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Additional Information

Effect of *in vitro* and *in vivo* conditions on development of parthenogenetic rabbit embryos after vitrification

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Abstract

Parthenote embryos offer multiple opportunities in biotechnological research, so it is important to analyse the possibilities for their cryopreservation in order to establish a biobank. The aim of this experiment was to determine the effect of culture conditions and vitrification on rabbit parthenogenetic embryos. Parthenotes were cultured under *in vivo* and *in vitro* conditions until day 3 (late morula/early blastocyst), when they were vitrified. Immediately after warming, they were newly cultured under *in vivo* and *in vitro* conditions till day 6 (blastocyst stage). Both culture conditions showed similar late morula/early blastocyst (0.39 ± 0.056 vs. 0.46 ± 0.043, for *in vivo* and *in vitro*, respectively) and blastocyst rates (0.12 ± 0.068 vs. 0.13 ± 0.070, for *in vivo* and *in vitro*, respectively). However, no parthenote was recovered when a combination of culture conditions was performed. To our best knowledge, this is the first demonstration of the ability of rabbit parthenogenetic embryos to develop after vitrification, with similar embryo development after *in vivo* or *in vitro* culture. Nevertheless, our results highlight the importance of culture conditions on the morphology of parthenote embryos. Therefore, we have described that special attention should be paid on culture conditions to generate parthenote embryos, with a view to their subsequent use, for example in embryonic stem cell production.

Key words: parthenogenesis, vitrification, rabbit, *in vivo*, *in vitro*

40 **Introduction**

41 Parthenogenesis does not occur naturally in mammals, although under the appropriate
42 stimuli, a parthenogenetic embryo development may occur, mimicking the early stage
43 of *in vivo* or *in vitro* embryo development [37]. Parthenogenetic embryos undergo a
44 normal morphological and functional embryo pre- and post-implantation development,
45 i.e. implantation ability, formation of an embryonic disk and extending the corpora lutea
46 lifespan of recipient females [14, 18, 30]. Therefore, embryos generated by
47 parthenogenetic activation can develop to blastocyst *in vitro* or implant and continue
48 with post-implantation development until different gestation times without developing
49 to term in different species [10, 16, 25, 31, 35, 45, 50].

50 Thus, parthenogenetic embryos have been used for several purposes, such as co-transfer
51 with cloned embryos as a critical step for somatic cell nuclear transfer [5, 17, 44], as an
52 object of study to help understand the fertilization and imprinting process [24, 36], as a
53 substitute to test new embryo technologies [8, 33] and to assess the quality of oocytes
54 matured *in vitro* [24]. In addition, they are being studied as an alternative source of
55 embryonic stem cells, which would avoid ethical concerns, as they are unable to
56 develop to term [3]. Embryonic stem cells (ESCs) are envisioned as a powerful source
57 of pluripotent cells from which desirable tissue can be derived for regenerative medicine
58 and cell therapy [19, 26].

59 With so many possible uses of parthenogenetic embryos, it is of practical importance to
60 establish a bank of parthenogenetic embryos based on cryopreservation.
61 Cryopreservation allows the different lines to be kept in stock and facilitates their
62 dissemination to different countries [11, 20, 21].

63 Embryo development rate has been proposed as a desirable criterion for evaluation of
64 the quality of embryos produced *in vitro* [46]. However, despite all the improvements
65 made in embryo *in vitro* culture, the process is still inefficient and resulting embryo
66 development differs from *in vivo* developmental patterns due to sub-optimal culture
67 conditions [9, 30, 34, 39, 40]. Hence, development to the blastocyst stage does not
68 necessarily ensure competence for further development [50]. So, the best criterion for
69 evaluating parthenogenetically produced embryos is their ability to undergo pre-
70 implantation and post-implantation development [16].

71 To date, parthenogenetic embryo vitrification has only been reported in the pig [8, 22,
72 23]. Therefore, the aim of the present study was to analyse the vitrification process in
73 rabbit parthenogenetic embryos by their ability to continue development post-warming
74 until the blastocyst stage under *in vivo* and *in vitro* culture conditions.

75

76

77 **Materials and methods**

78 All chemicals used in this study were purchased from Sigma-Aldrich Química S.A.
79 (Madrid, Spain) unless stated otherwise.

80 *Ethical Statement*

81 The experiment was performed in accordance with the principles of animal care
82 published by Spanish Royal Decree 53/2013. The animal studies were approved by the
83 Committee of Ethics and Animal Welfare of the Universidad Politécnica de Valencia.
84 Researchers involved in the work with animals held an animal experimentation license
85 issued by the Spanish authorities.

