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1 **CRYOSURVIVAL OF RABBIT EMBRYOS OBTAINED AFTER**
2 **SUPEROVULATION WITH CORIFOLLITROPIN ALFA WITH or WITHOUT**
3 **LH**

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18 **ABSTRACT**

19 The efficiency of an embryo bank depends on provision of optimal conditions for
20 recovery, cryopreservation and transfer to a breed or strain. In this sense, increasing the
21 number of embryos available using superovulation should improve the cryobank
22 efficiency. However, vagueness of response to conventional protocols to control or
23 increase ovarian response and the quality of oocytes and embryos and their
24 cryotolerance remain a challenge. The aim of our study was to evaluate the effect of
25 corifollitropin alpha (CTP) and a recombinant human FSH (rhFSH), alone or
26 supplemented with rhLH, on embryo cryosurvival by in vitro development and OCT4
27 and NANOG mRNA abundance at blastocyst stage and offspring rate. In vitro
28 development of vitrified embryos was not significantly affected by superstimulation
29 with or without rhLH supplementation, resulting in similar development rates to those
30 of the control groups (fresh and vitrified embryos from non-superstimulated donor
31 does). Blastocysts developed from vitrified embryos showed higher levels of OCT4
32 transcript abundance than fresh control, while NANOG transcript abundance was only
33 higher in the blastocysts developed from vitrified embryos after superstimulation
34 treatment in comparison with control groups. The implantation and offspring rates at
35 birth were negatively affected by supplementation with rhLH. Both rhFSH or CTP
36 vitrified embryo groups showed an implantation rate similar to those of the control
37 groups, but an offspring rate lower than control. In conclusion, embryos produced using
38 corifollitropin alpha did not compromise the cryosurvival of vitrified embryos in the
39 rabbit. In addition, this study points out the negative effect of rhLH supplementation in
40 terms of offspring rate on embryo vitrification.

41 **Keywords:** Superovulation, corifollitropin, rhLH, embryo, cryosurvival, rabbit.

42

43 **INTRODUCTION**

44 Embryo cryopreservation provides an important tool for animal breeding, enabling the
45 establishment of genome cryobanking and saving embryos for an unlimited time. The
46 efficiency of an embryo bank depends on the provision of optimal conditions for
47 recovery, cryopreservation and transfer for each breed or strain. To this end, different
48 laboratories have successfully developed embryo banks for a reduced number of breeds
49 or strains (main laboratory and livestock species). In this sense, increasing the number
50 of embryos using superovulation treatment will clearly reduce the number of donors,
51 improve the efficiency of animal production (Takeo and Nagata, 2015) and minimise the
52 number of animals in line with the 3Rs principle concept (Russell, 2005). Nevertheless,
53 controlling reproductive events in unusual species or breeds remains a challenge, given
54 the vagueness of response to conventional protocols to control or increase ovarian
55 response and the quality of obtained oocytes and embryos and their cryotolerance in
56 breeds or strains.

57 In rabbits, to ensure the maximum number of normal embryos recovered per donor, both
58 eCG and pituitary derived FSH have commonly been used to induce superovulation
59 (Kanayama et al. 1994, Kauffman et al. 1998, Hashimoto et al. 2004, Mehaisen et al.
60 2005, 2006, Salvetti et al. 2007). However, superovulation treatments may generate a
61 higher number of abnormal and immature follicles through either increasing the
62 cytogenetic defects or abnormal steroidogenesis pattern, affecting the oocyte
63 competence and consequently the future embryo development (Kennelly and Foote
64 1965, Fechheimer and Beatty 1974, Paufler et al. 1975, Taneja et al. 1990, Schmidt et
65 al. 1992, Cheng et al. 1999, Hashimoto et al. 2004; Cortell et al. 2015).

66 Specifically, both oocytes and embryos obtained after FSH treatments seem to have
67 advantages in terms of number and quality versus eCG, but due to the relatively short
68 elimination half-life of FSH and rapid metabolic clearance, two daily injections are
69 required to maintain the threshold level during ovarian superstimulation. Recently,
70 recombinant human FSH (rhFSH) and a long-acting recombinant FSH named
71 corifollitropin alpha (CTP) were successfully assayed in rabbit (Viudes-de-Castro et al.
72 2015 and 2017). CTP has a plasma half-life of approximately 65 hours and an almost
73 four-fold extended time to peak serum levels in a single injection (Duijkers et al. 2002,
74 Devroey et al. 2004, 2009) and provides both good superovulatory response and similar

75 in vivo survival rate to non superovulated embryos, especially when CTP is
76 supplemented with LH (Viudes-de-Castro et al., 2017).

