Effect of luteinizing hormone on rabbit ovarian superstimulation and embryo developmental potential

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Abstract

Assisted reproduction technologies require ovarian stimulation to increase the number of oocytes and embryos. Currently, superstimulation is achieved by gonadotropin treatment, but the embryo yield and quality are highly variable. Commonly, commercial preparations derived from pituitary and urinary origin are used to superovulate. Hence, ovarian superstimulation protocols have usually included both FSH and LH. The appearance of recombinant gonadotropins manufactured by genetic engineering techniques has ensured high quality and batch-to-batch consistency. Moreover, this enables us to assess the importance of LH in the ovarian stimulation. The main aim of this study was to evaluate the effect of recombinant human LH supplementation (10%) on embryonic development produced by rabbit does superovulated with low or high concentration (18.75 or 37.50 IU) of recombinant human FSH (rhFSH). Females treated with rhFSH increased the ovulation rate, and it was significantly higher when the high FSH dose was supplemented with LH. The superstimulation treatment used did not significantly affect in vitro development rate until the expanded blastocyst stage. The results of this study seem to suggest that, in terms of superovulatory response, when rabbit does are treated with 37.5-IU rhFSH, the use of LH supplementation allows an increase in the number of follicles recruited and the quality of embryos, in terms of ability to develop in vitro until blastocyst, and the expression profile of OCT4, NANOG, and SOX2 genes is not affected.

INTRODUCTION

Superstimulation of females from domestic animals is used to augment conventional genetic improvement pro- grams and facilitates advances in reproductive bio- technologies. Rabbit presents great interest as an experimental animal model for biomedical research, genetic modeling, and fur and meat production [1,2]. Super-stimulation is considered a fundamental method to estab- lish an abundant source of oocytes and embryos and requires the administration of exogenous gonadotropin. Although is well established that FSH alone can induce follicular growth, small amounts of LH are necessary to support the follicular development [3,4]. The action of LH on follicular development is not limited to providing androgen substrate for aromatization but also exerts a direct effect on the stimulation and modulation of folliculogenesis [5,6], and the influence and the amount of LH necessary for optimal follicular stimulation is still being investigated. Until recently, superovulation treatments in farm mammals were based on the use of eCG and FSH preparations derived from pituitary extracts (porcine, ovine, or equine), in which the variable LH concentration present may interfere the FSH activity [7,8], and it is one of the most important factors for the variability in the superstimulation response, affecting the ability of the ovary to respond to exogenous gonado- tropin treatments. The use of pituitary-derived products presents other problems including contamination from other hormones, inconsistencies within and among batches, and the possibility of the spread of disease-transmitting agents [8]. Several studies have attributed detrimental effects of an excessive LH content on follicular development, but these studies have not established an optimal dose of LH or an optimal ratio FSH:LH to maximize the super- stimulation response [9–11]. In rabbits, the effect of LH on superstimulation

66 has been studied using porcine FSH, obtaining highly variable results [11,12].

67 Because of break- throughs in recombinant technology, it is possible to easily

68 dispose of LH and FSH in an isolated manner, which facili- tates the study of the role

69 of LH in ovarian response.

70

71 On the other hand, the usual parameters to evaluate embryo quality are the

72 morphologic appearance at recov- ery or the blastocyst rate after IVC. However, it

73 has been reported that messenger RNA (mRNA) expression is related with the

74 embryo quality [13–18]. In addition, the early steps in embryo development can be

75 affected by the culture media and conditions as well as the production procedure

76 itself, which alters gene expression [13,14,16,19–21]. Therefore, the evaluation of

77 gene expression may help assess the developmental quality of the embryos. It is

78 known that the transcription factor octamer-binding 4 (OCT4), NANOG homeobox

79 (NANOG), and sex- determining region Y-box 2 (SOX2) have essential roles in early

80 development and are considered key regulators of the pluripotency maintenance

81 system [22]. Thus, changes in their expression might trigger failures in embryo devel-

82 opment and implantation. The implications of super- stimulation experience acquired

83 in animal studies and its possible short-term and long-term consequences should be

84 taken into account when proposing gonadotropin treat- ment to increase the number

85 of oocytes or embryos pro- duced for reproductive biotechnologies.

