 medium appeared significantly superior in *in vitro* post-warm survival rates than group
199 1 and group 2 media. No significant differences were observed regarding the other *in vitro* and *in*
200 *vivo* development rates.
201
202 **Mitochondrial activity assessment with JC-1**
203 Ratios of J-aggregate to J-monomer of cryopreserved expanded or hatching embryos,
204 cryopreserved with media containing animal products (group 1) or chemically defined products
205 (groups 2 and 3) are represented in Figure 2 and summarized in Figure 3. No significant
206 differences were observed between the three groups. After incubation with the CCCP control,
207 images showed no red fluorescence.
208
209 **4. Discussion**

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210 Over the last few decades, there have been important efforts to replace animal serum with defined
211 media containing no animal products for embryo cryopreservation. Numerous natural or synthetic
212 molecules have been used in slow cooling, as in rapid cooling media, to replace the biological and
213 the physical properties of animal albumin. Studies demonstrate that animal products can be
214 successfully replaced with products such as the silk protein sericin [24] and vegetal peptones [18]
215 during bovine embryo slow-freezing, or HA during murine [26,52], bovine [51], and ovine [26] slow
216 freezing.
217 The non-organic macromolecule polyvinyl alcohol has been used to slow-freeze and vitrify embryos
218 from different species, obtaining equivalent post-thaw development rates for murine [21,50,51] and
219 porcine [62] embryos. However, inferior development rates were also obtained for murine [12],
220 bovine [51,65,67] and ovine [38] embryos. Studies using polyvinylpyrrolidone tend to demonstrate
221 a negative effect on cryopreservation media [65], as well as inferior surfactant properties [21,65]
222 and toxicity to embryos [13,32].
223 Kuleshova *et al.* cryopreserved mouse embryos by rapid-cooling, using animal product free media
224 containing 35 % polymers (dextran or Ficoll) and 25 % of penetrating cryoprotectants (EG), using a
225 double straw arrangement to diminish contamination risk, obtaining *in vitro* development rates of
226 100 % blastocyst expansion and *in vivo* foetuses rates of 76 % [31]. One year later, these authors
227 obtained development rates (96 - 100% blastocyst expansion and 62 - 76 % live foetuses) after
228 vitrifying mouse embryos with 34 to 49 % (w/v) of macromolecules (Ficoll or dextran) and 11 to
229 27% (w/v) EG, in protein-free media [32]. However, these authors did not compare these protein-
230 free media with media containing animal products [32]. Another author evaluated the substitution of
231 foetal calf serum with Ficoll, on mouse embryo quick freezing, obtaining equivalent development
232 rates [21]. These studies suggest that these two molecules may be good candidates for
233 replacement of animal products.
234 In 1990, Palasz obtained equivalent post-thaw murine and bovine embryo development rates after
235 embryo slow-frezing with synthetic HA and with NCS (n = 206) [52]. Joly observed equivalent post-
236 thaw murine (n = 443) and ovine embryo (n = 120) *in vitro* development rates, after embryo slow-
237 freezing in media containing HA and BSA [26].

238 Bioniche Life Sciences Inc. developed synthetic holding and freezing media (SYNGRO®) for
239 bovine, equine, sheep and goat embryos, based on synthetic HA. However, few studies regarding
240 cryopreservation were published with these commercial products [22]. Some authors used these
241 media to slow-freeze equine embryos [3] and to slow-freeze and vitrify bovine embryos [30], but
242 these studies didn't aim to compare with animal derived product based media.

243 In our previous work, we showed that animal products could be successfully replaced with 20 %
244 Cryo3 in bovine [7] and rabbit embryo slow-freezing [8], where better *in vivo* development rates
245 were obtained with 20% Cryo3, compared to foetal calf serum [8].
246 In the present study, we evaluated the effect of replacing BSA in rapid-cooling solutions and in
247 warming solutions, using the same synthetic product as in our previous studies: Cryo3.
248 In *in vitro* experiments, significantly superior survival rates were observed in the 100% Cryo3
249 (group 3) compared to BSA (group 1) or 20% Cryo3 (group 2). No differences were found