77 The use of LH in superovulation treatments is controversial and unclear; the quality of
78 oocytes or embryos recovered after superovulation treatments varies, and one of the
79 reasons for this may be the variable LH:FSH ratio. Low LH levels might intensify FSH
80 sensitivity in granulosa cells by increasing androgen synthesis during the early stage of
81 folliculogenesis, and this activity is required for normal follicle and oocyte development
82 (Ruvolo et al. 2007, Durnerin et al. 2008). Moreover, high LH levels seem to be
83 detrimental for follicular growth. In rabbits, the effect of LH on superovulation has been
84 studied using purified porcine FSH, obtaining highly variable results (Hashimoto et al.
85 2004, Salvetti et al. 2007). An LH concentration window has been suggested in women
86 below which estradiol production is not adequate and above which LH may be
87 detrimental to follicular development (Borini and Dal Prato 2005, Balasch and
88 Fábregues 2006). Our studies with recombinant human gonadotropins suggested that in
89 the rabbit, the window of LH is FSH dose dependent, and the higher the concentration
90 of FSH used, the more the LH window shifts to higher concentrations. When the FSH
91 concentration used is low, 10% of LH supplement may exceed the LH requirements and
92 the fertilisation and embryonic development is negatively affected. In contrast, when the
93 FSH concentration used is high, 10% of LH supplementation is essential to increase the
94 number of follicles recruited during the selection phase (Viudes-de-Castro et al. 2015).

95 Another concerning aspect is the negative interaction between superovulation and
96 cryopreservation, making the embryos more sensitive to cryopreservation (Leoni et al.
97 2001, Belinger et al. 2004, Mehaisen et al. 2006, Forcada et al. 2011) and decreasing
98 their ability to produce offspring (Maurer et al. 1968, Carney and Foote 1990, Kauffman
99 et al. 1998, Mehaisen et al. 2006). Superovulated embryos seem to have a higher
100 sensitivity to low temperatures and this leads to a decrease in their subsequent potential
101 capacity for development after vitrification, based on a reduced number of trophoblastic
102 cells and inner cell mass per embryo (Vajta 2000, and Leoni et al. 2001). The surviving
103 blastomeres after vitrification procedures may be insufficient in number to permit the
104 re-expansion of the blastocoelic cavity and continue the physiological development.

105 The aim of the current study was to evaluate the effect of corifollitropin alpha (CTP)
106 and a recombinant human FSH (rhFSH), alone or supplemented with recombinant

107 human LH, on embryo cryosurvival by in vitro development and mRNA abundance of
108 OCT4 and NANOG at blastocyst stage and live offspring rate at birth.

109

110 **MATERIALS AND METHODS**

111 All chemicals, unless otherwise stated, were reagent-grade and purchased from Sigma-
112 Aldrich Química S.A. (Alcobendas, Madrid, Spain).

113 **Animals and ethical statement**

114 The research was carried out at the experimental farm of the Institute of Science and
115 Animal Technology (ICTA), Polytechnic University of Valencia. All the experimental
116 procedures used in this study were performed in accordance with Directive 2010/63/EU
117 EEC for animal experiments and reviewed and approved by the Ethical Committee for
118 Experimentation with Animals of the Polytechnic University of Valencia, Spain
119 (research code: 2015/VSC/PEA/00061).

120 One hundred and twenty nulliparous does 18-20 weeks old were used. Does belonged to
121 a New Zealand White line selected for litter size at weaning (Line A, Estany et al.
122 1989). Animals were housed in flat-deck cages, fed with a standard pellet diet ad
123 libitum and had free access to water. An alternating cycle of 16 h lights and 8 h of dark
124 was used.