86

87 This study aimed to evaluate the relative importance of LH in ovarian stimulation with

88 recombinant human FSH (rhFSH) and the effect on the development ability of

89 embryos in vitro and the expression of transcription factors OCT4, SOX2, and

90 NANOG.

2. MATERIAL AND METHODS

92

93   Unless stated otherwise, all chemicals in this study were purchased from

94 Sigma-Aldrich Química S.A (Madrid, Spain). The proteomic analysis was carried out

95 in the CSIE_university of Valencia Proteomics Unit, a member of ISCIII ProteoRed

96 Proteomics Platform.

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98

99 2.1. Animals

100 All animals were handled according to the principles of animal care published by

101 Spanish Royal Decree 53/2013 in the Official Spanish State Gazette (Boletín Oficial

102 del Estado) in 2013.The research was carried out at the experimental farm of the

103 Universidad Politécnica de Valencia. Forty-three nulliparous does and five bucks of

104 sexually mature New Zealand White rabbits were used. Animals were housed in flat-

105 deck cages, fed a standard pellet diet ad libitum, and had free access to water. An

106 alternating cycle of 16-hour light and 8-hour dark was used [23].

107

108 2.2. Hormonal treatment

109 Superstimulation was induced using rhFSH (GONAL-f 75; Serono Europe Ltd.,

110 London, UK) alone or in combination with recombinant human LH (rhLH; Luveris 75;

111 Serono Europe Ltd.). Does were treated intramuscularly with either 18.75 or 37.50 IU

112 rhFSH alone or in combination with 10% rhLH (1.87 and 3.75 IU, respectively)

113 dissolved in saline distributed in five equal doses at 12-hour intervals. Females were

114 assigned randomly to five treatment groups:

115

116   1. Control: females were not superstimulated but were treated with saline solution.

117   2. Low FSH: superstimulation was induced using 18.75-IU rhFSH.

118   3. Low FSH–LH: superstimulation was induced using 18.75-IU rhFSH þ 10% rhLH

119   (1.87 IU).

120   4. High FSH: superstimulation was induced using 37.50-IU rhFSH.

121   5. High FSH–LH: superstimulation was induced using 37.50-IU rhFSH þ 10% rhLH

122   (3.75 IU).

123

124   Does were inseminated with 0.5 mL of fresh heterospermic pool 10 to 12 hours after

125   the last gonadotropin injection. To prepare the heterospermic pool, ejaculates from

126   five males were collected using an artificial vagina. The 10-mL aliquot samples from

127   ejaculates were diluted 1:10 with a Tris-citrate-glucose extender (250-mM

128   Tris(hydroxymethyl)aminomethane, 83-mM citric acid, 50-mM glucose, pH 6.8–7.0,

129   300 mOsm/kg) for a prior motility rate evaluation. Ejaculates with an estimated

130   motility higher than 70% were pooled. From heterospermic pool, two aliquot samples

131   of 10 mL were taken; the first one was diluted 1:10 with the Tris-citrate-glucose

132   extender for motility rate evaluation in a computer-assisted analysis system, and the

133   second was diluted 1:10 with 1% of glutaraldehyde solution in PBS to calculate the

134   concentra- tion in a Thoma chamber and evaluate both the percent- ages of normal

135   intact acrosome and abnormal sperm by phase contrast at a magnification of 400.

136   Only hetero- spermic pools with more than 70% of motility rate, 85% of normal intact

137   acrosome, and less than 15% of abnormal sperm were used to inseminate the does.

138   Pooled semen was diluted to 40 million/mL by adding Tris-citrate-glucose extender to

139   prepare the seminal doses. Does were induced to ovulate with an intramuscularly

140   administration of 1-mg buserelin acetate at the same time as the insemination.