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250 regarding blastocyst formation, blastocyst expansion or blastocyst hatching development rates
251 between groups. In the literature, quite variable post-warm *in vitro* development can be found
252 (survival: 95.3 - 95.6 %, blastocyst formation: 56 – 91.7 %, hatching or expansion: 45 - 91.7 %)
253 [39,43,47,56]. This variability may depend on several factors, such as donor genetics, the housing
254 conditions of the animals and the embryo culture medium.
255 Our post-warm development rates were in the range of values found in the literature.
256 Embryos were not subjected to a pronase treatment to remove the mucin coat prior to culture.
257 Kasai compared the *in vitro* development with and without mucin coat digestion and observed that
258 approximately half of the non-treated embryos did not expand to a diameter more than twice that of
259 the morula (46 % non-treated vs 92 % treated embryos) [28]. Fischer observed that uterine
260 components are vital in the transformation of the extracellular coverings in the rabbit embryo. In
261 rabbit culture media lacking uterine components, the *zona pellucida* does not dissolve and loses
262 elasticity, leading to herniation of embryonic cells into the mucin coat, instead of expansion and
263 hatching [15]. Indeed, we observed slightly expanded embryos with embryonic cell herniation
264 (Figure 1) in cryopreserved and in non-cryopreserved groups. Considering these findings, we
265 pooled slightly expanded herniated blastocysts with expanded and hatching blastocysts.
266 To evaluate mitochondrial activity between cryopreservation groups, we only used developed
267 embryos. If we had randomly picked embryos from all developmental stages, the development
268 rates would have influenced the total mitochondrial activity and, therefore, confound our results.
269 We obtained equivalent mitochondrial activity between the three groups, suggesting that all the
270 developed embryos had the same energetic capacity of continuing further development. The
271 obtained JC-1 ratios are equivalent to ratios found in the literature for fresh mouse blastocysts
272 (approx. 1.35) [1]. Images obtained with the CCCP control demonstrated the JC-1 ratio is
273 dependent on mitochondrial potential variations.
274 In our *in vivo* experiments, no statistically significant difference was observed between fresh and
275 cryopreserved embryos. No difference was found regarding pregnancy rates of the three rapid
276 cooling groups, even if group 3 rates tended to be superior. Equivalent implantation rates and live-
277 birth rates were obtained with the three rapid cooling media groups. As in *in vitro* development
278 studies, post-transfer *in vivo* development rates found in the literature can considerably vary
279 (pregnancy: 56 - 100%, implantation: 8.7 – 65 %, live foetuses: 6.4 – 57.1 %) [25,28,43,48,59]. *In*
280 *vivo* development rates may depend on the cryopreservation medium, cooling device and
281 technique, transfer conditions (laparotomy / endoscopy, surgeon), and the housing conditions of
282 the animals. The *in vivo* development rates obtained in our study were in the range and close to the
283 superior limit for pregnancy and implantation rates.
284 Regarding cryopreservation effects on embryos, a difference was found between fresh (expansion
285 or hatching rate 97.5 %) and cryopreserved embryos during *in vitro* development, but this
286 difference was no longer observed after *in vivo* transfers. A possible explanation would be that
287 cryopreservation partially impairs embryos, and this damage can be reversible if embryos return to
288 physiological conditions after cryopreservation. Both *in vitro* and *in vivo* experiments in this study
289 indicate that animal products can be replaced by both concentrations (20% and 100%) of Cryo3.

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290 Ménézo and Khatchadourian observed that non-defined peptides could bond to albumin, with
291 subsequent deleterious effects on embryo post-thaw survival [46]. When using cryopreservation
292 media entirely composed of synthetic chemically defined products, such as Cryo3, these
293 interactions are avoided.
294 Moreover, the use of a commercial synthetic medium for embryo cryopreservation, prepared
295 industrially under rigorous quality control instead of laboratory-made media, avoids preparation
296 variability, and increases reproducibility and standardization of the cryopreservation process.
297

298 **5. Conclusion**

299 The results from this study seem to demonstrate that a chemically defined substitute (STEM
300 ALPHA.Cryo3) can successfully replace BSA in rabbit embryo rapid-cooling and warming media.
301 The elimination of animal products of embryo cryopreservation media may improve procedure
302 standardisation, by avoiding variability in media composition and, consequently, more variable
303 results. Additionally, it would avoid sanitary concerns inherent to animal-derived products.
304 To improve sanitary conditions, we have replaced BSA with 100% Cryo3 medium in rabbit embryo
305 rapid-cooling media in the French National CryoBank.
306

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312

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521 Table 1. *In vitro* and *in vivo* rabbit embryo development rates after rapid cooling with media
522 containing animal products (group 1) or chemically defined products (group 2 and group 3).
523