125 **Hormonal treatment**

126 Ovarian stimulation was induced using corifollitropin alpha (CTP, Elonva, Merck Sharp
127 & Dohme S.A.; Spain) and recombinant human FSH (rhFSH, Gonal-F 75; Serono
128 Europe Ltd., London, United Kingdom) either alone or in combination with
129 recombinant human LH (rhLH, Luveris 75; Serono Europe Ltd., London, United

130 Kingdom). Rabbit donors, weighing 3.9 to 4.2 kg, were assigned randomly to five
131 experimental groups:

132 -Group CTP: 12 rabbit does were subcutaneously treated once with 3 µg of
133 corifollitropin alpha.

134 -Group CTP+rhLH: 12 rabbit does were subcutaneously treated once with 3 µg of
135 corifollitropin alpha and intramuscularly treated with a 10% of recombinant human LH,
136 distributed in five equal doses at 12-hour intervals.

137 -Group rhFSH: 12 rabbit does were intramuscularly treated with 3 µg of recombinant
138 human FSH, distributed in five equal doses at 12-hour intervals.

139 -Group rhFSH+rhLH: 12 rabbit does were intramuscularly treated with 3 µg of
140 recombinant human FSH in combination with a 10% of recombinant human LH,
141 distributed in five equal doses at 12-hour intervals.

142 -Group non-superovulated donor does: 36 receptive rabbit does without stimulation
143 treatment were used to obtain fresh and vitrified embryos. Fresh embryos from non-
144 superovulated does were used as control.

145 Superstimulated donor does were inseminated with 1 mL of sperm pool from fertile
146 males of the same line 60 h after the first gonadotropin injection, and ovulation was
147 induced with 1 µg buserelin acetate (Suprefact; Hoechst Marion Roussel, S.A., Madrid,
148 Spain) given intramuscularly. Control group females were inseminated with the same
149 sperm pool and ovulation was induced with 1 µg buserelin acetate. Sperm pool was
150 constituted by 5-6 ejaculates from different males exhibiting a white colour and with a
151 motility rate higher than 70% and morphological abnormality rate lower than 25%. The
152 seminal dose used per donor doe was around 20-30 million sperm.

153 **Embryo recovery**

154 Females were euthanised 72 h after artificial insemination with an intravenous injection
155 of 0.6 g pentobarbital sodium (Dolethal; Vetoquinol, Madrid, Spain) and the
156 reproductive tract was immediately removed. Embryos were recovered by perfusion of
157 each oviduct and uterine horn with 10 mL Dulbecco's phosphate buffered saline
158 (HyClone™ DPBS liquid without Calcium, Magnesium, Phenol Red; HyClone
159 Laboratories, Logan, Utah, USA) containing 0.2% bovine serum albumin
160 (AMRESCO® Albumin Bovine, (BSA); Solon, USA), 0.133 g/L $CaCl_2$, 0.100 g/L
161 $MgCl_2$ and antibiotics (100 IU/mL Penicillin and 0.01 mg/mL Streptomycin, Sigma-
162 Aldrich Quimica S.A., Spain). The recovered fluid was collected into sterile Petri dishes
163 for examination under a stereomicroscope. Embryos were scored by morphologic
164 criteria according to International Embryo Transfer Society classification (IETS).
165 Briefly, only embryos in morula or early blastocyst stages with homogenous cellular
166 mass and spherical mucin coat and zona pellucida were catalogued as normal embryos.
167 In superstimulated groups, only embryos from donor does with more than 30 normal
168 embryos were vitrified to avoid using non-superovulated embryos. Normal embryos
169 from control group were split to immediately culture or transfer as fresh embryos or to
170 vitrify.

171 A total of 1061 normal embryos were used, 171 non-superovulated fresh embryos and
172 890 as vitrified from different experimental groups.

173 **Embryo vitrification procedure**

174 Vitrification was carried out in 12 batches. A total of 890 embryos were vitrified and de-
175 vitrified using the methodology described by Vicente et al. (1999). The vitrification
176 procedure was carried out in two steps at 20 °C. In the first step, embryos were placed

177 for 2 min in a vitrification solution consisting of 10% (v/v) dimethyl-sulphoxide (1.75M
178 DMSO, Sigma) and 10% (v/v) ethylene glycol (2.23 M EG, Sigma) in DPBS
179 supplemented with 0.2% (w/v) of BSA. In the second step, embryos were suspended for
180 1 min in a solution of 20% (v/v) DMSO and 20% EG in DPBS supplemented with 0.2%
181 (w/v) of BSA. Then, embryos suspended in the vitrification medium were loaded into
182 0.25 ml plastic straws (IMV, L'Aigle, France) between two drops of DPBS separated by
183 air bubbles. Finally, the straws were sealed and directly plunged into liquid nitrogen.

