MASTER INTERUNIVERSITARIO EN MEJORA GENÉTICA ANIMAL Y BIOTECNOLOGÍA DE LA REPRODUCCIÓN

Effect of ovarian stimulation treatments on the embryo quality in rabbit

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ABSTRACT

Superovulation in animals is used to produce a maximum number of transferable embryos per donor, in order to support both genetic improvement programs and *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, its administration mode, the donor and the environment. Nowadays the use of gonadotropins such as FSH, LH, hCG and eCG has enabled us to achieve the superovulation in different species successfully. However, the posology and the effect of both gonadotropins when used simultaneously (e.g. FSH and LH) it is not yet clearly defined. The superovulation in rabbit does and in other species, has had a great advance in recent years, changing the eCG use by the recombinant gonadotropins and jumping the morphological classification to the molecular analysis of embryos. The objective of this study was to evaluate the effect of long-acting FSH-CTP and rhFSH alone or supplemented with rhLH on ovarian stimulation in rabbit does and determine the impact of its stimulation on *in vitro* and *in vivo* development of fresh and cryopreserved embryos.

Our outcomes showed that both the ovulation rate and the mean of recovered transferable embryos per donor were similar among the different ovarian stimulation groups (FSH-CTP, FSH-CTP+rhLH, rhFSH, rhFSH+rhLH), but much higher than the control group.

In vitro viability of superovulated embryos from FSH-CTP group was similar to the embryos from the control group until blastocysts stage at 48 hours, but it was higher at expanded blastocyst stage for FSH-CTP group in relation to the control group at 48 hours. The embryo development at any stage was affected by the vitrification process. However, it seems that ovarian stimulation did not affect the SOX2 and OCT4 gene expression. NANOG gene overexpression was observed in embryos from FSH-CTP, FSH-CTP+rhLH and rhFSH in concordance with the higher *in vitro* embryo development of these groups.

In vivo viability of embryos from both FSH-CTP and FSH-CTP+rhLH experimental groups was similar to the embryos viability from the control group. The *in vivo* embryo viability at implantation stage was affected by the vitrification process, but foetal losses and birth rate was not affected. Nevertheless, it seems that the vitrification did not affect the *in vivo* viability of embryos from FSH-CTP and rhFSH groups in relation to the control group.

There was no difference in the litter size and weight at birth and weaning among the experimental groups. The vitrification did not affect the litter size at birth and weaning but the pup weight who came from the vitrified embryos was five per cent higher than the one in the fresh groups. By contrast, weight at weaning was lower than the fresh group.

In conclusion, the results of this study suggest that the use of FSH-CTP (3 µg/doe) is enough to superovulate rabbit does without compromising the quantity and quality of embryos, either fresh or vitrified. In addition, the use of this hormone reduces the frequency of animal handling, improving the animal welfare too.

Keywords: superovulation, long acting FSH-CTP, rhFSH, rhLH, gene expression, embryo viability, vitrification.

RESUMEN

La superovulación en animales se emplea para producir el máximo número de embriones transferibles por donante, bien con el fin de apoyar a los programas de mejora genética y la conservación *ex situ* o bien para la optimización de otras biotecnologías. A lo largo del tiempo, el uso de esta biotecnología ha mostrado resultados muy variables como consecuencia de varios factores tales como el origen de las hormonas exógenas, el modo de aplicación de las mismas, las hembras donantes y el ambiente. Hoy en día el uso de gonadotropinas como la FSH, LH, hCG y eCG permite conseguir satisfactoriamente la superovulación de hembras de diferentes especies, sin embargo, aún no está claramente definida la posología y la consecuencia de usar dos gonadotropinas conjuntamente (por ejemplo, FSH y LH). Recientemente la superovulación en conejas ha tenido grandes avances, cambiando el uso de la eCG por gonadotropinas recombinantes y evaluando la calidad embrionaria a través del análisis molecular de los embriones, al igual que en otras especies. El objetivo de este estudio fue evaluar el efecto de la FSH-CTP de larga acción y la rhFSH solas o suplementadas con rhLH sobre la estimulación ovárica en conejas y determinar el impacto de dicha estimulación sobre el desarrollo *in vitro* e *in vivo* de embriones frescos y criopreservados.

Nuestros resultados mostraron que la tasa de ovulación fue similar entre los grupos de estimulación ovárica (FSH-CTP, FSH-CTP+rhLH, rhFSH, rhFSH+rhLH), al igual que la media de embriones transferibles recuperados por donante.