		Group 1 (0.4 % BSA)	**Group 2 (20% Cryo3)**	**Group 3 (Cryo3)**	**Control (Fresh)**
In vitro development	**% Survival**	90.8%[a] (168/185)	85.6 %[a] (131/153)	94.9 %[b] (188/198)	
	% Blastocyst	85.1 %[a] (143/168)	77.9 %[a] (102/131)	83.3 %[a] (156/188)	97.5 %[b] (39/40)
	% Expansion*, or Hatching	63.1 %[a] (106/168)	63.4 %[a] (83/131)	58.0%[a] (109/188)	97.5 %[b] (39/40)
In vivo development	**% Pregnancy rate**	80.0%[NS] (24/31)	68.0%[NS] (17/24)	95.2 %[NS] (20/23)	83.3 %[NS] (5/6)
	% Implantation	40.5 %[NS] (117/303)	45.9 %[NS] (84/183)	44.8%[NS] (94/234)	46.9 %[NS] (23/49)
	% Live birth	35.6 %[NS] (103/303)	35.5 %[NS] (65/183)	38.1 %[NS] (80/234)	40.8%[NS] (20/49)

524 Different letters in the same row indicate a statistically significant difference ($p < 0.05$). NS
525 indicates no statistically significant difference was observed.
526 % Survival: number of morphologically intact embryos after freezing per frozen embryos.
527 % Pregnancy rate: number of does positive to pregnancy diagnosis per recipient
528 % Implantation: number of born kits (alive and dead) per transferred embryos on pregnant females
529 % Live birth: number of live-born kits per transferred embryos on pregnant females
530 *Slightly expanded blastocysts with herniation of embryonic cells comprised in this group
531

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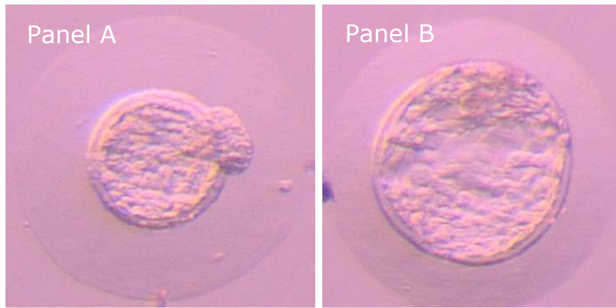
1 Figure 1. Stereoscopic pictures of rabbit embryos.



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3 Panel A) A slightly expanded blastocyst with embryonic cell herniation.

4 Panel B) An expanded blastocyst.

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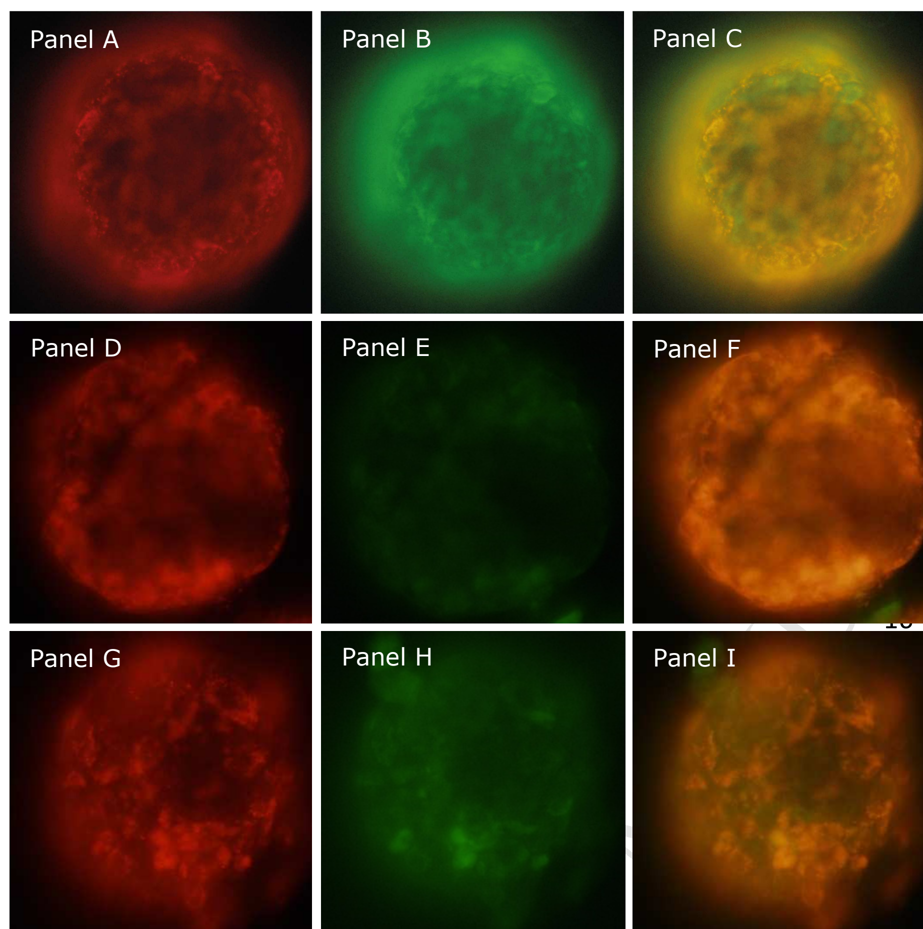
1 Figure 2. Epifluorescence photomicrographs of rabbit embryos stained with JC-1.