184 De-vitrification procedure was performed by placing the French ministraws 10 cm from
185 nitrogen vapour until the onset of ice formation in the vitrified fraction (milky
186 appearance) and thawing by submerging the straws into a water bath at 20 °C for 10 sec.
187 The vitrification medium was removed in two steps. In the first step, the embryos were
188 expelled with the medium into a solution of DPBS with 0.33M sucrose for 5 min, and in
189 the second step the embryos were washed in a DPBS solution for another 5 min.

190 Devitrified embryos were scored and only undamaged embryos were catalogued as
191 transferable.

192 **In vitro culture until blastocyst stage**

193 In vitro culture was performed in 6 batches. A total of 536 embryos were cultured for 48
194 h in Tissue Culture Medium 199 (TCM199) + 10% Foetal Bovine Serum (FBS, Sigma-
195 Aldrich Quimica S.A., Spain) supplemented with antibiotics (100 IU/mL Penicillin and
196 0.01 mg/mL Streptomycin, Sigma-Aldrich Quimica S.A., Spain) at 38.5 °C, 5% CO_2
197 and saturated humidity. The in vitro development ability of embryos to hatched state
198 (more than 50% of mass cell extruded to zona pellucida, Figure 1) was recorded.

199

200 **mRNA expression of OCT4 and NANOG genes. Extraction and quantitative PCR**
201 **analysis**

202 PolyA RNA was extracted from pools consisting of 13 to 15 in vitro cultured embryos
203 using the Dynabeads kit (Life Technologies, Carlsbad, CA, USA), following the
204 manufacturer's instructions. Four independent embryo pools were used for each
205 experimental group. Then, reverse transcription was carried out using qScriptTMcDNA
206 Synthesis kit (Quantabio, Beverly, MA, USA), according to the manufacturer's
207 instructions. Real-time polymerase chain reaction (PCR) reactions were conducted in an
208 Applied Biosystems 7500 system (Applied Biosystems). Every PCR was performed
209 from 5-mL diluted 1:10 complementary DNA (cDNA) template, 250-nM of forward and
210 reverse specific primers (Table 1), and 15 mL of Power SYBR Green PCR Master Mix
211 (Fermentas Gmbh, Madrid, Spain) in a final volume of 20 mL. The PCR protocol
212 included an initial step of 50 °C (2 minutes), followed by 95 °C (10 minutes), and 42
213 cycles of 95 °C (15 seconds) and 60 °C (30 seconds). After quantitative PCR, a melting
214 curve analysis was performed by slowly increasing the temperature from 65 °C to 95
215 °C, with continuous recording of changes in fluorescent emission intensity. The
216 specificity was confirmed by melting curve analysis.

217 Relative expression levels were calculated by $2^{-\Delta\Delta Ct}$ method adjusted for PCR efficiency
218 (Livak and Schmittgen, 2001), applying the geometric average of H2AFZ (H2A histone
219 family member Z (Mamo et al. 2008) and GAPDH glyceraldehyde- 3-phosphate
220 dehydrogenase (Llobat et al., 2012) as a housekeeping normalisation factor. Relative
221 expression of cDNA pool from all samples was used as the calibrator to normalise all
222 samples within one PCR run or between several runs. List of primers used for
223 quantitative real-time polymerase chain reaction is shown in table 1.

224

225 **Embryo transfer**

226 A total of 491 embryos were transferred into 36 recipient females induced to ovulate 72
227 hours before transfer with 1 µg of buserelin acetate injected intramuscularly (Hoechst,
228 Marion Roussel, Madrid, Spain). Synchronous females were anaesthetised by
229 intramuscular injection of 16 mg of xylazine (Rompún, Bayer AG, Leverkusen,
230 Germany) following intravenous injection of 16-20 mg ketamine hydrochloride
231 (Imalgène, Merial SA, Lyon, France). Oviductal embryo transfer was performed using
232 the laparoscopic technique described by Besenfelder and Brem (1993). The number of
233 embryos transferred per recipient doe was from 12 to 15. At the end of the transfer,
234 rabbit does were intramuscularly injected with 0.5 mL/doe of enrofloxacin (Baytril 5%,
235 Bayer, Barcelona, Spain), brought back to the flat deck cages and fed a standard pellet
236 diet ad libitum, having free access to water.

