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EFFECT OF CORIFOLLITROPIN ALFA SUPPLEMENTED WITH OR WITHOUT LH ON OVARIAN STIMULATION AND EMBRYO VIABILITY IN RABBIT

María Pilar Viudes-de-Castro[a,*], Francisco Marco-Jiménez[b], José Isaí Cedano-Castro[b], José Salvador Vicente[b]

[a]Centro de Investigación y Tecnología Animal (CITA), Instituto Valenciano de Investigaciones Agrarias (IVIA), 12400 Segorbe (Castellón), Spain

[b]Institute of Science and Animal Technology (ICTA), Universitat Politècnica de València, 46022 Valencia, Spain

* Corresponding author: MP Viudes de Castro. Tel.: +34-964-712-166.

E-mail address: viudes_mar@gva.es

Abstract:

There is increasing interest in using rabbits for research as a laboratory model as well as for industrial production of meat, wool and fur. Superovulation in animals is used to produce a maximum number of transferable embryos per donor, in order to either support genetic improvement programs, *ex situ* conservation or to optimize other biotechnologies. Over time, the use of this biotechnology has shown variable outcomes as a consequence of several factors, such as the origin of exogenous hormone, posology and the effect of gonadotropins used simultaneously, the donor and the environment. The aim of this study was to compare the efficacy of a single injection of corifollitropin alfa (CTP), alone or supplemented with LH, versus a FSH standard protocol of five equal doses administered twice daily to superovulate rabbit does (20 per group and 29 control females). We determined: 1) the impact of this stimulation on *in vitro* development and mRNA expression at blastocyst stage and 2) *in vivo* embryo development and viability rate at birth of transferred embryos. Our outcomes showed that the ovulation rate was similar among the different ovarian stimulation groups, reaching more than fourfold the ovulation rate of a control doe. While rates of embryos developing to the blastocyst stage after 48h of *in vitro* culture were similar between groups, the hatched blastocyst rate was higher for superovulated embryos from CTP group. Moreover, no significant differences among mRNA expression of *OCT4*, *SOX2* and *NANOG* genes were detected. Nevertheless, embryos from ovarian stimulated does with CTP+LH showed significantly higher implantation rates and survival at birth among the different ovarian stimulation groups and similar to those in the control group. In conclusion, the results of this study suggest that a single injection of long acting corifollitropin alfa can be effectively used in rabbits to elicit a more than fourfold increase in ovulation rate compared to control animals. In addition, the LH

supplementation allows us to obtain similar *in vivo* embryo development results as in the control group.

Keywords: superovulation, long acting FSH, LH, gene expression, embryo viability.

1. Introduction

Despite extensive research efforts in the past 60 years, variability in the superovulation response among individuals is the major problem in all species. Several factors affect the outcome of ovarian superstimulation, such as gonadotropin preparation and dosage, the administration mode, donor characteristics, the environment, etc. Although considerable progress has been made in the study of folliculogenesis, manipulation of ovarian function, gonadotropin biochemistry and factors inherent to the donor animal, the application of superovulation remains a challenge [1-4].

Superovulation protocols in farm animals have usually relied on the use of gonadotropins extracted from animals (chorionic or pituitary extracts). Specifically, in rabbits, in order to ensure the maximum number of normal embryos recovered per donor, both equine chorionic gonadotrophin (eCG) and pituitary derived FSH (ovine and porcine pituitary extracts) have commonly been used to induce superovulation [5-10]. It is known that eCG prolongs plasma half-life and can negatively affect embryonic development [11]. An alternative to pituitary-derived FSH, which has been made available by biotechnology, is recombinant FSH. The use of this hormone might reduce the variation of pituitary-derived FSH [12], and when the exogenous recombinant FSH is from the same species, it may prevent the humoral immune response and transmission of diseases across species [13]. The use of FSH has advantages over eCG, but due to its