141   2.3. Embryo recovery

142   Females were slaughtered 38 to 40 hours after artificial insemination with an

143 intravenous injection of 0.6-g pentobarbital sodium (Dolethal; Vetoquinol, Madrid,

144 Spain), and the reproductive tract was immediately removed. Embryos were

145 recovered by perfusion of each oviduct with 5-mL Dulbecco's PBS without

146 calcium2.3.1. Seminal plasma preparation chloride and supplemented with 0.1% of

147 BSA. Embryos were scored by morphologic criteria according to Interna- tional

148 Embryo Transfer Society classification. Only embryos in the eight- to 16-cell stage

149 without morphologic abnor- malities in mucin coat, ZP, and embryo cells were

150 cataloged as normal embryos (Fig. 1).

151   The ovulation rate, number of embryos, and number of oocytes were

152 recorded. The recovery rate was calculated as the number of embryos and oocytes

153 recovered divided by the ovulation rate, multiplied by 100. The fertilization rate was

154 calculated as the number of embryos divided by the number of embryos and oocytes

155 recovered, multiplied by 100.

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157   2.4. In vitro culture

158   A total of 429 embryos were cultured for 48 hours in medium TCM-199

159 (Sigma, St. Louis, MO, USA) supple- mented with 10% fetal bovine serum and

160 antibiotics (penicillin G sodium, 300,000 IU; penicillin G procaine, 700,000 IU; and

161 dihydrostreptomycin sulfate, 1250 mg; Penivet 1; Divasa Farmavic, Barcelona,

162 Spain) at 38.5 C, 5% CO_2, and saturated humidity. The in vitro development ability

163 of embryos until blastocyst stage was recorded for subsequent analysis (Fig. 1).

164   2.5. RNA extraction and quantitative PCR analysis

165   PolyA RNA was extracted from pools consisting of 13 to 15 embryos using the

166   Dynabeads kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer's

167   in- structions. Six independent embryo pools were produced for each experimental

168   group. Then, reverse transcription was carried out using SuperScript III Reverse

169   Transcriptase (Life Technologies AS, Oslo, Norway) according to the manufacturer's

170   instructions. Real-time polymerase chain reaction (PCR) reactions were conducted in

171   an Applied Biosystems 7500 system (Applied Biosystems). Every PCR was

172   performed from 5-mL diluted 1:10 complementary DNA (cDNA) template, 250-nM of

173   forward and reverse specific primers (Table 1), and 10 mL of Power SYBR Green

174   PCR Master Mix (Fermentas Gmbh, Madrid, Spain) in a final volume of 20 mL. The

175   PCR protocol included an initial step of 50   C (2 minutes), followed by 95   C (10

176   minutes), and 42 cycles of 95   C (15 seconds) and 60   C (30 seconds). After

177   quantitative PCR, a melting curve analysis was performed by slowly increasing the

178   temperature from 65   C to 95   C, with continuous recording of changes in

179   fluorescent emission intensity. To avoid detection of genomic DNA (gDNA), at least

180   one primer per pair was designed to span an exon–exon boundary. All the primers

181   were tested on gDNA and RNA to confirm that potentially contaminant gDNA was not

182   amplified. The specificity was confirmed by melting curve analysis, gel

183   electrophoresis, and sequencing of the quantitative PCR products. Serial dilutions of

184   cDNA pool made from several samples were done to assess PCR efficiency. A ddCt

185   method adjusted for PCR efficiency was used, using the geometric average of

186   H2AFZ (H2A histone family member Z [24]) and GAPDH (glyceraldehyde- 3-

187   phosphate dehydrogenase [25]) as a housekeeping normalization factor [26].

188   Relative expression of cDNA pool from various samples was used as the calibrator to

189 normalize all samples within one PCR run or between several runs. SOX2 data were

190 normalized by a Napierian logarithm transformation.