237 **Embryo survival rate**

238 Eleven days after ovulation induction, laparoscopy was performed on the recipient does
239 and the number of implanted embryos per female was recorded. Animals were
240 anaesthetised as described above. In vivo implantation rate was calculated as the
241 successful implantation of the total transferred embryos per each recipient. At birth,
242 litter size and individual pup weight were recorded. Offspring rate at birth was defined
243 as the number of pups at birth related to the number of transferred embryos.

244 **Statistical analysis**

245 For development to hatched blastocyst, implantation and offspring rates at birth, a probit
246 link with binomial error distribution was used, including the treatment group as fixed
247 effect. Data on relative mRNA abundance were analysed by ANOVA using a GLM
248 including the treatment group as fixed effect. NANOG data were normalised by an

249 Arctangent transformation for its subsequent analysis. All statistical analyses were
250 performed with SPSS software (SPSS 16.0 software package; SPSS Inc., 2002,
251 Chicago, IL, USA). Results were reported as least-square means (LSM) with standard
252 error of the mean. LSM were separated using Fisher's protected least significant
253 difference test, with treatment effect declared significant at $P < 0.05$.

254 **RESULTS**

255 Of the 890 vitrified embryos, 856 (96.1%) had no damage to their covers and were
256 cultured or transferred.

257 **Effects of superstimulation treatment on in vitro development, implantation and**
258 **offspring rates**

259 As shown in Table 2, In vitro development of vitrified embryos was not significantly
260 affected by superstimulation with or without rhLH supplementation, resulting in similar
261 development rates to those of the control groups (fresh and vitrified).

262 The implantation and offspring rates at birth were negatively affected by
263 superstimulation treatment supplemented with rhLH. Both rhFSH or CTP vitrified
264 embryo groups showed an implantation rate similar to those of the vitrified and fresh
265 control groups, but a survival rate at birth lower than the fresh control group (Table 2).

266 **Effects of superstimulation treatment on OCT4 and NANOG relative expression**

267 Blastocysts developed from vitrified embryos showed higher levels of OCT4 transcript
268 abundance than fresh control, while NANOG transcript abundance was only higher in
269 the blastocysts developed from vitrified embryos after superstimulation treatment, in
270 comparison with the fresh and vitrified control groups (Table 2).

271

272 **DISCUSSION**

273 This study showed that rabbit embryos derived from a long acting rhFSH (CTP) provide
274 a cryosurvival similar to that of fresh embryos derived from unstimulated donors in
275 terms of implantation rate and similar survival rate at birth to those of vitrified embryos
276 derived from unstimulated donors. In addition, the results indicate that superovulation
277 treatment with CTP provides similar cryosurvival to that found when daily rhFSH is
278 administered. Therefore, our results showed that superovulation treatment using CTP is
279 a successful strategy for the establishment of GRBs in rabbit, reducing the level of
280 distress for the animal and making its application more practical and efficient (Viudes-
281 de-Castro et al., 2017).

282 When CTP or rhFSH was supplemented with rhLH, implantation and offspring rate
283 were lower. Nevertheless, no negative effects were detected either in the in vitro
284 development or in the transcript abundance analysis of two candidate genes related to
285 embryo development and cell pluripotentiality. The usual in vitro parameters to evaluate
286 oocyte or embryo quality are based on morphological appearance at the recovery
287 moment, development rate, metabolites and some gene expression levels such as factor
288 octamer-binding 4 (OCT4), NANOG homeobox (NANOG) and SOX2, but they may
289 not be enough. Changes in gene expression might trigger failures in development and
290 implantation of the embryos and, consequently, in pregnancy loss. In this study, 72h
291 superovulated vitrified embryos cultured for 48h had OCT4 and NANOG up-regulation
292 versus non-superovulated fresh embryos, showing signs of alterations induced by
293 vitrification process in these embryos, although no changes of in vitro developing rates
294 were observed. These altered expressions were not observed when these genes were
295 analysed in superovulated and non-superovulated fresh embryos (Viudes-de-Castro et
296 al., 2017). However, these results agree with the epigenetic changes in developing