La viabilidad *in vitro* de los embriones procedentes del tratamiento con FSH-CTP fue similar al grupo control hasta el estadio de blastocisto a las 48 horas, sin embargo, se observó un mayor número de blastocistos expandidos en el grupo FSH-CTP frente al control. Sin embargo, parece que la estimulación ovárica no afectó la expresión de los genes SOX2 y OCT4, mientras que hubo una sobreexpresión del gen NANOG, en concordancia con el mayor desarrollo in vitro de los embriones de los grupos FSH-CTP, FSH-CTP+rhLH y rhFSH.

El proceso de vitrificación afectó el desarrollo *in vitro* e incrementó la expresión génica de SOX2, OCT4 y NANOG en los embriones. Sin embargo, parece que la vitrificación no afectó la viabilidad *in vivo* de los embriones de los grupos FSH-CTP y rhFSH.
La viabilidad *in vivo* de los embriones de los grupos experimentales FSH-CTP y FSH-CTP+rhLH fue similar al grupo control.

El proceso de vitrificación afectó a la viabilidad de los embriones hasta la implantación, pero las pérdidas fetales y la tasa de partos no se vieron afectadas. Sin embargo, la vitrificación no afectó a la viabilidad in vivo de los embriones provenientes de los tratamientos FSH-CTP y rhFSH en relación al grupo control.
El tamaño y peso de la camada fue similar entre los distintos grupos de superovulación estudiados. El proceso de superovulación no afectó al tamaño de camada al nacimiento y destete. Sin embargo, mientras que el peso al nacimiento de los gazapos provenientes de embriones vitrificados fue un cinco por ciento mayor que los provenientes de embriones frescos, en el momento del destete su peso fue menor.

En conclusión, los resultados de este estudio sugieren que el uso de FSH-CTP (3 µg/coneja) es suficiente para superovular conejas sin comprometer la cantidad y la calidad de los embriones, ya sean frescos o vitrificados. Además, el uso de esta hormona reduce el manejo de los animales, mejorando el bienestar animal.

Palabras clave: superovulación, FSH-CTP de acción prolongada, rhFSH, rhLH, expresión génica, viabilidad embrionaria, vitrificación.

INDEX

INDEX OF TABLES

INDEX OF FIGURES

1. INTRODUCTION

Superovulation is the artificially induced release of a larger-than-normal number of oocytes from the ovary promoted by exogenous hormones administration (Gibbons et al. 1981, and Park et al. 2015). In animals it is common to use this reproductive technology to produce the maximum number of transferable embryos per donor (Taylor et al. 2007) in order to support both genetic improvement programs and *ex situ* conservation, or to optimize other biotechnologies such as culture, cloning, cryopreservation or transgenesis (Salvetti et al. 2007, and Viudes-de-Castro et al. 2009).

Despite extensive research efforts in the past 60 years, variability in the superovulation response among individuals is the major problem in all species. Diverse factors influence the success of superovulation such as the gonadotropin preparation, its administration mode, the ovarian physiological status, the donor and the environment (Taylor et al. 2007). 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.

1.1. Gonadotropin biochemistry

Follicle-stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG) and equine chorionic gonadotropin (eCG) are members of the gonadotropin family. They are glycoprotein hormones which are characterized by a heterodimeric structure composed of α-subunit no-covalently linked to a specific β-subunit. The α-subunit is common to all gonadotropin family members and contains 92 amino acid residues, with five disulphide bonds contributing to its tertiary structure. The β-subunit is specific for each hormone although they all show some degree of homology because they bind to a common α -subunit. LH and hCG share common biological properties and their β-subunits possess a high degree of sequence homology (Combarnous 1988, and Ryan et al. 1988).

The FSH and LH are secreted by the pituitary gland, while hCG and eCG are secreted by the placenta in pregnant women and mares, respectively. There are pharmaceutical companies that produce and distribute eCG, hCG, FSH from porcine, ovine, equine or bovine pituitary and recombinant FSH and LH (Leao and Esteves 2014).

