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

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A B S T R A C T

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.

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1. Introduction

Superstimulation of females from domestic animals is used to augment conventional genetic improvement programs and facilitates advances in reproductive biotechnologies. Rabbit presents great interest as an experimental animal model for biomedical research, genetic modeling, and fur and meat production [1,2]. Superstimulation is considered a fundamental method to establish 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

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the variability in the superstimulation response, affecting the ability of the ovary to respond to exogenous gonadotropin 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 superstimulation response [9–11]. In rabbits, the effect of LH on superstimulation has been studied using porcine FSH, obtaining highly variable results [11,12]. Because of breakthroughs in recombinant technology, it is possible to easily dispose of LH and FSH in an isolated manner, which facilitates the study of the role of LH in ovarian response.

On the other hand, the usual parameters to evaluate embryo quality are the morphologic appearance at recovery or the blastocyst rate after IVC. However, it has been reported that messenger RNA (mRNA) expression is related with the embryo quality [13–18]. In addition, the early steps in embryo development can be affected by the culture media and conditions as well as the production procedure itself, which alters gene expression [13,14,16,19–21]. Therefore, the evaluation of gene expression may help assess the developmental quality of the embryos. It is known that the transcription factor octamer-binding 4 (*OCT4*), NANOG homeobox (*NANOG*), and sex-determining region Y-box 2 (*SOX2*) have essential roles in early development and are considered key regulators of the pluripotency maintenance system [22]. Thus, changes in their expression might trigger failures in embryo development and implantation. The implications of superstimulation experience acquired in animal studies and its possible short-term and long-term consequences should be taken into account when proposing gonadotropin treatment to increase the number of oocytes or embryos produced for reproductive biotechnologies.

This study aimed to evaluate the relative importance of LH in ovarian stimulation with recombinant human FSH (rhFSH) and the effect on the development ability of embryos *in vitro* and the expression of transcription factors *OCT4*, *SOX2*, and *NANOG*.

2. Materials and methods

Unless stated otherwise, all chemicals in this study were purchased from Sigma Aldrich Química S.A. (Madrid, Spain).

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 + 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 + 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-μL 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 μL 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 concentration in a Thoma chamber and evaluate both the percentages of normal intact acrosome and abnormal sperm by phase contrast at a magnification of $\times$ 400. Only heterospermic 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-μg buserelin acetate at the same time as the insemination.

2.3. Embryo recovery

Females were slaughtered 38 to 40 hours 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 oviduct with 5-mL Dulbecco's PBS without calcium

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