297 vitrified mouse embryos at promoter region of three pluripotency genes, including OCT-
298 4, observed by Wang et al. (2010) and Zhao et al. (2012). Only vitrified embryos from
299 non-superovulated donors did not have NANOG over-expressed. In any case, the in
300 vitro results were indicative of OCT4 and NANOG disturbances and this could explain
301 the minor viability at birth of the vitrified embryos versus fresh control embryos, but did
302 not support the lower viability of vitrified embryos obtained from superstimulation
303 treatment supplemented with rhLH. It has been demonstrated that in vitro culture
304 systems do not mimic the uterine environment and in vitro developed embryos differ
305 from their in vivo counterparts, even after vitrification (Corcoran et al., 2006; Saenz-de-
306 Juano et al., 2010, Asku et al., 2012, Marco-Jiménez et al., Vicente et al., 2013).

307 Vitrification and transfer procedures provide a sub-optimal condition and stressful
308 environments to which the embryos must adapt (Saenz-de-Juano et al., 2016). This
309 could contribute to the embryo-foetal programming and have consequences even in the
310 neonatal period or adulthood (Pluess and Belsky, 2011). Previous studies in rabbit
311 revealed that vitrification causes developmental differences in gastrulation, expression
312 gene and proteomic disturbances in placental tissues with consequences on foetal
313 mortality and differences in postnatal growth patterns (Mocé et al, 2010, Vicente et al,
314 2013, Saenz-de-Juano et al., 2014, 2015). The present study shows the difficulties of
315 rabbit vitrified embryos in surviving until the end of gestation, but especially if embryos
316 come from superstimulation treatment supplemented with rhLH. Some authors have
317 shown beneficial effects of LH on ovarian response, oocyte maturation and in vivo
318 survival (Sirard et al. 2006, Ruvolo et al. 2007, Viudes-de-Castro et al. 2015 and 2017
319 in rabbit). Viudes-de-Castro et al. (2017) showed that fresh embryos obtained from
320 donors superstimulated with CTP and supplemented with rhLH reached similar
321 implantation and birth survival rates to those of control embryos, suggesting a greater

322 embryo competence to implant than the other superstimulated groups with rhFSH with
323 or without LH supplementation. However, in the present study, when CTP+rhLH was
324 associated with the cryopreservation process, the in vivo cryosurvival or offspring rate
325 of vitrified embryos was lower than for CTP alone and the vitrified control group. In the
326 same way, rhFSH+rhLH group showed these detrimental effects on in vivo
327 cryosurvival.

328 Taking into account the modifications in the expression of the genes and the highest
329 percentage of implanted embryos observed after the transfer of vitrified embryos from
330 CTP and rhFSH groups (66 and 73%), it will be necessary to define adequate
331 asynchrony or the recipient doe strain in order to improve survival through the reduction
332 of foetal losses. Marco-Jiménez et al (2013) observed a higher rate of embryos
333 developed to term when 12h asynchrony between vitrified embryo and recipients was
334 used. In this study, we decided to test the cryosurvival of superovulated rabbit embryos
335 in a synchronic approach.

336 In conclusion, our findings indicate that embryos produced using corifollitropin alpha
337 superovulation treatment do not compromise the cryosurvival of vitrified embryos in the
338 rabbit. In addition, the present study clearly highlights the negative effect of rhLH
339 supplementation in rhFSH superstimulation treatment in terms of offspring at birth,
340 whereas there was no effect on in vitro embryo developmental rates. Therefore,
341 corifollitropin alpha is a successful strategy for the establishment of genetic resource
342 banks in the rabbit, reducing the level of distress for the animal and making its
343 application more practical and efficient.