191

192   2.6. Statistical analysis

193 The effect of superstimulation treatment (control, low FSH, low FSH–LH, high FSH,

194 or high FSH–LH) on the ovulation rate, number of recovered embryos, recovery rate,

195 and fertilization rate was analyzed by ANOVA using a general linear model

196 procedure. For the developmental rate, a probit link with binomial error distribution

197 was used. Data of relative mRNA abundance were normalized by a Napierian

198 logarithm transformation and analyzed using a general linear model. All statistical

199 analyses were performed with SPSS software (SPSS 16.0 software pack- age; SPSS

200 Inc., 2002, Chicago, IL, USA). Results were reported as least-square means with

201 standard error of the mean. Means were separated using Fisher's protected least

202 significant difference test, with treatment effect declared significant at $P < 0.05$.

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205

206 **3. RESULTS**

207   Effects of superstimulation treatments on recovery variables are shown in

208 Table 2. All the females treated with rhFSH increased the ovulation rate. However,

209 the lack of statistical difference among the low FSH concentration groups and control

210 may be due to the small sample size. Ovulation rate in high FSH concentration

211 groups was significantly higher than that in the control group. Addi- tionally, the

212 ovulation rate was significantly higher when the high FSH dose was supplemented

213 with LH. The number of recovered embryos showed a similar evolution to the

9

214 ovulation rate. A clear trend toward an increase in the number of recovered embryos

215 was observed in FSH-treated females, although there was no significant difference

216 among low FSH groups and the control. The high FSH groups showed significantly

217 higher numbers of recovered embryos than the control group, although the results

218 from high and low FSH groups without LH supplement were not statistically different.

219 The recovery rate was not affected by superstimulation treatment. Finally, the

220 fertilization rate

221   was significant decreased in the low FSH group supplemented with LH.

222   As shown in Table 3, the superstimulation treatment did not significantly affect

223 in vitro development rate until the expanded blastocyst stage. The proportion of

224 embryo development was very high.

225   Results of superstimulation treatment on embryos OCT4, NANOG, and SOX2

226 gene expression are shown in Figure 2. No significant differences among relative

227 values of OCT4, NANOG, and SOX2 gene expression from different superstimulation

228 treatment were detected.

229

230 **4. DISCUSSION**

231   In the present study, the ovulation rates of females treated with 37.50-IU

232 rhFSH supplemented with 10% rhLH were 3.5 times higher than those observed for

233 the control group and twice of those observed for 18.75-IU rhFSH alone or with 10%

234 rhLH groups. Similar results were reported by Ju et al. [27] with porcine FSH.

235 Nevertheless, the undefined LH concentration present in pituitary extract used by

236 these authors hampered the comparison of trials. In women, an LH concentration

237 window has been described [6,28] below which estradiol production is not adequate

238 and above which LH may be detrimental to follicular development. In a work

239   designed to simplify hormone administration and reduce the stress of treatment and

240   animal handling in rabbits, 25-IU rhFSH alone or in combination with rhLH was

241   diluted in a slow-release polymer (polyvinylpyrrolidone). It was shown that the

242   superovulatory response of does with

243

244   the rhFSH dose used was not dependent on rhLH supple- mentation [29], and

245   it was concluded that the endogenous LH concentration in rabbits seemed enough to

246   duplicate the ovulation rate of does treated with this FSH dose. A similar ovulation

247   rate was reported previously by other authors using FSH from pituitary glands

248   [11,12,30,31]. Therefore, results of the present work showed that 37.50-IU rhFSH

249   supplemented with 3.75-IU rhLH treatment was significantly more efficient than the

250   other treatments tested, showing an ovulation rate and a number of embryos

251   recovered three times higher than control females. During a normal follicular wave,

252   subordinate follicles regress because of decreasing concentration of circulating FSH.

