A SIGLE INJECTION OF CORIFOLLITROPIN ALFA SUPPLEMENTED WITH hCG INCREASE FOLLICULAR RECRUITEMENT AND HIGH-QUALITY EMBRYOS IN THE RABBIT

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Running title: Corifollitropin-α superstimulation with hCG addition

Abstract: Superovulation protocols are designed to achieve maximum embryo yields. Nevertheless, ovarian response control and the quality of obtained embryos is still a challenge. On the other hand, to save the superovulated embryos until their subsequent use, it is usual to cryopreserve them, so it is also crucial to assess their cryotolerance. The aim of this study was to compare the efficacy of a single injection of corifollitropin alfa (FSH-CTP) alone or supplemented with hCG and to determine the impact of this stimulation on in vitro and in vivo development of fresh or devitrified embryos. Our outcomes showed that ovulation rate and recovered embryos were significantly increased when hCG was used. In vitro development of fresh and devitrified embryos and survival at birth was not significantly affected by superstimulation treatment. Results of this study suggest that a single injection of long acting FSH-CTP supplemented with hCG can be effectively used in rabbits to elicit an increase in ovulation rate and number of recovered embryos. Furthermore, we demonstrated that hCG supplementation had no negative effects in embryo cryosurvival and development, showing similar survival rate at birth than FSH-CTP alone group.

Keywords: superovulation, long acting FSH, hCG, embryo viability, vitrification.

INTRODUCTION

Superovulation protocols are designed to ensure the maximum number of transferable embryos per donor. Most of the embryos produced by superovulation are cryopreserved until they are subsequently used, which allows saving embryos for an unlimited time. The human gonadotropins, follicle-stimulating hormone (FSH), luteinizing hormone (LH) and human chorionic gonadotropin (hCG) are commonly used for superovulation in humans and animals. The effectiveness of superovulation treatments with gonadotropins is dependent on maintenance of adequate daily levels of FSH throughout the process. The short elimination half-life and rapid metabolic clearance of the traditional FSH require twice daily treatments, which increases the donor handling and the possibility of errors in giving the treatments. The introduction of corifollitropin alfa (FSH-CTP), a long-acting recombinant FSH, has giving an opportunity to simplify superstimulation protocols, reducing the number of injections and consequently improving the overall donor management. On the other hand, results of superovulation treatments vary, and one of the reasons for this may be the variable LH:FSH ratio. Although LH has essential and well-established roles in ovarian steroid synthesis and ovulation (Chappel and Howles, 1991; Wallach *et al.* 1995; Sen and Caiazza, 2013), 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 (Ruvolo *et al.* 2007, Durnerin *et al.* 2008). Moreover, a high LH level seems to be detrimental for follicular growth. In some clinical studies, where endogenous LH was absent or inactive, recombinant human FSH (rhFSH) alone allowed follicle development but with an inadequate estradiol concentration (Lévy *et al.* 2000) and embryo quality and implantation rates were significantly higher in recipients

of embryos from donors receiving rhFSH in combination with recombinant human LH (rhLH) compared with rhFSH alone (Acebedo *et al.* 2004; Ruvolo *et al.* 2007; Franco *et al.* 2009). It has been controversy over whether rhLH or hCG should be used for superovulation treatments. While hCG has LH-like activity it differs compared with LH in its potency and duration of action (Ezcurra and Humaidan, 2014). Although the α-subunits of LH and hCG show a high degree of similarity, the β-subunit, in the case of hCG, has five glycosylation sites more than that of LH. The extra glycosylation sites give hCG a longer half-life (Le Cotonnec *et al.* 1998; Mannaerts *et al.* 1998). In rabbits, the effect of LH on superovulation has been studied using purified porcine FSH, obtaining highly variable results (Hashimoto *et al.* 2004, Salvetti *et al.* 2007). Our studies with recombinant human gonadotropins (rhFSH either alone or in combination with rhLH) suggested that the window of LH in rabbits is FSH dose dependent (Viudes-de-Castro *et al.* 2015). It seems that the endogenous LH concentration is enough to duplicate the ovulation rate of does treated with low FSH doses (Viudes-de-Castro *et al.* 2009), but is insufficient to increase follicular recruitment when higher doses of FSH are used (Viudes-de-Castro *et al.* 2015). Recently, we evaluated the ovarian stimulation response in rabbit does treated with FSH-CTP versus a traditional rhFSH, either alone or in combination with rhLH (Viudes-de-Castro *et al.* 2017). Results suggested that embryos from does treated with FSH-CTP supplemented with LH reached similar in vivo development as embryos from no superovulated does. Due to the relatively and rapid metabolic clearance of recombinant LH, is a more time-consuming protocol, requiring one daily injections to maintain the threshold level during ovarian superstimulation. Since the circulating half-life of hCG is 80-fold longer than that of LH (Cole, 2010), a single injection of FSH-CTP plus hCG could simplify the superovulation application treatment and reduce the donor distress.