344

345

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351

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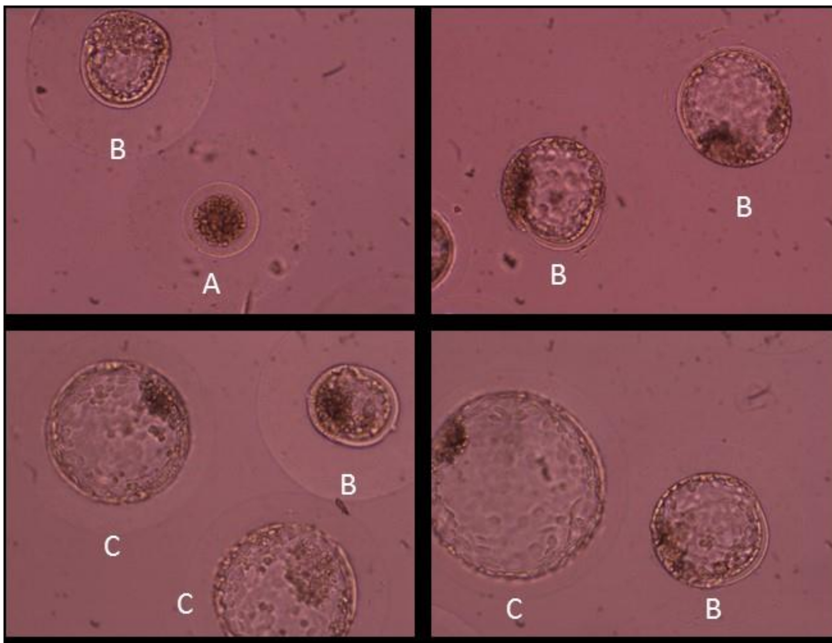
505 

506 Figure 1. Non-developed morula (A), hatching (B) and hatched (C) rabbit blastocyst

507 embryos after 48h of in vitro culture

508 Table 1. List of primers used for quantitative real-time polymerase chain reaction.

Gene symbol	Forward primer	Reverse primer	Fragment (bp)
OCT4	CGAGTGAGAGGCAACTTGG	CGGTTACAGAACCACACACG	125
NANOG	CCAGGTGCCTCTTACAGACA	TCACTACTCTGGGACTGGGA	104
H2AFZ	AGAGCCGGCTGCCAGTTCC	CAGTCGCGCCCACACGTCC	85
GAPDH	GCCGCTTCTTCTCGTGCAG	ATGGATCATTGATGGCGACAACAT	144

509

510 *H2AFZ:* H2A histone family member Z; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase;

511 *OCT4*: transcription factor octamer-binding 4; *NANOG*: NANOG homeobox.

512

513

514 Table 2. Effect of ovarian stimulation treatments on in vitro and *in vivo* development (least square mean ± standard error).

				In vitro				In vivo	
Embryo type	Experimental groups	N	CE	Hatched blastocyst rate	*OCT4*	*NANOG*	TE	Implantation rate	Offspring rate at birth
Vitrified	FSH-CTP	165	82	0.55 ± 0.06	0.87 ± 0.083[a]	0.55 ± 0.084[a]	83	0.66 ± 0.05[ab]	0.48 ± 0.06[b]
	FSH-CTP+LH	176	95	0.48 ± 0.05	0.85 ± 0.083[a]	0.54 ± 0.084[a]	81	0.38 ± 0.05[c]	0.33 ± 0.05[c]
	FSH	175	98	0.60 ± 0.05	0.79 ± 0.083[a]	0.62 ± 0.084[a]	77	0.73 ± 0.05[a]	0.53 ± 0.06[b]
	FSH+LH	176	87	0.46 ± 0.05	0.76 ± 0.083[a]	0.54 ± 0.084[a]	89	0.42 ± 0.05[c]	0.31± 0.05[c]
	Non-superovulated	164	86	0.42 ± 0.05	0.73 ± 0.083[a]	0.23 ± 0.084[b]	78	0.53 ± 0.06[bc]	0.44 ± 0.06[bc]
Fresh	Non-superovulated	171	88	0.57 ± 0.05	0.48 ± 0.083[b]	0.28 ± 0.084[b]	83	0.78 ± 0.05[a]	0.75 ± 0.05[a]

515 CTP: Corifollitropin α single-injection; CTP+rhLH: A single-injection of corifollitropin α and five equal doses at 12-hours interval of
516 recombinant human LH; rhFSH: Five equal doses at 12-hour intervals of recombinant human FSH. rhFSH+rhLH: Five equal doses at 12-hours
517 interval of recombinant human FSH plus recombinant human LH; Non-superovulated group: Five doses at 12-hours interval of saline solution;
518 N: Number of embryos; CE: Number of cultured embryos; TE: Number of transferred embryos. [a,b]Values in the same column with different
519 superscripts are statistically different (P<0.05).

520