253   Small follicles require FSH to continue their growth. In the superstimulation protocol,

254   the depressed endogenous FSH caused by the secretory products of dominant

255   follicles is replaced by exogenous FSH. However, a larger number of recruited

256   follicles implies an increase in the LH require- ment of mural granulosa cells and,

257   consequently, a higher level of circulating FSH would result in an increased de- mand

258   of LH as a requirement to improve the number of follicles recruited. Ovulation rate

259   observed with 37.50-IU rhFSH was significantly higher when it was supple- mented

260   with 3.75-IU rhLH. It seems that endogenous LH levels from females treated with

261   37.5-IU rhFSH alone were insufficient to increase follicular recruitment to the values

262   achieved when this concentration of FSH was sup- plemented with LH. This suggests

263   that the window of LH in rabbits is FSH dose dependent, and the higher the con-

264 centration of FSH used, the more the LH window shifts to higher concentrations.

265 When the FSH concentration used is low, 10% of LH supplement may exceed the LH

266 requirements and the fertilization and embryonic devel- opment is negatively

267 affected. In contrast, when the FSH concentration used is high, 10% of LH

268 supplementation is essential to increase the number of recruited follicles dur- ing the

269 selection phase. These results are in agreement with a study in women using rFSH

270 and rLH which suggest that LH actually sensitizes follicles to FSH and thereby

271 encour- ages follicular development and hormone secretion. In this study, the

272 number of developing follicles was much reduced or delayed in the group treated

273 with a fixed dose of rFSH without rLH [3]. The present findings seem to be consistent

274 with those of Filicori et al. [32], who found variable effects of LH and FSH on follicles

275 of different sizes. They showed that FSH alone affected the growth dynamic of small

276 follicles and the LH supplementation affected the medium or large follicles,

277 suggesting that expression of LH receptors may provide an additional source of

278 follicular support. In fact, at nonsaturating concentrations of FSH and LH, the

279 response is additive. Therefore, once the appro- priate stage of follicular

280 development in response to treatment with FSH is reached, granulosa cells become

281 receptive to LH stimulation and LH becomes capable of exerting its actions on both

282 theca and granulosa cells [33].

283 Regarding embryo quality, to provide a more complete idea of the

284 development potential of the embryos in the present work, in addition to the in vitro

285 development ability of embryos, the OCT4, NANOG, and SOX2 gene expressions

286 were studied. Results of the present work showed that embryo quality, in terms of

287 ability to develop in vitro from

288   eight- to 16-cell stage to the blastocyst stage and OCT4, NANOG, and SOX2

289   gene expression, was not affected by superovulation treatment, with results similar to

290   nontreated females. In cows, it was shown that hormonal superstimulation with

291   porcine or ovine FSH, with high and low LH percentage, respectively, may affect the

292   mRNA profile of some genes [20,34]. Mundim et al. [20] showed differences in gene

293   expression profile of a group of genes related to embryo development between

294   superovulated and control embryos, but no differences were observed compared to

295   embryos produced in vitro. Chu et al. [34] observed that although the number of

296   genes influenced seemed low, the mRNA expression profile of potential fac- tors of

297   developmental competence in the bovine oocyte was affected by follicular

298   stimulation. It is necessary to study whether the source of FSH may affect the

299   expression profile of these genes.

300   In conclusion, results from the present study showed that the treatment of

301   rabbit does with 37.5-IU rhFSH alone or supplemented with rhLH was effective in

302   stimulating superovulation without affecting the embryos ability to develop in vitro

303   until blastocyst and the embryonic expression of OCT4, NANOG, and SOX2 genes.

304   On the other hand, the present study suggests that the rhLH supple- mentation at

305   this rhFSH concentration increased the number of follicles recruited and the

306   superovulatory response in rabbits.

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310   **Acknowledgements**

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References

317

318   [1] Fischer B, Chavatte-Palmer P, Viebahn C, Navarrete Santos A, Duranthon V.

319   Rabbit as a reproductive model for human health. Reproduction 2012;144:1–10.

320   [2] Lavara R, Baselga M, Vicente JS. Does storage time in LN2 influence survival

321   and pregnancy outcome of vitrified rabbit embryos? Theriogenology 2011;76:652–7.

