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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.

Keywords: rabbit, aminopeptidase activity; dilution rate, male genotype, reproductive performance.

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

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain). The proteomic analysis was carried out in the CSIE_university of Valencia Proteomics Unit, a member of ISCIII ProteoRed Proteomics Platform.

2.1. Animals

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 in the Official Spanish State Gazette (Boletín Oficial del Estado) in 2013.The research was carried out at the experimental farm of the Universidad Politécnica de Valencia. Forty-three nulliparous does and five bucks of sexually mature New Zealand White rabbits were used. Animals were housed in flat-deck cages, fed a standard pellet diet ad libitum, and had free access to water. An alternating cycle of 16-hour light and 8-hour dark was used [23].

2.2. Hormonal treatment

Superstimulation was induced using rhFSH (GONAL-f 75; Serono Europe Ltd., London, UK) alone or in combination with recombinant human LH (rhLH; Luveris 75; Serono Europe Ltd.). Does were treated intramuscularly with either 18.75 or 37.50 IU rhFSH alone or in combination with 10% rhLH (1.87 and 3.75 IU, respectively) dissolved in saline distributed in five equal doses at 12-hour intervals. Females were assigned randomly to five treatment groups:

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

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

3. Low FSH–LH: superstimulation was induced using 18.75-IU rhFSH þ 10% rhLH (1.87 IU).

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

5. High FSH–LH: superstimulation was induced using 37.50-IU rhFSH þ 10% rhLH (3.75 IU).

Does were inseminated with 0.5 mL of fresh heterospermic pool 10 to 12 hours after the last gonadotropin injection. To prepare the heterospermic pool, ejaculates from five males were collected using an artificial vagina. The 10-mL aliquot samples from ejaculates were diluted 1:10 with a Tris-citrate-glucose extender (250-mM Tris(hydroxymethyl)aminomethane, 83-mM citric acid, 50-mM glucose, pH 6.8–7.0, 300 mOsm/kg) for a prior motility rate evaluation. Ejaculates with an estimated motility higher than 70% were pooled. From heterospermic pool, two aliquot samples of 10 mL were taken; the first one was diluted 1:10 with the Tris-citrate-glucose extender for motility rate evaluation in a computer-assisted analysis system, and the second was diluted 1:10 with 1% of glutaraldehyde solution in PBS to calculate the concentra- tion in a Thoma chamber and evaluate both the percent- ages of normal intact acrosome and abnormal sperm by phase contrast at a magnification of 400. Only hetero- spermic pools with more than 70% of motility rate, 85% of normal intact acrosome, and less than 15% of abnormal sperm were used to inseminate the does. Pooled semen was diluted to 40 million/mL by adding Tris-citrate-glucose extender to prepare the seminal doses. Does were induced to ovulate with an intramuscularly 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.

156

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% CO2, 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

PolyA RNA was extracted from pools consisting of 13 to 15 embryos using the Dynabeads kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer's in- structions. Six independent embryo pools were produced for each experimental group. Then, reverse transcription was carried out using SuperScript III Reverse Transcriptase (Life Technologies AS, Oslo, Norway) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) reactions were conducted in an Applied Biosystems 7500 system (Applied Biosystems). Every PCR was performed from 5-mL diluted 1:10 complementary DNA (cDNA) template, 250-nM of forward and reverse specific primers (Table 1), and 10 mL of Power SYBR Green PCR Master Mix (Fermentas Gmbh, Madrid, Spain) in a final volume of 20 mL. The PCR protocol included an initial step of 50 □ C (2 minutes), followed by 95 □ C (10 minutes), and 42 cycles of 95 □C (15 seconds) and 60 □C (30 seconds). After quantitative PCR, a melting curve analysis was performed by slowly increasing the temperature from 65 □ C to 95 □ C, with continuous recording of changes in fluorescent emission intensity. To avoid detection of genomic DNA (gDNA), at least one primer per pair was designed to span an exon–exon boundary. All the primers were tested on gDNA and RNA to confirm that potentially contaminant gDNA was not amplified. The specificity was confirmed by melting curve analysis, gel electrophoresis, and sequencing of the quantitative PCR products. Serial dilutions of cDNA pool made from several samples were done to assess PCR efficiency. A ddCt method adjusted for PCR efficiency was used, using the geometric average of H2AFZ (H2A histone family member Z [24]) and GAPDH (glyceraldehyde- 3-phosphate dehydrogenase [25]) as a housekeeping normalization factor [26]. Relative expression of cDNA pool from various samples was used as the calibrator to

normalize all samples within one PCR run or between several runs. SOX2 data were normalized by a Napierian logarithm transformation.