2



23

24 Figure 2a. A, D, G) Regions of high MMP are indicated by red fluorescence (emission ~590 nm).

25 B, E, H) Depolarized regions are indicated by green fluorescence (emission ~529 nm).

26 C, F, I) Merged images.

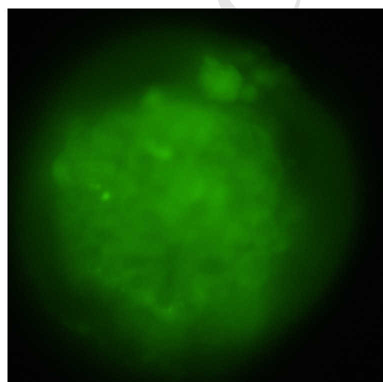
27 A, B, C) Embryo vitrified with a medium containing 0.4% BSA.

28 D, E, F) Embryo vitrified with a medium containing 20% CRYO3.

29 G, H, I) Embryo vitrified with a medium containing 100% CRYO3.

30

31



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34 Figure 2b. After CCCP control (merged images). No regions of high MMP are visible.

35

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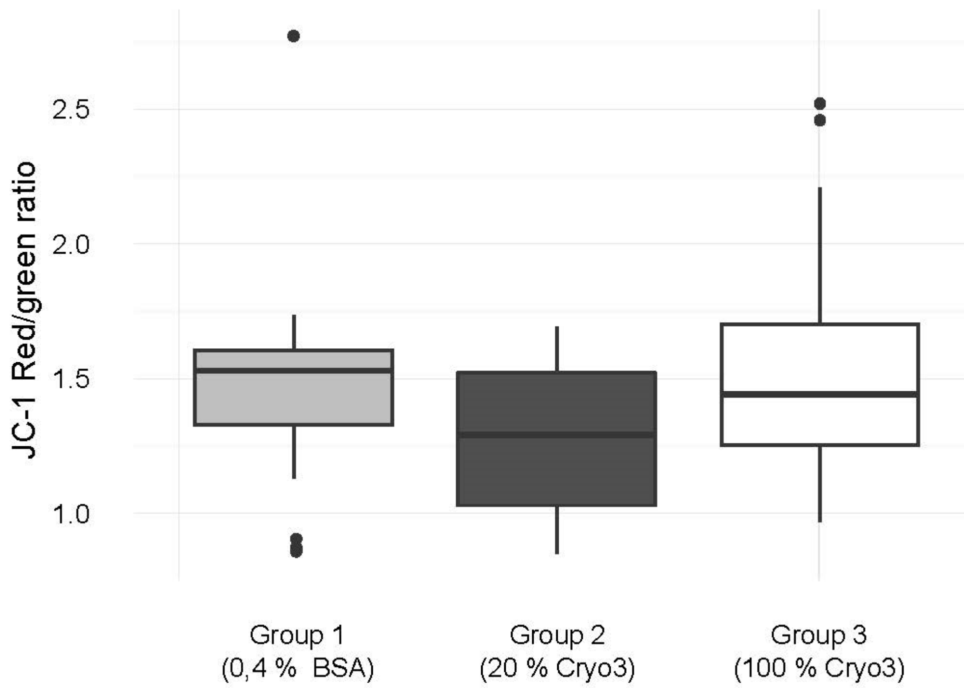
1 Figure 3. JC-1 staining: red/green ratio of cryopreserved expanded or hatching blastocysts vitrified
2 with media containing animal products (group 1) or chemically defined products (group 2 and group 3).



3

4 Red/green ratio of embryos vitrified with group 1 (n = 31), group 2 (n = 27), or group 3 (n = 31),
5 obtained with epifluorescence microscopy. No significant difference was observed between groups.
6

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Highlights

- Embryo cryopreservation media usually contain animal-derived products.
- These products present an undefined variable composition and a contamination risk.
- We aimed to replace BSA with a synthetic medium in rapid cooling “freezing” media.
- Cryo3 can replace BSA in rabbit embryo rapid cooling “freezing” and warming media.

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