86

87 *Animals*

88 New Zealand white rabbit females, 5 months old, from the experimental farm at the
89 Universidad Politécnica de Valencia, were used as oocyte donors (n=60), embryo
90 donors (n=3) and recipients (n=14). The rabbits were kept in conventional housing (with
91 an alternating cycle of 16 light hours and 8 dark hours and under controlled
92 environmental conditions: average daily minimum and maximum temperature of 17.5
93 °C and 25.5 °C, respectively). All had free access to fresh food and water.

94 *Oocyte collection*

95 Cumulus oocyte complexes at the metaphase II stage were collected from donor females
96 induced to ovulate by an intramuscular dose of 1 µg of buserelin acetate (Suprefact;
97 Hoechst Marion Roussel, S.A., Madrid, Spain). Cumulus oocyte complexes were
98 collected 14-15 hours after ovulation induction by flushing each oviduct with
99 Dulbecco's phosphate-buffered saline (DPBS) without calcium chloride and
100 supplemented with 0.1% (wt/vol) of Bovine serum albumin (BSA). Cumulus cells were
101 removed by incubation of oocytes for 15 minutes at room temperature with 0.1%
102 (wt/vol) hyaluronidase.

103 *Parthenogenetic oocyte activation*

104 Oocyte activation was performed as previously described by Naturil-Alfonso et al. [30].
105 Briefly, oocytes were submitted to two sets 1 h apart of two DC electrical pulses of 3.2
106 kv/cm for 20 µs at 1 sec apart in an activation medium (0.3 M mannitol supplemented
107 with 100 µM MgSO4 and 100 µM CaCl2), followed by 1h incubation in TCM199
108 medium supplemented with 5 µg/µL of cycloheximide and 2mM of 6-DMAP.

109 *Embryo development until Day 3*

110 For *in vivo* culture, presumptive parthenotes were transferred immediately after
111 activation by ventral midline laparoscopy into oviducts of synchronized receptive does,
112 which ovulation was induced 24 h before as previously described [1, 28]. Briefly,
113 recipients were sedated by intramuscular injection of 16 mg xylazine (Rompun; Bayer
114 AG, Leverkusen, Germany). As surgical preparation for laparoscopy, anesthesia was
115 performed by intravenous injection of 16 to 20 mg of ketamine hydrochloride
116 (Imalgene; Merial, S.A., Lyon, France) into the marginal ear vein. During laparoscopy,
117 12 mg of morphine hydrochloride (Morfina; B. Braun, Barcelona, Spain) was
118 administered intramuscularly. First, the abdominal region was shaved, and the animals
119 were then placed on an operating table in a vertical position (head down at 45° angle).
120 Only an endoscope trocar was inserted into the abdominal cavity. When the trocar was
121 removed, the abdomen was insufflated with CO2 and the endoscope was then inserted.
122 For embryo transfer, embryos were aspirated in a 17-gauge epidural catheter (Vygon
123 Corporate, Paterna, Valencia) introduced into the inguinal region with an epidural
124 needle and then inserted in the oviduct through the infundibulum. After surgery, does
125 were treated with antibiotics (0.1 mL/kg of procaine penicillin, Duphapen Strep; Pfizer,
126 S.L.) and buprenorphine hydrochloride (0.08 mg every 12 hours for 3 days, Buprex;
127 Esteve, Barcelona, Spain). Females were euthanized 3 days later and parthenogenetic
128 embryos were recovered by perfusion of uterine horns with 20 mL of Dulbecco
129 Phosphate Buffered Saline (DPBS) supplemented with 0.1% of BSA.

130 For *in vitro* culture, presumptive parthenotes were cultured in TMC199 supplemented
131 with 10% Foetal Bovine Serum (FBS) until Day 3. Culture was performed in 500 µL of
132 medium layered under paraffin oil at 38.5ºC in 5% CO2 and saturated humidity.

133 *Control embryo production*

134 Control embryos were produced using receptive does artificially inseminated with
135 0.5mL of freshly collected, pooled semen diluted 1:5 with tris-citric-glucose diluent and
136 with ≥ 70% motility [49]. Immediately after insemination, ovulation was induced by an
137 intramuscular injection of 1µg of Buserelin Acetate.