2.6. Statistical analysis

The effect of superstimulation treatment (control, low FSH, low FSH–LH, high FSH, or high FSH–LH) on the ovulation rate, number of recovered embryos, recovery rate, and fertilization rate was analyzed by ANOVA using a general linear model procedure. For the developmental rate, a probit link with binomial error distribution was used. Data of relative mRNA abundance were normalized by a Napierian logarithm transformation and analyzed using a general linear model. All statistical analyses were performed with SPSS software (SPSS 16.0 software pack- age; SPSS Inc., 2002, Chicago, IL, USA). Results were reported as least-square means with standard error of the mean. Means were separated using Fisher's protected least significant difference test, with treatment effect declared significant at $P < 0.05$.

3. RESULTS

Effects of superstimulation treatments on recovery variables are shown in Table 2. All the females treated with rhFSH increased the ovulation rate. However, the lack of statistical difference among the low FSH concentration groups and control may be due to the small sample size. Ovulation rate in high FSH concentration groups was significantly higher than that in the control group. Addi- tionally, the ovulation rate was significantly higher when the high FSH dose was supplemented with LH. The number of recovered embryos showed a similar evolution to the

ovulation rate. A clear trend toward an increase in the number of recovered embryos was observed in FSH-treated females, although there was no significant difference among low FSH groups and the control. The high FSH groups showed significantly higher numbers of recovered embryos than the control group, although the results from high and low FSH groups without LH supplement were not statistically different. The recovery rate was not affected by superstimulation treatment. Finally, the fertilization rate

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

As shown in Table 3, the superstimulation treatment did not significantly affect in vitro development rate until the expanded blastocyst stage. The proportion of embryo development was very high.

Results of superstimulation treatment on embryos OCT4, NANOG, and SOX2 gene expression are shown in Figure 2. No significant differences among relative values of OCT4, NANOG, and SOX2 gene expression from different superstimulation treatment were detected.

4. DISCUSSION

In the present study, the ovulation rates of females treated with 37.50-IU rhFSH supplemented with 10% rhLH were 3.5 times higher than those observed for the control group and twice of those observed for 18.75-IU rhFSH alone or with 10% rhLH groups. Similar results were reported by Ju et al. [27] with porcine FSH. Nevertheless, the undefined LH concentration present in pituitary extract used by these authors hampered the comparison of trials. In women, an LH concentration window has been described [6,28] below which estradiol production is not adequate 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-

centration of FSH used, the more the LH window shifts to higher concentrations. When the FSH concentration used is low, 10% of LH supplement may exceed the LH requirements and the fertilization and embryonic devel- opment is negatively affected. In contrast, when the FSH concentration used is high, 10% of LH supplementation is essential to increase the number of recruited follicles dur- ing the selection phase. These results are in agreement with a study in women using rFSH and rLH which suggest that LH actually sensitizes follicles to FSH and thereby encour- ages follicular development and hormone secretion. In this study, the number of developing follicles was much reduced or delayed in the group treated with a fixed dose of rFSH without rLH [3]. The present findings seem to be consistent with those of Filicori et al. [32], who found variable effects of LH and FSH on follicles of different sizes. They showed that FSH alone affected the growth dynamic of small follicles and the LH supplementation affected the medium or large follicles, suggesting that expression of LH receptors may provide an additional source of follicular support. In fact, at nonsaturating concentrations of FSH and LH, the response is additive. Therefore, once the appro- priate stage of follicular development in response to treatment with FSH is reached, granulosa cells become receptive to LH stimulation and LH becomes capable of exerting its actions on both theca and granulosa cells [33].

Regarding embryo quality, to provide a more complete idea of the development potential of the embryos in the present work, in addition to the in vitro development ability of embryos, the OCT4, NANOG, and SOX2 gene expressions were studied. Results of the present work showed that embryo quality, in terms of ability to develop in vitro from

eight- to 16-cell stage to the blastocyst stage and OCT4, NANOG, and SOX2 gene expression, was not affected by superovulation treatment, with results similar to nontreated females. In cows, it was shown that hormonal superstimulation with porcine or ovine FSH, with high and low LH percentage, respectively, may affect the mRNA profile of some genes [20,34]. Mundim et al. [20] showed differences in gene expression profile of a group of genes related to embryo development between superovulated and control embryos, but no differences were observed compared to embryos produced in vitro. Chu et al. [34] observed that although the number of genes influenced seemed low, the mRNA expression profile of potential fac- tors of developmental competence in the bovine oocyte was affected by follicular stimulation. It is necessary to study whether the source of FSH may affect the expression profile of these genes.

In conclusion, results from the present study showed that the treatment of rabbit does with 37.5-IU rhFSH alone or supplemented with rhLH was effective in stimulating superovulation without affecting the embryos ability to develop in vitro until blastocyst and the embryonic expression of OCT4, NANOG, and SOX2 genes. On the other hand, the present study suggests that the rhLH supple- mentation at this rhFSH concentration increased the number of follicles recruited and the superovulatory response in rabbits.

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