322   [3] European Recombinant Human LH Study Group. Recombinant human luteinizing

323   hormone (LH) to support recombinant human follicle stimulating hormone (FSH)-

324   induced follicular development in LH- and FSH-deficient anovulatory women: a dose-

325   finding study. J Clin Endocrinol Metab 1998;83:1507–14.

326   [4] Shoham Z. The clinical therapeutic window for luteinizing hormone in controlled

327   ovarian stimulation. Fertil Steril 2002;77:1170–7.

328   [5] Filicori M, Cognigni GE, Samara A, Melappioni S, Perri T, Cantelli B,

329   et al. The use of LH activity to drive folliculogenesis: exploring un- charted territories

330   in ovulation induction. Hum Reprod Update 2002;8:543–57.

331   [6] Balasch J, Fábregues F. LH in the follicular phase: neither too high nor too low.

332   Reprod Biomed Online 2006;12:406–15.

333 [7] Aggarwal BB, Farmer SW, Papkoff H, Seidel Jr GE. Biochemical properties of

334 equine chorionic gonadotropin from two different pools of pregnant mare sera. Biol

335 Reprod 1980;23:570–6.

336 [8] Hesser MW, Morris JC, Gibbons JR. Advances in recombinant gonadotropin

337 production for use in bovine superovulation. Reprod Domest Anim 2011;46:933–42.

338 [9] Donaldson LE, Ward DN, Glenn SD. Use of porcine follicle- stimulating hormone

339 after chromatographic purification in super- ovulation of cattle. Theriogenology

340 1986;25:747–57.

341 [10] Zelinski Wooten MB, Hutchison JS, Hess DL, Wolf DP, Stouffer RL. Follicle-

342 stimulating hormone alone supports follicle growth and

343 oocyte development in gonadotropin-releasing hormone

344 antagonist-treated monkeys. Hum Reprod 1995;10:1658–66.

345 [11] Hashimoto S, Kuramochi T, Aoyagi K, Takahashi R, Ueda M, Hirao M, et al.

346 Refined porcine follicle-stimulating hormone promotes the responsiveness of rabbits

347 to multiple-ovulation treatment. Exp

348 Anim 2004;53:395–7.

349 [12] Salvetti P, Theau-Clément M, Beckers JF, Hurtaud J, Guerin P, Neto V,

350 et al. Effect of the luteinizing hormone on embryo production in

351 superovulated rabbit does. Theriogenology 2007;67:1185–93.

352 [13] Lonergan P, Rizos D, Kanka J, Nemcova L, Mbaye AM, Kingston M, et al.

353 Temporal sensitivity of bovine embryos to culture environ- ment after fertilization and

354 the implications for blastocyst quality.

355 Reproduction 2003;126:337–46.

356 [14] Gutiérrez-Adán A, Rizos D, Fair T, Moreira PN, Pintado B, De La

357 Fuente J, et al. Effect of speed of development on mRNA expression pattern in early

358 bovine embryos cultured in vivo or in vitro. Mol Reprod Dev 2004;68:441–8.

359 [15] Balasubramanian S, Son WJ, Mohana Kumar B, Ock SA, Yoo JG, Im GS, et al.

360 Expression pattern of oxygen and stress-responsive gene transcripts at various

361 developmental stages of in vitro and in vivo preimplantation bovine embryos.

362 Theriogenology 2007;68: 265–75.

363 [16] Wrenzycki C, Herrmann D, Niemann H. Messenger RNA in oocyte and embryos

364 in relation. Theriogenology 2007;68:77–83.

365 [17] Rizos D, Clemente M, Bermejo-Alvarez P, De La Fuente J, Lonergan P,

366 Gutierrez-Adán A. Consequences of in vitro culture conditions on embryo

367 development and quality. Reprod Domest Anim 2008;43: 44–50.

368 [18] Purpera MN, Giraldo AM, Ballard CB, Hylan D, Godke RA, Bondioli KR. Effects

369 of culture medium and protein supplementa- tion on mRNA expression of in vitro

370 produced bovine embryos. Mol Reprod Dev 2009;76:783–93.