1.1.1. Equine chorionic gonadotropin (eCG)

eCG has a high affinity for both LH and FSH receptors in the ovaries and stimulates follicular growth and estradiol and progesterone secretion.

eCG is characterized by a heterodimeric structure composed by a common α-subunit (15 kDa, 96 amino acids) non-covalently linked to a specific β-subunit (30 kDa), consists of 149 amino acids, and it has the most heavily glycosylated carboxyl terminal peptide region among all known hormones. These residues are responsible of its prolonged plasma half-life (McIntosh et al. 1975, Sugino et al. 1987, and Murphy and Martinuk 1991), causing an extended ovarian stimulation which may provoke associated problems such as anovulated follicles, abnormal endocrine profiles, disturbing oocyte and embryo transport and reduced embryo quality (Moor et al. 1984). Additionally, the use of repeated treatment with high doses of the eCG has shown to induced a humoral immune response (Baruselli et al. 2011).

1.1.2. Human chorionic gonadotropin (hCG)

hCG is the major human pregnancy glycoprotein hormone and the first hormonal message from the placenta towards the mother, contributing to maternal recognition of the developing human embryo at about 6-8 weeks of pregnancy (Cole 2010). It is produced by the trophoblast of the embryo and by the villous syncytiotrophoblast of the placenta during pregnancy (Fournier et al. 2015), and the maternal concentration and glycan structure change along this period. Indeed, hCG and its free β-subunit are detectable in maternal blood as early as two days after implantation and is used in pregnancy diagnosis. Their levels increase until reaching a peak at about 10 - 12 weeks of pregnancy and then decrease gradually, while its free α subunit increases progressively up to term (Jaffe et al. 1969).

Human Chorionic Gonadotropin is a highly glycosylated molecule of 57 kDa. The seventy per cent of its structure is represented by the peptide, while the other 30% is carbohydrate residue (De Medeiros and Norman 2009). hCG is composed by a non-covalently α-subunit (92 amino acids) and the β-subunit (134 amino acids) which has two sites of N-glycosylation and four sites of O-glycosylation (Figure 1), that gives a half-life greater than other gonadotropins, several days versus hours (De Rensis et al. 2010) (Rull et al. 2008, and Kumar and Sharma 2014). In domestic animals, hCG has a wide range of applications. It is used for ovulation induction in horses (Ginther et al.

2009), rabbits (Mehaisen et al. 2006), sheep and goats (Wani et al. 1997), and also in fish (Kahkesh et al. 2010).

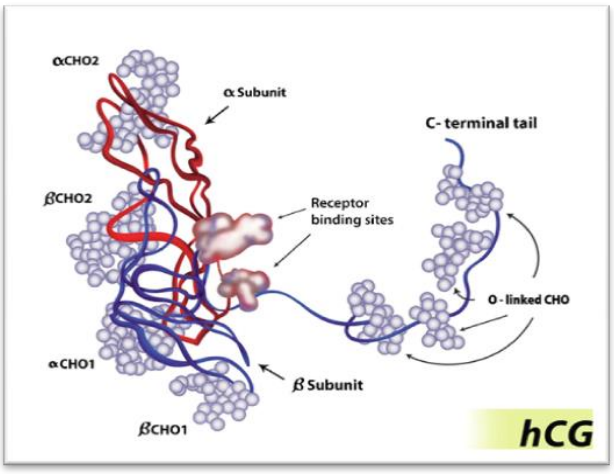

Figure 1. human Chorionic Gonadotropin molecule. The α and β-subunits are represented by red and blue strands, respectively, whereas the carbohydrate chains are represented by light blue balls (Leao and Esteves 2014).

1.1.3. Follicle-stimulating hormone (FSH)

FSH stimulates follicular development in the ovary. This hormone has two subunits, the α and β-subunit. The α-subunit has 92 amino acids, while the β-subunit has 111-112 amino acids (Figure 2) (Duijkers et al. 2002). Most molecular weight estimates for FSH are around 33 kDa. (Cupps 1991).

The biological half-life of FSH is a few hours (about 4 or 5 hours or less in cows) and taking this into account it is possible to obtain a superstimulation response with the administration of FSH twice daily over 3 – 4 days, gaining follicular growth only with this hormone (Loumaye et al. 2003, and Bó and Mapletoft 2014). Pituitary-derived FSH presents some drawbacks, such as the contamination with other hormones (LH) which could cause the premature activation of the oocytes (Moor et al. 1984) and variations within and among batches. In addition, the repeated use of exogenous FSH might induce a humoral immune response, at least in goats, rabbits and marsupials (Viudes-de-Castro et al. 2009, Hesser et al. 2011, and Carvalho et al. 2014).