138

139 *Vitrification and warming procedure*

140 Parthenotes and control embryos at late morula/early blastocyst stage were vitrified
141 using the methodology described by Vicente et al. [47]. For parthenote vitrification, 11
142 receptive does were euthanized 72-h after transfer and 3-day-old embryos were
143 recovered by perfusion of each uterine horn with 10 ml of DPBS supplemented with
144 0.2% (w/v) of BSA. A total of 37 parthenogenetic embryos from *in vivo* culture and 66
145 parthenogenetic embryos from *in vitro* culture and 23 control embryos were vitrified.
146 Briefly, the vitrification procedure was carried out in two steps at 20°C. In the first step,
147 embryos were placed for 2 min in a vitrification solution consisting of 12.5% (v/v)
148 DMSO and 12.5% (v/v) ethylene glycol (EG) in DPBS supplemented with 0.2% (w/v)
149 of BSA. In the second step, embryos were suspended for 30 s in a solution of 20% (v/v)
150 DMSO and 20% (v/v) EG in DPBS supplemented with 0.2% (w/v) of BSA. Then,
151 embryos suspended in this latter medium were loaded into 0.125-ml plastic straws
152 (French ministraw, IMV Technologies), and two sections of DPBS were added at either
153 end of each straw, separated by air bubbles. Finally, straws were sealed with a plugging
154 rod and plunged directly into liquid nitrogen.

155 Warming was performed by horizontally placing the straw 10 cm from liquid nitrogen
156 for 20–30 s; when the crystallization process began, the straws were immersed in a
157 water bath at 20°C for 10–15 s. The vitrification medium was removed by placing the

158 embryos for 5 min into a solution of DPBS with 0.2% (w/v) of BSA and sucrose at
159 0.33 M and then into a solution of DPBS with 0.2% (w/v) of BSA for another 5 min.

160 *Embryo development post-warming until blastocyst stage (Day 6)*

161 A diagram of the experimental design is represented in Figure 1. All parthenotes from *in*
162 *vivo* culture were transferred into oviducts (Group 1), as previously described (n=24).
163 Parthenotes from *in vitro* culture were divided into two groups; Group 2 was assigned to
164 *in vivo* culture by laparoscopic transfer, as previously described (n=22), while Group 3
165 continued *in vitro* culture as previously described (n=23). Additionally, two control
166 groups were defined: fresh embryos of 3-day-old vitrified-warmed, transferred and
167 recovered on Day 6, Group 4 and fresh embryos allowed to undergo 6 *in vivo*
168 development until Day, Group 5.

169

170 The ability of parthenotes and control embryos to reach the blastocyst stage was
171 assessed at Day 6 of development. To do this, receptive does were euthanized and *in*
172 *vivo* embryos were recovered by perfusion of each uterine horn with 10 ml of DPBS
173 supplemented with 0.2% (w/v) of BSA (Groups 1, 2, 4 and 5).

174

175 *Statistical analysis*

176 To compare embryo development after activation according to the type of embryo (*in*
177 *vivo* or *in vitro*) as a fixed factor, a generalized linear model was performed. The error
178 was designated as having a binomial distribution using the probit link function.
179 Binomial data for activation rate and late morula/early blastocyst stage were assigned a
180 value of one if cleavage occurred after activation or the late morula/early blastocyst
181 stage was reached, respectively. Failure to cleave or develop to the late morula/early

182 blastocyst stage resulted in a score of zero. Differences of $p<0.05$ were considered
183 significant. The same analysis was performed for development ability of vitrified
184 embryos with the experimental groups as fixed factor. Binomial data for post-warming
185 development were assigned a value of one if it reached the blastocyst stage, or zero if it
186 did not. Data are shown as means ± standard error means (S.E.M.). All analyses were
187 performed with SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002).

188 **Results**

189 *Parthenogenetic embryo development to late morula/early blastocyst stage*

190 A total of 245 activated oocytes were transferred and developed *in vivo*, while 223 were
191 activated and cultured *in vitro*. Activation rate was different for each developmental
192 condition, with more divided embryos under *in vitro* culture than *in vivo* (0.61 ± 0.033
193 vs. 0.31 ± 0.029, for *in vitro* and *in vivo*, respectively). However, no differences in late
194 morula/early blastocyst rate was found between groups at Day 3 (0.46 ± 0.043 vs. 0.39
195 ± 0.056, for *in vitro* and *in vivo*, respectively).