relatively short elimination half-life and rapid metabolic clearance is a more time-consuming protocol, requiring two daily injections to maintain the threshold level during ovarian superstimulation. A long-acting recombinant FSH, corifollitropin alfa, was approved by the European Medicines Agency in January 2010. This kind of recombinant FSH comprises an α-subunit which is identical to that of FSH and a hybrid β-subunit which is produced by fusion of the carboxyterminal peptide from the β-subunit of hCG to the β-subunit of FSH [14,15]. This aminoacid-residue of the carboxyterminal peptide extended the FSH half-life thanks to four additional O-linked carbohydrate side chains terminating with a sialic acid residue. Corifollitropin alfa (CTP) has an approximately two-fold longer half-life (65-hours plasma half-life) and an almost four-fold extended time to peak serum levels [16,17]. Hence, in human a single injection can replace the pharmacokinetic profile of first seven daily standard gonadotropin injections and support multi-follicular growth for an entire week [18,19]. Sustained-follicle-stimulating hormones have been used successfully in women [14,15,20,21] and cattle [13].

On the other hand, results of superovulation treatments vary, and one of the reasons for this may be the variable LH:FSH ratio. In some clinical studies where endogenous LH was absent or inactive, recombinant human FSH alone allowed follicle development but with an inadequate estradiol concentration [22]. Although LH has essential and well-established roles in ovarian steroid synthesis and ovulation [23-25], the use of LH in superovulation treatments is controversial and unclear. Low LH levels might intensify FSH sensitivity in granulosa cells by increasing androgen synthesis during the early stage of folliculogenesis and this activity is required for normal follicle and oocyte development [26,27]. Moreover, high LH levels seems to be detrimental for follicular growth. In rabbits, the effect of LH on superovulation has been studied using purified

porcine FSH, obtaining highly variable results [7,10]. Our studies with recombinant human gonadotropins (rhFSH either alone or in combination with rhLH) suggested that the window of LH effect in rabbits is FSH dose dependent [28]. It seems that the endogenous LH concentration is enough to duplicate the ovulation rate of does treated with low FSH doses [29], but it is insufficient to increase follicular recruitment when higher doses of FSH are used [28].

The current study was performed to compare the efficacy of a single injection of corifollitropin alfa (CTP), alone or supplemented with LH, versus a rhFSH standard protocol of five equal doses administered twice daily to superovulate rabbit does, in order to improve the female distress by the use of a single dose. We determined: 1) the impact of this stimulation on *in vitro* development and mRNA expression at blastocyst stage and 2) *in vivo* embryos development and viability rate at birth of transferred embryos.

2. Materials and Methods

2.1. Animals and ethical statement

The research was carried out at the experimental farm of the Institute of Science and Animal Technology (ICTA), Polytechnic University of Valencia. All animals were handled in accordance with the principles of animal care published by Spanish Royal Decree 53/2013 (BOE 2013). The experiments were approved by the Committee of Ethics and Animal Welfare Committee of the Polytechnic University of Valencia (procedure 2015/VSC/PEA/00061).

One hundred thirty-three nulliparous does 18-20 weeks old were used. Does belonged to a New Zealand White line selected for litter size at weaning [30]. Animals were housed

in flat-deck cages, fed with a standard pellet diet *ad libitum* and had free access to water. An alternating cycle of 16 h lights and 8 h of dark was used.

2.2. Hormonal treatment

Ovarian stimulation was induced using Corifollitropin alfa (Elonva, Merck Sharp & Dohme S.A.; Spain) and recombinant human FSH (Gonal-F 75; Serono Europe Ltd., London, United Kingdom) either alone or in combination with recombinant human LH (Luveris 75; Serono Europe Ltd., London, United Kingdom). In a previous work [29] using Gonal-F 75 as recombinant FSH, we established that 0.75 μg of FSH/Kg live weight showed a good superovulatory response. In the present work the FSH dose was fitted to 3μg according to the weight of females rabbit used (3.9 to 4.2 kg). The dose used for both recombinant FSH hormones (Elonva and Gonal-F 75) was the same Rabbit donors, were assigned randomly to five experimental groups (Figure 1):

-Group CTP: 20 rabbit does were subcutaneously treated once with 3 μg of Corifollitropin alfa.