Recent studies in cows reported that lengthening the FSH treatment protocol to 7 days and maintaining the amount of FSH administered, improve the results by 2.5 times more transferable embryos per animal than the regular 4-days FSH treatment protocol (Dias et al. 2013). However

many practitioners prefer decreasing FSH dose schedules (Bó and Mapletoft 2014), and are also searching for other protocols requiring less handling (Carvalho et al. 2014).

An alternative to the pituitary-derived FSH, which has been made available by biotechnology, is the recombinant FSH. The use of this hormone might reduce the variation of the pituitary-derived FSH (Hesser et al. 2011), and when the exogenous recombinant FSH is from the same species, it may prevent the humoral immune response and transmission of diseases across species (Carvalho et al. 2014).

However, in some clinical studies where endogenous LH was absent or inactive, recombinant FSH alone allowed follicle development but with an inadequate estradiol concentration (Lévy et al. 2000). Thus, it seems that FSH and LH are both essential in the process of folliculogenesis and steroidogenesis.

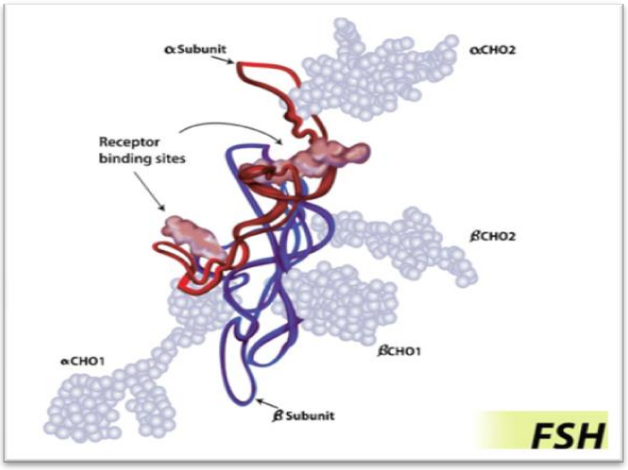

Figure 2. Follicle-stimulating hormone (Leao and Esteves 2014).

On the other hand, since 2010 the industry has been producing long-acting recombinant FSH (FSH-CTP, corifollitropin alfa) (Leao and Esteves 2014). This kind of recombinant FSH is a novel hybrid molecule developed by combining an □-subunit (rhFSH) with a □-subunit which is the hCG C-terminal peptide (CTP) (Campbell 2005, and Verbost et al. 2011), using site-directed mutagenesis and gene transfer techniques (Fauser et al. 2009). Its longer half-life is due to the hCG CTP, which includes four additional O-linked carbohydrate side chains, each with two terminal sialic acid residues which exclusively interacts with FSH receptors. Corifollitropin alfa has an approximately two-fold longer half-life (65 hours plasma half-life) and an almost four-fold extended time to peak

serum levels (Duijkers et al. 2002, and Devroey et al. 2009). Therefore a single injection can replace the pharmacokinetic profile of first seven daily standard gonadotropin injections and support multi-follicular growth for an entire week (Balen et al. 2004, and Devroey et al. 2009).

1.1.4. Luteinizing hormone (LH)

Luteinizing hormone has essential and well-established roles in ovarian steroid synthesis and ovulation (Shoham et al. 1995, Fischer 2007, and Sen and Caiazza 2013). LH is a heterodimeric glycoprotein with a molecular mass of 28 kDa, comprising an α (92 amino acids) and a β-subunit (Figure 3). The β-subunit consists of 120 amino acids with important changes in the glycosylation regulating bioavailability and function (Ezcurra and Humaidan 2014).

It exists a compilation of isoforms that differs with respect to post-translational modification (predominantly glycosylation) and state of degradation, which influences their biological activity and half-life in circulation (Bergendah and Veldhuis 2001). Indeed, LH has an essential function on follicles development and controls the hormonal production through the steroid pathway (Loumaye et al. 2003), stimulating the biosynthesis of androgens, which are the substrate for estradiol (Hillier 2001). Thus the ovarian steroidogenesis can be driven by activation of a low number of LH receptors (around 1%) (Doody et al. 2010)