196 *Blastocyst developmental ability after vitrification*

197 After warming, only non-damaged embryos with intact zona pellucida were considered
198 to be transferable embryos (93.1% and 83.3%, for *in vivo* and *in vitro* culture,
199 respectively). Results of development ability after warming are shown in Table 1. After
200 3 additional days of development (Day 6), no differences were observed in blastocyst
201 rate between groups of both culture conditions (0.12 ± 0.068 vs. 0.13 ± 0.070, for Group
202 1 and Group 3, respectively). In contrast, no embryos from Group 2 reached the
203 blastocyst stage. Moreover, blastocyst rate was similar between controls (0.68 ± 0.107
204 vs. 0.69 ± 0.128, for Group 4 and Group 5, respectively), but were higher than

205 blastocyst rates of parthenotes. Additionally, recovered parthenote embryos from *in*
206 *vitro* culture (Group 3, Figure 2) were smaller than *in vivo* ones (Group 1, Figure 2). *In*
207 *vivo* parthenotes were similar in size to control vitrified embryos (Group 4, Figure 2),
208 but smaller than control fresh embryos (Group 5, Figure 2).

209

210 **Discussion**

211 Parthenote embryos offer multiple possibilities in biotechnological research, so the
212 establishment of a parthenogenetic embryo bank would allow their long-term
213 maintenance in stock and enable their international distribution [11, 20]. To our
214 knowledge, only three studies in parthenogenetic embryo vitrification have been done
215 [8, 22, 23]. The studies were carried out using pig embryos under *in vitro* conditions. A
216 previous study performed in our laboratory suggested that parthenogenetic embryos
217 developed *in vivo* are not equivalent to their *in vivo* counterparts [30]. For this reason,
218 we studied the effect of culture environment, *in vivo* and *in vitro*, on preimplantation
219 development of rabbit parthenote embryos vitrified on Day 3.

220 The results from the current study are similar to the outcomes of our previous work
221 [30], as development rate to late morula/early blastocyst stage at Day 3 post-activation
222 was similar between *in vivo* and *in vitro* derived parthenotes. This pattern continued
223 after vitrification to blastocyst stage, as Group 1 and Group 3 parthenotes recovered at
224 Day 3 post-warming (Day 6 of development) presented similar blastocyst rate. The
225 vitrification procedure was performed at Day 3, as we had seen previously that this is
226 the best developmental stage for embryo vitrification in rabbit, reaching proper
227 development stages of around 45% after warming [27]. Thus, despite performing
228 optimal vitrification and transfer procedures, the results of the control embryos were

229 similar to previous ones in our laboratory [27, 41]. Our results are lower than those
230 obtained in the pig, which showed about 70% of live embryos *in vitro* after vitrification
231 [8, 22, 23]. This may be due higher sensitivity to vitrification of rabbit parthenogenetic
232 embryos. In fact, previous studies have reported that rabbit oocytes are extremely
233 sensitive to the high levels of cryoprotectants used in vitrification [13, 42, 48].
234 However, the developmental rates of parthenogenetic rabbit vitrified embryos to
235 blastocyst stage of about 12-13% resulting in the present study are in accordance with
236 the implantation rate reached by non-vitrified rabbit parthenogenetic embryos (10.2%,
237 [30]). Nevertheless, no parthenote embryo from Group 2 developed to the blastocyst
238 stage, which might be due to the absence of the mucin coat in *in vitro* developed
239 embryos. This mucin coat is specific to rabbit embryos and has been shown to be
240 relevant for the contact between embryonic and maternal tissues [7, 12, 38]. Rabbit
241 embryos developed *in vitro* for more than 48 h did not develop and survive when
242 transferred into the uterus of synchronize recipients [2, 29, 43].

243 On the other hand, in *vitro* culture after vitrification was shown to affect embryo
244 development, as parthenogenetic blastocysts of Group 3 were smaller than their *in vivo*
245 counterparts. Moreover, despite different vitrification survival, Group 1 parthenotes and
246 Group 4 embryos developed into blastocysts that were similar in size (Figure 2).
247 Differences between *in vivo* and *in vitro* developed parthenotes and more similarities
248 between *in vivo* derived parthenotes and normal fertilized embryos have been also
249 addressed previously [30, 31, 32]. So, as recommended by these previous studies and by
250 Brevini et al. [4] and from the results revealed in this work, the consideration of
251 parthenote embryos to generate embryonic stem cells for therapeutic use should be
252 studied with caution.