371 [19] Rizos D, Lonergan P, Boland MP, Arroyo-Garcia R, Pintado B, de la Fuente J, et

372 al. Analysis of differential mRNA expression between bovine blastocysts produced in

373   different culture systems: implica- tions for blastocyst quality. Biol Reprod

374   2002;66:589–95.

375   [20] Mundim TC, Ramos AF, Sartori R, Dode MA, Melo EO, Gomes LF, et al.

376   Changes in gene expression profiles of bovine embryos pro- duced in vitro, by

377   natural ovulation, or hormonal superstimulation. Genet Mol Res 2009;8:1398–407.

378   [21] Saenz-De-Juano MD, Naturil-Alfonso C, Vicente JS, Marco-Jimenez F. Effect of

379   different culture systems on ARNm expression in devel- oping rabbit embryos.

380   Zygote 2013;21:103–9.

381   [22] Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, et al. Core

382   transcriptional regulatory circuitry in human embryonic stem cells. Cell

383   2005;122:947–56.

384   [23] Theau-Clement M. Preparation of the rabbit doe to insemination: a review. World

385   Rabbit Sci 2007;15:61–80.

386   [24] Mamo S, Gal AB, Polgar Z, Dinnyes A. Expression profiles of the pluripotency

387   marker gene POU5F1 and validation of reference genes in rabbit oocytes and

388   preimplantation stage embryos. BMC Mol Biol 2008;9:67.

389   [25] Navarrete-Santos A, Tonak S, Kirstein M, Kietz S, Fischer B. Two insulin-

390   responsive glucose transporter isoforms and the insulin receptor are developmentally

391   expressed in rabbit preimplantation embryos. Reprod Res 2004;128:503–15.

392   [26] Weltzien FA, Pasqualinib C, Vernierb P, Dufoura S. A quantitative real-time RT-

393   PCR assay for European eel tyrosine hydroxylase. Gen Comp Endocrinol

394   2005;142:134–42.

395 [27] Ju JC, Chang YC, Huang WT, Tang PC, Cheng SP. Superovulation and

396 transplantation of demi- and aggregated embryos in rabbits. Asian Australas J Anim

397 Sci 2001;14:455–61.

398 [28] Borini A, Dal Prato L. Tailoring FSH and LH administration to indi- vidual

399 patients. Reprod Biomed Online 2005;11:283–93.

400 [29] Viudes-de-Castro MP, Cortell C, Mocé E, Marco-Jiménez F, Joly T, Vicente JS.

401 Effect of recombinant gonadotropins on embryo quality in superovulated rabbit does

402 and immune response after repeated treatments. Theriogenology 2009;72:655–62.

403 [30] Kauffman RD, Schmidt PM, Rall WF, Hoeg JM. Superovulation of rabbits with

404 FSH alters in vivo development of vitrified morulae. Theriogenology 1998;50:1081–

405 92.

406 [31] Mehaisen GM, Viudes-De-Castro MP, Vicente JS, Lavara R. In vitro and in vivo

407 viability of vitrified and non-vitrified embryos derived from eCG and FSH treatment in

408 rabbit does. Theriogenology 2006; 65:1279–91.

409 [32] Filicori M, Cognigni GE, Taraborelli S, Spettoli D, Ciampaglia W, Tabrelli de Fatis

410 C, et al. Luteinizing hormone activity supplementation en- hances follicle-stimulating

411 hormone efficacy and improves ovulation induction outcome. J Clin Endocrinol Metab

412 1999;84:2659–63.

413 [33] Oliveira JB, Mauri AN, Petersen CG, Martins AM, Cornicelli J, Cabaña M, et al.

414 Recombinant luteinizing hormone supplementation to recombinant follicle-stimulation

415 hormone during induced ovarian stimulation in the GnRH-agonist protocol: a meta-

416 analysis. J Assist Reprod Genet 2007;24:67–75.

417   [34] Chu T, Dufort I, Sirard MA. Effect of ovarian stimulation on oocyte gene

418   expression in cattle. Theriogenology 2012;77:1928–38.