-Group CTP+LH: 20 rabbit does were subcutaneously treated once with 3 μg of Corifollitropin alfa and intramuscularly treated with a 10% of recombinant human LH distributed in five equal doses at 12-hours interval.

-Group FSH: 20 rabbit does were intramuscularly treated with 3 μg of recombinant human FSH distributed in five equal doses at 12-hours intervals.

-Group FSH+LH: 20 rabbit does were intramuscularly treated with 3 μg of recombinant human FSH in combination with a 10% of recombinant human LH distributed in five equal doses at 12-hours intervals.

-Control group: 26 females were treated intramuscularly with saline solution (0.2 mL) at the same time as the other groups.

Does were inseminated with 1 mL of pooled sperm from fertile males of the same line 60 h after the first gonadotropin injection, and ovulation was induced with 1 µg buserelin acetate (Suprefact; Hoechst Marion Roussel, S.A., Madrid, Spain) given intramuscularly.

2.3. Embryo recovery

Females were euthanized 72 h after artificial insemination with an intravenous injection of 0.6 g pentobarbital sodium (Dolethal; Vetoquinol, Madrid, Spain), and the reproductive tract was immediately removed. Embryos were recovered by perfusion of each uterine horn with 10 mL Dulbecco´s phosphate buffered saline (HyClone™ DPBS liquid Without Calcium, Magnesium, Phenol Red; HyClone Laboratories, Logan, Utah, USA) containing 0.2% bovine serum albumin (AMRESCO® Albumin Bovine, (BSA); Solon, USA), 0.133 g/L CaCl2, 0.100 g/L MgCl2 and antibiotics (100 IU/mL Penicillin and 0.01 mg/mL streptomycin, Sigma-Aldrich Quimica S.A., Spain). The recovered fluid was collected into sterile Petri dishes for examination under a stereomicroscope. Embryos were scored by morphologic criteria according to International Embryo Transfer Society classification (IETS). Briefly, only embryos in morula or early blastocyst stages with homogenous cellular mass, and spherical mucin coat and zona pellucida were catalogued as normal (transferable) embryos. The following parameters were evaluated:

-Ovulation induction rate: proportion of treated does with corpora lutea

-Donor does rate: proportion of donor females with at least one normal embryo

-Ovulation rate: number of corpora lutea

-Recovery rate: (number of embryos + oocytes recovered/number of corpora lutea) x 100

-Normal embryo development rate: (number of normal embryos/ number of embryos + oocytes recovered) x 100

2.4. Experiments

2.4.1. Experiment 1: Effects of superstimulation treatment on *in vitro* development and mRNA expression at blastocyst stage

2.4.1.1. *In vitro* culture until blastocyst stage

In vitro culture was performed in 8 batches. Fifty to eighty embryos were cultured in each batch. Embryos were placed in four-well dishes (a maximum of 10 embryos per well in 0.5ml of medium). A total of 530 embryos were cultured for 48 h in Tissue Culture Medium 199 (TCM199) + 10% Fetal Bovine Serum (FBS, Sigma-Aldrich Quimica S.A., Spain) supplemented with antibiotics (100 IU/mL Penicillin and 0.01 mg/mL streptomycin, Sigma-Aldrich Quimica S.A., Spain) at 38.5 °C, 5% CO2 and saturated humidity. To evaluate the developmental potential of embryos we considered expanding (diameter>134mm) and hatching (cell mass extruding through zona pellucida) blastocysts stages. So the *in vitro* development ability of embryos was assessed on the basis of the blastocyst rate (proportion of expanded blastocyst + hatching blastocyst at 48h of culture from total cultured embryos) and hatched rate (proportion of embryos with more than 50% of mass cell extruded to zona pellucida at 48h of culture from total cultured embryos).

2.4.1.2. mRNA expression of the three core pluripotency factors (OCT4, Nanog and SOX2)

On alternate embryo culture batches, developed embryos were used to mRNA expression determination. Four independent embryo pools were used for each experimental group. After embryo culture till blastocyst (48 hours), polyA RNA was extracted from pools consisting of 13 to 15 embryos of each ovarian stimulation treatment using the Dynabeads kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. Then, reverse transcription was carried out using qScript^{TM}cDNA Synthesis kit (Quanta Biosciences, Beverly, MA, USA) following the manufacturer's instructions.