253 In conclusion, rabbit parthenote embryos are able to continue embryo development
254 after vitrification both under *in vivo* and *in vitro* conditions, but with low development
255 rates, which although low are those expected for rabbit parthenogenetic embryos. Thus,
256 it is possible to establish a parthenogenetic embryo bank for their future uses. However,
257 *in vitro* culture seems to be sub-optimal, generating a reduction in blastocyst size when
258 compared with *in vivo* development. Further studies on rabbit embryo vitrification will
259 be needed to improve vitrification procedures and increase post-warming survival rate.
260 Special attention should be paid to culture conditions of parthenotes for their subsequent
261 use in areas such as embryonic stem cell production.

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268

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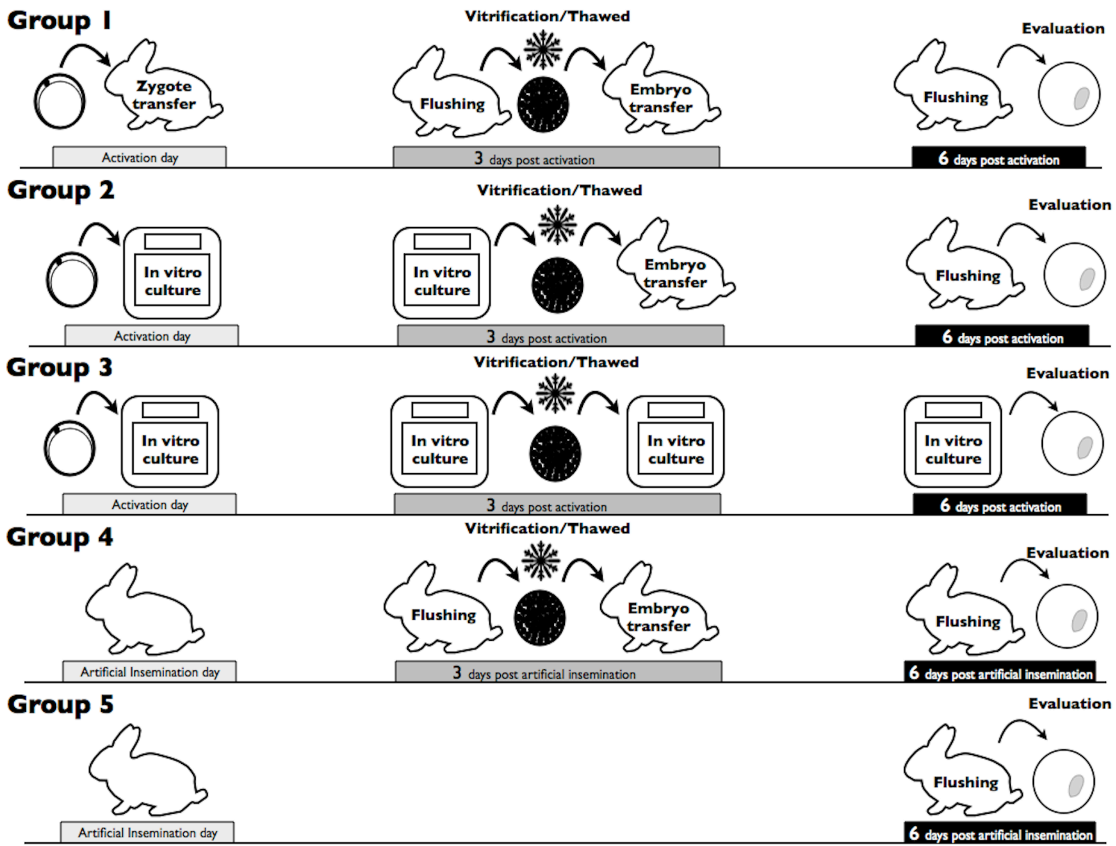
414

415 **Figure 1.** Experimental design diagram.

416

417 **Figure 2.** Importance of culture conditions, *in vivo* vs. *in vitro*, on the morphology of
418 parthenote blastocysts. Scale bar=1000 µm.