OBJECTIVES

The current study was performed to compare the efficacy of a single injection of corifollitropin alfa (FSH-CTP) combined with hCG versus a FSH-CTP alone to superovulate rabbit does, and to determine the impact of this stimulation combined with embryo cryopreservation on *in vitro* and *in vivo* embryo development.

MATERIALS AND METHODS

Animals and ethical statement

The research was carried out at the experimental farm of the Institute of Science and Animal Technology (ICTA), Universitat Politècnica de València. All animals were handled in accordance with the principles of animal care published by Spanish Royal Decree 53/2013 (BOE 2013) and the Directive 2010/63/EU EEC. The experiments were approved by the Ethics and Animal Welfare Committee of the Universitat Politècnica de València (procedure 2015/VSC/PEA/00061).

Ninety-nine nulliparous rabbit does belonging to a New Zealand White line selected for litter size at weaning were used (Line A, Estany *et al.* 1992). 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.

Hormonal treatment

Ovarian stimulation was induced using Corifollitropin alfa (Elonva, Merck Sharp & Dohme S.A.; Spain) either alone or in combination with hCG (Coriogan, Laboratorios Ovejero S. A.; León, Spain). Rabbit donors, weighing 3.9 to 4.2 kg, were assigned randomly between experimental groups:

-Group FSH-CTP: 40 rabbit does were subcutaneously treated once with 3 µg of Corifollitropin alfa.

-Group FSH-CTP+hCG: 40 rabbit does were subcutaneously treated once with 3 µg of Corifollitropin alfa plus 7.5 UI of hCG.

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.

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 ovulation rate was estimated counting the ovarian follicles with scar under the microscope stereoscope. The number of oocytes and the normal and abnormal embryos were recorded.

Embryo vitrification procedure

Vitrification was carried out in 8 batches. A total of 340 embryos were vitrified and de-vitrified using the methodology described by Vicente *et al.* (1999). The vitrification procedure was carried out in two steps at 20 °C. In the first step, embryos were placed for 2 min in a vitrification solution consisting of 10% (v/v) dimethyl-sulphoxide (1.75M DMSO, Sigma-Aldrich Quimica S.A., Spain) and 10% (v/v) ethylene glycol (2.23 M EG, Sigma-Aldrich Quimica S.A., Spain) in DPBS supplemented with 0.2% (w/v) of BSA. In the second step, embryos were suspended for 1 min in a solution of 20% (v/v) DMSO and 20% EG in DPBS supplemented with 0.2% (w/v) of BSA. Then, embryos suspended in the vitrification medium were loaded into plastic straws (French ministraws, IMV, L'Aigle, France) between two drops of DPBS separated by air bubbles. Finally, the straws were sealed and directly plunged into liquid nitrogen.

De-vitrification procedure was performed by placing the straws to 10 cm from vapour nitrogen until vitrified fraction begin to ice formation (milking aspect 20-30 sec) and thawed by submerging the straws into a water bath at 20 °C for 10 sec. The vitrification medium was removed in two steps. In the first step, the embryos were expelled with the

medium into a solution of DPBS with 0.33M sucrose for 5 min, and in the second step the embryos were washed in a solution of DBPS for another 5 min. Devitrified embryos were scored and only undamaged embryos were catalogued as transferable (334 undamaged embryos, 98.2% transferable embryos).