RNA expression was assessed using real-time polymerase chain reaction (PCR) assay to measure *OCT4*, *NANOG* and *SOX4* mRNA transcript abundance. Real time PCR reactions were conducted in an Applied Biosystems 7500 system (Applied Biosystems). Every PCR was performed from 5-μL diluted 1:10 complementary DNA (cDNA) template, 250-nM of forward and reverse specific primers (Table 1), and 15 μL of Power SYBR Green PCR Master Mix (Fermentas Gmbh, Madrid, Spain) in a final volume of 20 μL. 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. The specificity was confirmed by melting curve analysis.

Relative gene expression was calculated via ΔΔCt method adjusted for PCR efficiency, applying the geometric average of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and the H2A histone family member Z (*H2AFZ*) housekeeping genes as normalization factor [28]. The expression of a cDNA pool from various samples was

used as a calibrator to normalize all samples within one PCR run or between several runs.

2.4.2. Experiment 2: Effects of superstimulation treatment on implantation rate and survival rate at birth

2.4.2.1. Embryo transfer

A total of 324 normal embryos were transferred into 27 recipient females. Ovulation was induced in the receptive females (according to the turgidity and color of the vulva) with 1 µg of buserelin acetate (Hoechst, Marion Roussel, Madrid, Spain) given intramuscularly 72 hours before transfer. Synchronous females were anaesthetized by intramuscular injection of 16 mg of xylazine (Rompún, Bayer AG, Leverkusen, Germany) following intravenous injection of 16-20 mg ketamine hydrochloride (Imalgène, Merial SA, Lyon, France). Oviductal embryo transfer was performed using the laparoscopic technique described by Besenfelder and Brem [31]. The number of embryos transferred per recipient does was from 10 to 13. At the end of the transfer, rabbit does were intramuscular injected with 0.5 mL/doe of enrofloxacin (Baytril 5%, Bayer, Barcelona, Spain) brought back to the flat deck cages, and fed a standard pellet diet *ad libitum*, having free access to water.

2.4.2.2. *In vivo* embryo development and viability rate at birth

Eleven days after ovulation induction, recipient does were laparoscopized and implanted embryos per female were recorded [32]. Animals were anesthetized as described above. Implantation rate was calculated as the successful implantation of the total transferred embryos per each recipient. Survival rate at birth was calculated as the proportion of pups born respect to the embryos transferred per each recipient. At birth, litter size and individual pup weight were recorded.

2.5. Statistical analysis

Embryo donor rate, ovulation rate, number of recovered embryos, recovery and normal embryo development rate were analyzed by ANOVA using a general linear model (GLM) procedure, included the ovarian stimulation treatment as a fixed effect. Also, data of relative mRNA abundance were analyzed by ANOVA using a GLM including as fixed effect the ovarian stimulation treatment group. *NANOG* data were normalized by an Arctangent transformation for its subsequent analysis.

For blastocyst rate, hatched rate, implantation rate and survival rate at birth, a probit link with binomial error distribution was used, including as fixed effect the treatment. Finally, pups weight at birth was analyzed by ANOVA using a GLM including as fixed effect the ovarian stimulation treatment and the covariate litter size at birth. All statistical analyses were performed with SPSS software (SPSS 16.0 software package; SPSS Inc., 2002, Chicago, IL, USA). Results were reported as least-square means (LSM) with standard error of the mean. LSM were separated using Fisher´s protected least significant difference test, with treatment effect declared significant at $P < 0.05$.

3.- Results

3.1. Evaluation of ovarian stimulation treatment on the ovarian response and recovery rates

The ovarian stimulation treatment did not significantly affect ovulation induction, donor does or recovery rates (Table 2). All groups subjected to ovarian stimulation treatments showed a significant increase in ovulation rate related to control group (Table 2). There was not statistical difference among the ovarian stimulation groups with respect to the ovulation and recovery rate and 19 ovarian stimulated does (23.7%), failed to produce

embryos. However, fertilization rate of CTP+LH and FSH groups was lower than control group (Table 2). Regardless of the ovarian stimulation treatment, all groups presented a more than threefold increase in transferable embryos per donor over control group (Table 2).