Group 1
Zygote transfer
Activation day
Vitrification/Thawed
Flushing
Embryo transfer
3 days post activation
Evaluation
Flushing
6 days post activation
Group 2
In vitro culture
Activation day
Vitrification/Thawed
In vitro culture
Embryo transfer
3 days post activation
Evaluation
Flushing
6 days post activation
Group 3
In vitro culture
Activation day
Vitrification/Thawed
In vitro culture
In vitro culture
3 days post activation
Evaluation
In vitro culture
6 days post activation
Group 4
Artificial Insemination day
Vitrification/Thawed
Flushing
Embryo transfer
3 days post artificial insemination
Evaluation
Flushing
6 days post artificial insemination
Group 5
Artificial Insemination day
Evaluation
Flushing
6 days post artificial insemination

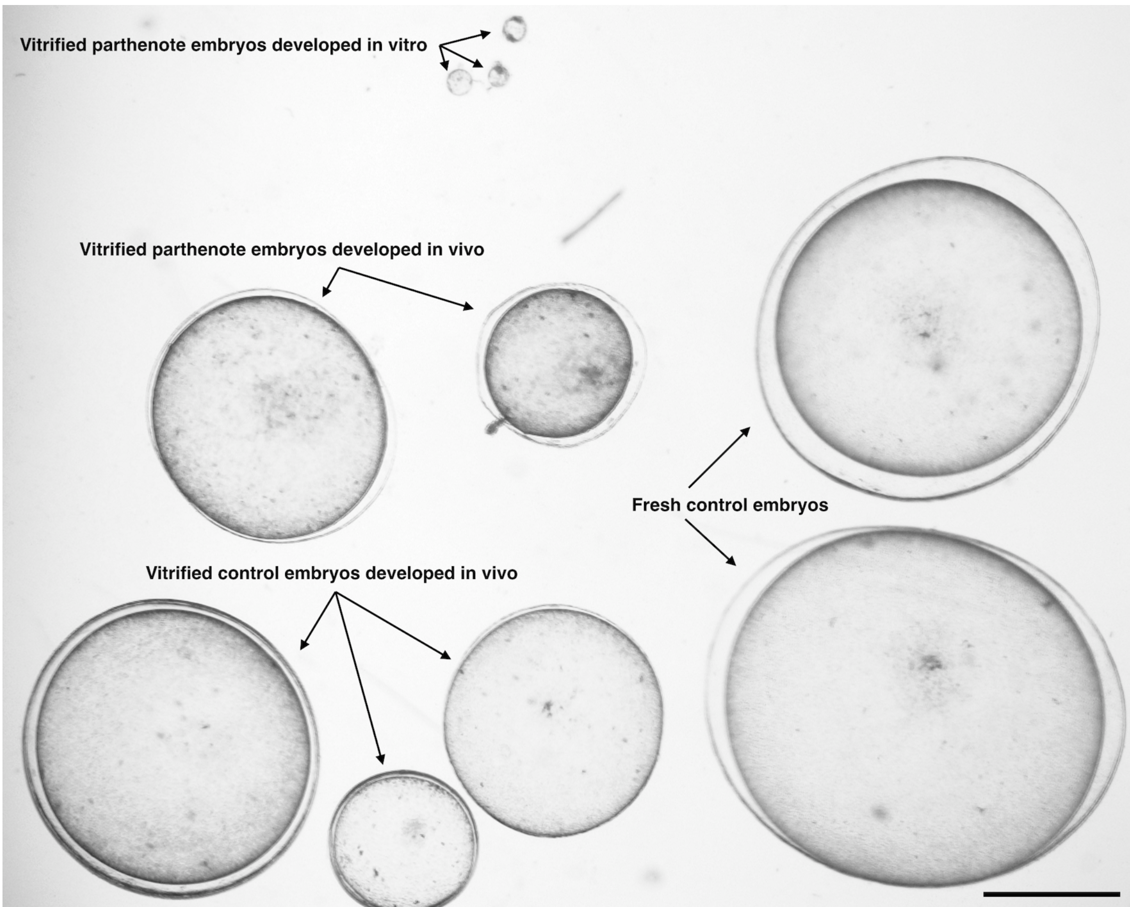

Vitrified parthenote embryos developed in vitro
Vitrified parthenote embryos developed in vivo
Fresh control embryos
Vitrified control embryos developed in vivo

Table 1. Parthenotes blastocyst development rates at Day 6 after vitrification procedure under different culture conditions.

Type of embryo	Procedure	Culture conditions		Transferred embryos	Blastocysts
		0-3 days	3-6 days		
Parthenotes	Vitrified	In vivo	In vivo	24	0.12±0.07[b]
	Vitrified	In vitro	In vivo	22	0.00±0.00[c]
	Vitrified	In vitro	In vitro	23	0.13±0.07[b]
Control	Vitrified	In vivo	In vivo	19	0.68±0.11[a]
	Fresh	In vivo		13	0.69±0.13[a]

Values with different superscripts in the same column are statistically different ($p < 0.05$)