In vitro culture until blastocyst stage

A total of 390 embryos were cultured (192 and 198 embryos from FSH-CTP and FSH-CTP+hCG group, respectively, of which 173 were fresh and 217 devitrified embryos). Eight embryo culture replicates were performed. Embryos were cultured for 48 h in Tissue Culture Medium 199 (TCM199) plus 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. The *in vitro* development ability of embryos to hatched state (more than 50% of mass cell extruded to zona pellucida) was recorded.

Embryo transfer

A total of 297 embryos were transferred into 19 recipient females (143 and 154 embryos from FSH-CTP and FSH-CTP+hCG group, respectively, of which 117 were fresh and 180 devitrified embryos). 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 (1993). The number of embryos transferred per recipient does was from 12 to 15. 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.

Statistical analysis

Ovulation rate, number of recovered embryos, abnormal embryos, oocytes and blastocysts were analyzed by ANOVA using a general linear model (GLM) procedure including the ovarian stimulation treatment (FSH-CTP and FSH-CTP+hCG) as fixed effect. For ovulation induction rate, development to blastocyst rate, hatching blastocyst rate and survival rate at birth, a probit link with binomial error distribution was used, including as fixed effects the ovarian stimulation treatment (FSH-CTP and FSH-CTP+hCG) and the embryo preservation state (fresh and devitrified). All statistical analyses were performed with SPSS software (SPSS 21.0 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 $P < 0.05$.

RESULTS

Evaluation of ovarian stimulation treatment on the ovarian response and recovery variables

As shown in Table 1, the ovarian stimulation treatment did not significantly affect ovulation induction rate. However, FSH-CTP+hCG group showed a significant increase in ovulation rate and recovered embryos in comparison with FSH-CTP alone group (51.1±2.50 vs 42.5±2.72 ovulation rate and 41.4±2.96 vs 28.2±3.23 recovered embryos for FSH-CTP+hCG and FSH-CTP, respectively; $P<0.05$, Table 1). Nevertheless, there was not statistical difference in the number of abnormal embryos, oocytes and blastocysts between both stimulation treatments.

Evaluation of ovarian stimulation treatment and preservation method on *in vitro* development

Both FSH-CTP and FSH-CTP+hCG treatments resulted in similar development to blastocyst rate, regardless the embryos were fresh or devitrified. Fresh embryos showed higher development to blastocyst rate compared with devitrified embryos (Table 2). However, the hatching blastocyst rate was similar among superovulation or preservation treatments.

Evaluation of embryo viability after transfer

FSH-CTP+hCG treatment showed similar viability rate at birth than FSH-CTP alone. Moreover, embryos of both superstimulation treatments showed the same cryotolerance (Table 2).

DISCUSSION

The FSH-CTP introduction reduces the number of injections needed for ovarian stimulation and permits a simpler approach. FSH-CTP has been used successfully in women, cattle and rabbits (Devroey *et al.* 2009; Fauser *et al.* 2010; Carvalho *et al.* 2014, Boostanfar *et al.* 2015, Viudes de Castro *et al.* 2017; Vicente *et al.* 2018). Although FSH is the main regulator of ovarian follicle growth and maturation, LH induce key changes in both oocyte and follicular cells, that contribute to suitable oocyte competence acquisition, with a prominent role in the process of ovulation and in subsequent fertilization and implantation process. Therefore, it is common that in controlled ovarian stimulation protocols, in an attempt to mimic folliculogenesis, FSH is usually administered in combination with LH. In a previous study by our group (Viudes de Castro *et al.* 2017), when treatments with rhFSH and FSH-CTP alone or supplemented with rhLH were compared, no differences were observed among treatments in the number of transferable embryos. Therefore we found that only the FSH-CTP with LH supplementation group reached the implantation rate of the non stimulated does. Absence of rhLH seemed to compromise the in vivo viability, showing lower implantation and survival rate when FSH-CTP alone was used, suggesting a higher embryo competence to implant on FSH-CTP supplemented with LH group. With an effective half-life of approximately one day, rhLH is suitable for once-daily SC injection, so when FSH-CTP was supplemented with LH, the opportunity to reduce the number of injections and consequently the donor stress in ovarian stimulation protocol disappeared, main advantage of FSH-CTP introduction. Therefore, a more donor-friendly approach should be used. LH and hCG share structural similarities and function thought the same receptor. In addition, hCG exhibits a markedly longer half-life than LH due to the higher number of both glycosylation sites and sialic acid residues (Campbell, 2005). In the current work a single dose of FSH-CTP supplemented with