3.2. Experiment 1: Effects of superstimulation treatment on *in vitro* development and mRNA expression at blastocyst stage

In vitro development was significantly affected by superstimulation treatment (Table 3). Both CTP and FSH resulted in similar rates to blastocyst to those of the control group (Table 3). However, embryos from ovarian stimulated does with CTP showed higher hatched rates compared with the other experimental groups (Table 3).

The pattern of mRNA expression of *OCT4*, *SOX2* and *NANOG* was not significantly affected by superstimulation treatment (Figure 2).

3.3. Experiment 2: Effects of superstimulation treatment on implantation rate and survival rate at birth

The implantation rate and offspring rate at birth were significantly affected by superstimulation treatment. Embryos from ovarian stimulated does with CTP+LH showed similar implantation rates and viability rates at birth to those in control embryos, with both groups presenting higher results than the other experimental groups (Table 3).

The weight at birth was not significantly affected by the ovarian stimulation treatment (59.7±1.9, 52.4±2.0, 54.2±2.3, 55.1±2.6 and 53.8±1.7 g for CTP, CTP+LH, FSH, FSH+LH and Control; respectively).

4.- Discussion

The domestication and breeding experiments led to the establishment of animals with new characteristics which do not depend on competition for survival and are less influenced by environmental factors. This study focused on both the improvement of stimulation treatment and the evaluation of embryonic viability. In the present work, the results indicate that superovulation treatment with the long acting FSH alone or in combination with 10% LH induces a superovulatory response similar to that found when daily FSH is administered. Rabbit does were effectively stimulated to produce more than a fourfold increase in ovulation rate over control animals with all the treatments used. The ovulation rate and number of embryos recovered by donor are similar to or greater than those in the other works previously published. In rabbit, the superovulatory response with FSH treatments seems to be better than that obtained with eCG treatments (ovulation rates from 19 to 56 *vs* 16 to 40, and normal embryos from 12 to 34 *vs* 11 to 24, respectively) [6,8-10,28,29,33]. In general, a common method for induction of superovulation is treatment with a combination of LH and FSH in an attempt to mimic folliculogenesis. Although low dose LH optimizes folliculogenesis through the LH receptor expressed in granulosa cells in larger antral follicles, the addition of high-dose seems to be detrimental for follicular growth [34]. Some authors have shown beneficial effects of LH on ovarian response, oocyte maturation and oocyte/embryo quality [26,28,35]. However, nowadays the use of LH in superovulation treatments is not yet fully understood. In the present study, we evaluated two types of recombinant human FSH alone or supplemented with LH. In contrast to previous studies where the FSH supplementation with LH was studied [28], in the current work the difference in ovarian response observed between treatments with FSH with or

without LH was not significant, although the LH supplemented groups showed higher numbers of ovulated follicles per doe. Previous works with recombinant gonadotropins suggested that the LH window in rabbits seemed to be FSH dose dependent, and the higher the concentration of FSH used, the more the LH window shifts to higher concentrations [28,29]. It is necessary to highlight that ovarian stimulation treatments can also trigger anovulatory processes in some donors, as well as donor ovulation without normal embryos in other cases. In our study, 19 ovarian stimulated does (23.7%), failed to produce embryos, the results being similar to those observed by Mehaisen et al. [9] with eCG and ovine FSH; Salvetti et al. [10] with purified porcine FSH alone or with LH, and higher than Viudes-de-Castro et al. [29] with recombinant human FSH alone or in combination with LH. The lack of ovulation also appears in spontaneous ovulation species treated with gonadotropins [36]. These findings support that the use of exogenous gonadotropins in superovulation could alter the endogenous LH release due to an increase of estradiol and progesterone plasmatic concentration [37-40] and interfere in positive feedback of the estradiol on the LH secretion, blocking the ovulation process. In addition, Holmes et al. [41] reported that high exogenous progesterone values markedly reduced the number of ovaries with ovulated follicles after LH stimulation in rabbits. Similarly, Salvetti et al. [10] observed that the mean of preovulatory follicles in non-ovulated rabbit does was higher than in ovulated does (16.4 and 10.2, respectively), suggesting that the follicle development was carried out by the exogenous gonadotropins but the ovulation mechanism was suppressed.

When ovarian stimulation treatments are applied, both *in vitro* and *in vivo* viability of embryos could be compromised. Exogenous gonadotropins can induce changes in oocyte maturation and metabolism [42] and negative adjustments to fertilization environments and early embryo development, the latter as a consequence of

steroidogenic alterations from anaovulatory and hemorrhagic follicles usually associated with these treatments [8,10,43,44]. Some authors have shown beneficial effects of LH on ovarian response, oocyte maturation and oocyte/embryo quality in women [26,] and rabbits [28], althought the use of LH in combination with FSH in ovarian superstimulation remains controversial. Quality evaluation of oocytes or embryos *in vitro* is usually performed by morphologic criteria at the time of recovery, evaluating its rate of development and/or the expression levels of some genes. The factor octamer-binding 4 (*OCT4*), *NANOG* homeobox (*NANOG*) and Sex determining region Y-box 2 (*SOX2*) are three core pluripotency factors with essential roles in early development and are considered to be key regulators of the pluripotency maintenance system [45], and changes in their expression might trigger failures in the development and implantation of the embryos and, consequently, in pregnancy loss. In the present work, *in vitro* development is accompanied by a similar gene expression profile among the experimental groups, with results similar to nontreated females. These results might suggest no modifications of development patterns or disturbances in the necessary synchrony between uterine environment and embryos. These results corroborate the findings of previous works [28] with recombinant FSH, where no changes in the expression patterns of these genes or in development rates were found in blastocysts derived from 8-16 cell cultured embryos. In cows, porcine FSH has been reported to induce changes in the mRNA profile of genes related to embryo development between *in vivo* superovulated embryos and control embryos, but no differences compared to *in vitro* produced embryos were shown [46]. Furthermore, Chu et al. [47] using ovine FSH observed that although the number of genes influenced seemed low, the mRNA expression profile of potential factors of developmental competence in the bovine oocyte was affected by follicular stimulation.

On the other hand, the *in vivo* results showed that implantation and survival rate at birth were affected by ovarian stimulation treatment. We found that only the CTP with LH supplementation group reached the implantation rate of the control group and similar survival rates, suggesting a higher embryo competence to implant than the other superstimulated groups. The LH supplementation of CTP seems to better mimic the events occurring in non-stimulated does. Superovulation treatments with FSH or CTP alone did not affect *in vitro* embryos development, while absence of LH compromised the *in vivo* viability, showing lower implantation and survival rate, in agreement with the meta-analysis over a population of 6443 women performed by Lehert et al [48], who found an increase in pregnancy rate with LH supplementation versus FSH alone. These findings support the results observed in rabbit when FSH preparations from pituitary or chorionic origin were used. Mehaisen et al. [9] reported that embryos from eCG and ovine FSH showed similar *in vivo* survival rates than non-superovulated embryos (44 to 49%), and Salvetti et al.[10] did not observe differences in birth rate among embryos from porcine FSH with or without LH and non-superovulated groups (49.0, 43.9 and 52.3%, respectively).

The results of the present study suggest that the use of 3 µg/doe CTP in a single injection is enough to superovulate rabbit does without compromising the quantity and quality of embryos. At the same time, the implantation and survival of embryos at birth for CTP supplemented with 10% of LH was similar to that found in control embryos. Therefore, these results offer the possibility of developing new ovarian stimulation strategies associating the long-acting CTP regimen with hCG or a recombinant long-acting LH, reducing the level of distress for the animal and making its application more practical and efficient.