hCG is more effective in ovarian stimulation than FSH-CTP alone, maximizing the number of transferable embryos recovered and providing similar embryo development rate in vitro and survival rate at birth to that of FSH-CTP alone. In contrast to our previous study with rhFSH and FSH-CTP alone or supplemented with rhLH where no differences in the number of transferable embryos was observed (Viudes de Castro *et al.* 2017). These results are similar to those observed by Drakakis *et al.* (2009) in women, who demonstrated the superiority of hCG over rhLH in ovarian stimulation with rhFSH, showing significantly higher number of transferable embryos, implantation and pregnancy rates when hCG was used. In addition, it seemed that hCG allowed a highly effective and more stable occupancy of LH/hCG receptors than rhLH. Moreover, our results indicate that superovulation treatment using hCG supplementation do not compromise the in vivo embryo cryosurvival, contrary to what was observed in a previous study using rhLH supplementation (Vicente *et al.* 2018). On the other hand, regardless the morphological assessment of the embryo can provide certain clues about the quality, the potential for in vitro development and certainly the survival rate at birth are determinant to identify an effective ovarian stimulation protocol. In the present work, embryos obtained from donors superstimulated with FSH-CTP supplemented with hCG reached a similar developmental rate in vitro and the survival rate at birth to those of FSH-CTP alone. On the other hand, in vitro and in vivo cryosurvival of embryos was not affected by the superstimulation treatment.

Although it is common for ovarian stimulation treatments to trigger anovulatory processes and ovulation of donors without normal embryos, in the present study the percentage of females that did not respond to ovarian stimulation treatment with FSH-CTP supplemented with hCG was very low (5%), while a 20% of females who received FSH-CTP alone treatment were not induced to ovulate. Additionally, of the seventy

females induced to ovulate only two donors did not produce normal embryos, one per treatment.

In conclusion, the use of corifollitropin alfa supplemented with hCG maximize the number of transferable embryos without affecting their cryosurvival rate at birth and reduce the female stress. Therefore, FSH-CTP supplemented with hCG provide an effective and more donor-friendly ovulation stimulation protocol.

Author contributions

MPVC and JSV conceived and supervised the study, analyzed the data and drafting the article, FMJ assisted in the interpretation of data and manuscript development, AMP, XGD and AMT assisted in the acquisition of data and also participated in revising critically the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Table 1: Effect of ovarian stimulation treatment on recovery variables. Results are presented as least square means with standard errors.

TRAITS	FSH-CTP	FSH-CTP+hCG
Number of females	40	40
Ovulation induction rate	0.80±0.063	0.95±0.034
Ovulation rate	42.5±2.72[a]	51.1±2.50[b]
Recovered embryos	28.2±3.23[a]	41.4±2.96[b]
Abnormal embryos	3.2±0.92	3.1±0.85
Oocytes	1.6±0.48	1.3±0.44
Blastocysts	0.5±0.44	0.7±0.40

[a,b] Values in the same row with different superscripts are statistically different (P<0.05).

Table 2: Effect of ovarian stimulation treatment on embryo development. Results are presented as least square means with standard errors.

Experimental group	Development to blastocyst rate		
	n^1	Fresh	Devitrified
CTP	192	0.95 ± 0.026^{a}	0.82 ± 0.044^{b}
CTP+hCG	198	0.99 ± 0.014^{a}	0.79 ± 0.041^{b}
	Hatching blastocyst rate		
	n^1	Fresh	Devitrified
CTP	192	0.54±0.058	0.38±0.056
CTP+hCG	198	0.45±0.050	0.47±0.050
	Survival rate at birth		
	n^2	Fresh	Devitrified
CTP	143	0.60 ± 0.053^{x}	0.39 ± 0.064^{y}
CTP+hCG	154	0.62 ± 0.050^{x}	0.45 ± 0.064^{y}

CTP: 3 µg Corifollitropin □; CTP+hCG: Corifollitropin □ plus 7.5 UI of hCG;

n^1: number of cultured embryos; n^2: number of transferred embryos.

^{a,b} and ^{x,z} values in the same column and row with different superscripts are statistically different (P<0.05).