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Table1. 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
SOX4	AGCATGATGCAGGAGCAG	GGAGTGGGAGGAAGAGGT	270
H2AFZ	AGAGCCGGCTGCCAGTTCC	CAGTCGCGCCCACACGTCC	85
GAPDH	GCCGCTTCTTCTCGTGCAG	ATGGATCATTGATGGCGACAACAT	144

Abbreviations: *OCT4*: transcription factor octamer-binding 4; *NANOG*: NANOG homeobox; *SOX2*: sex-determining region Y-box 2; *H2AFZ*: H2A histone family member Z; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Effect of ovarian stimulation treatments on recovery variables (least square mean ± standard error).

Groups	N	Ovulation induction rate	Donor does rate	Ovulation rate	Recovery rate (%)	Normal embryo development rate(%)	Transferable embryos/Doe
CTP	20	0.80 ± 0.09	0.75 ± 0.10	52.9 ± 4.6[a]	81.0 ± 6.0	91.3 ± 5.8[ab]	37.9 ± 4.7[a]
CTP+LH	20	0.90 ± 0.07	0.85 ± 0.08	59.8 ± 4.4[a]	82.1 ± 5.7	84.5 ± 5.5[b]	37.2 ± 4.5[a]
FSH	20	0.85 ± 0.08	0.70 ± 0.10	47.8 ± 4.5[a]	71.3 ± 5.8	77.8 ± 5.8[b]	27.7 ± 4.6[a]
FSH+LH	20	0.85 ± 0.08	0.75 ± 0.10	55.7 ± 4.5[a]	64.6 ± 5.8	93.0 ± 6.0[ab]	35.7 ± 4.6[a]
Control	26	1.00 ± 0.00	0.96 ± 0.38	12.4 ± 3.6[b]	80.1 ± 4.7	99.7 ± 4.6[a]	9.6 ± 3.7[b]

CTP: Corifollitropin α ; CTP+LH: Corifollitropin alfa with a 10% of recombinant human LH; FSH: recombinant human FSH; FSH+LH: recombinant human FSH plus recombinant human LH; Control group: saline solution; N: number of does; Ovulation induction rate: proportion of treated does with corpora lutea; Donor does rate: proportion of donor females with at least one normal embryo; Ovulation rate: number of corpora lutea; Recovery rate: (number of embryos + oocytes recovered/corpora lutea) x 100; Normal embryo development rate: (number of normal embryos/ number of embryos + oocytes recovered) x 100; [a,b]Values in the same column with different superscripts are statistically different ($P<0.05$).

555 Table 3: Effect of ovarian stimulation treatments on *in vitro* and *in vivo* development (least square mean ± standard
556 error).

		In vitro		*In vivo*		
Groups	N	Blastocyst rate	Hatched rate	TE (RD)	Implantation rate	Survival rate at birth
CTP	102	0.98 ± 0.01^{a}	0.75 ± 0.04^{a}	70 (6)	0.63 ± 0.06^{a}	0.53 ± 0.06^{abc}
CTP+LH	89	0.90 ± 0.03^{b}	0.58 ± 0.05^{bc}	77 (6)	0.86 ± 0.04^{b}	0.66 ± 0.05^{ab}
FSH	107	0.99 ± 0.09^{a}	0.62 ± 0.05^{c}	60 (5)	0.60 ± 0.06^{a}	0.43 ± 0.06^{c}
FSH+LH	130	0.89 ± 0.03^{b}	0.51 ± 0.04^{bc}	60 (5)	0.63 ± 0.06^{a}	0.45 ± 0.06^{c}
Control	102	0.98 ± 0.01^{a}	0.47 ± 0.05^{b}	57 (5)	0.81 ± 0.05^{b}	0.75 ± 0.06^{a}

557 CTP: Corifollitropin α ; CTP+LH: Corifollitropin alfa plus recombinant human LH; FSH: recombinant human FSH;
558 FSH+LH: recombinant human FSH plus recombinant human LH; Control group: saline solution; N: number of
559 cultured embryos; TE: number of transferred embryos; RD: number of recipient does; Implantation rate: implanted
560 embryos/total transferred embryos. Survival rate at birth: proportion of pups born respect to the transferred embryos;
561 [a,b,c]Values in the same column with different superscripts are statistically different ($P<0.05$).

562 Figure 1. Experimental design.

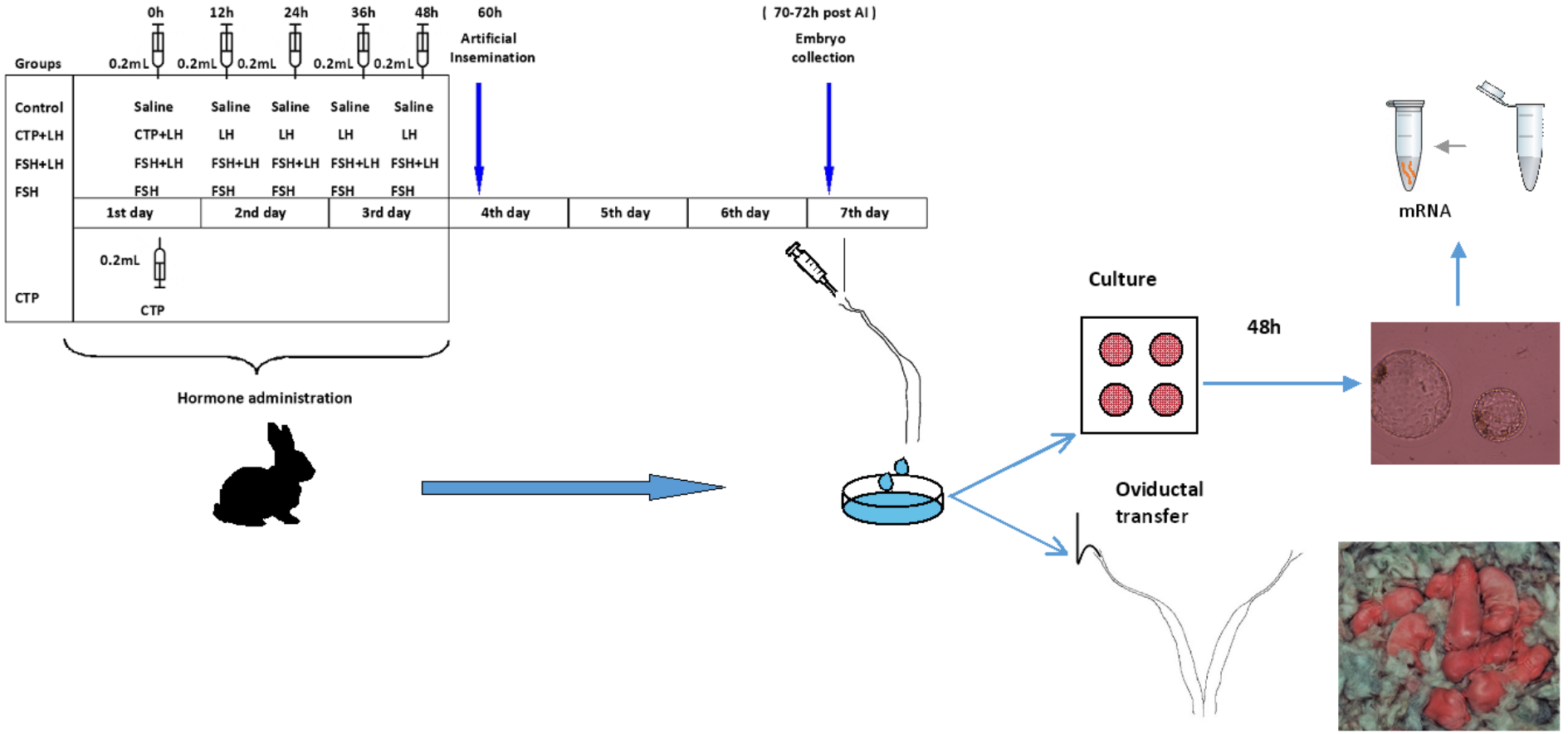

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Figure 2. Analysis of mRNA transcription levels (mean±SEM) for OCT4, SOX2 and NANOG in blastocysts/hatched blastocysts after *in vitro* culture for 48 hours. Values from real-time PCR were normalized to geometric average of H2AFZ and GAPDH.