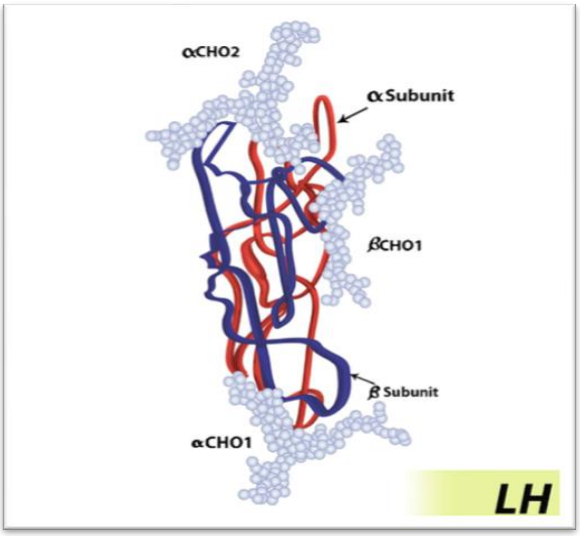

Figure 3. Luteinizing hormone (Leao and Esteves 2014).

Several clinical studies in women have shown that LH might intensificate granulosa cells FSH sensitivity by increasing androgen synthesis during the early stage of folliculogenesis (Durnerin et al. 2008). It becomes clear that some LH activity is required for a normal follicle and oocyte development (Ruvolo et al. 2007, and Franco et al. 2009).

Historically, superstimulation protocols have included both LH and FSH in an attempt to mimic the folliculogenesis. However, nowadays the use of LH in superovulation treatments is controversial and unclear. Although low-dose LH optimizes the folliculogenesis through the LH receptor expressed on granulosa cells in larger antral follicles, the addition of high-dose seems to be detrimental for follicular growth (Hillier 1994).

1.2. Rabbit reproductive physiology

The short reproductive cycle and the precise timing of ovulation of rabbit provide advantages for documenting the moment of embryo development, apposition and attachment in maternal uterus and facilitate the evaluation of nutritional, environmental or pharmaceutical assays on reproduction (Hoffman et al. 1998, Tao and Niemann 2000, and Lee and DeMayo 2004).

Female rabbits are considered as induced ovulators, because ovulation takes place after mating (Bakker and Baum 2000). Hence, rabbits do not have a regular cycle as other mammals (Harkness et al. 2010). Rabbits display oestrous at 4 - 6 days intervals (Milligan 1982). The receptive sexual behavior is consequence of the estrogen, which acts on the brain (Bakker and Baum 2000). In this stage, the rabbit females show different signs, as lordosis at the male presence (Theau-Clément et al. 2005) or red vulva color (Rodriguez and Ubilla 1988). When the follicles degenerate, the secretion of estrogen decrease and rabbit does enter into a non-receptive period (Harcout-Brown 2002).

When mating occurs, genital somatosensory receptor is stimulated (Bakker and Baum 2000) which evoked signals funnel, via neural pathways along the spinal cordon (Lin and Ramirez 1991), resulting in a massive release of GnRH with peaks within 1 – 2 h after mating. That is possible because the coital signal in the rabbit females involves sequential events in the brainstem and hypothalamus (Centeno et al. 2004), after that, the preovulatory LH surge takes place, provoking the ovulation (11 – 12 h after mating) (Bakker and Baum 2000).

Rabbit female reproduction requires a finely tuned coordination of the brain, pituitary, and ovaries to control folliculogenesis, oestrous cycle, sexual behavior, and ovulation via a series of complex feedback mechanisms (Bakker and Baum 2000, and Boiti et al. 2006).

On the other hand, after ovulation induction in rabbits, if the pregnancy does not occur, a particular phase, called pseudopregnancy, takes place in the female. The corpora lutea reached maximum size 10 -12 days after mating, when the serum progesterone concentration reaches the high level, at day 14 begins the luteal regression which is completed around day 18, when progesterone declines to basal value (Browning et al. 1980). Prostaglandin F2 α (PGF2α) secretion by the uterine endometrium reaches its highest level at day 17 of pseudopregnancy (Lytton and Poyser 1982).

In the rabbit, the sperm is deposited in the vagina. As in other mammals, fertilization occurs in the ampulla about 14 hours after mating or insemination. The stages of embryo development prior to implantation are influenced by the oviductal and uterine environments. In the first 68 – 72 h embryo segmentation occurs and blastocyst formation begins. Around the third day of development, the embryo reaches the uterine horn, and in the following three days, before initiating the attachment process, blastocysts cause uterine decidual reaction (6 – 7 days). Subsequently, rabbit trophectoderm creates large knob-like projections that adhere and fuse to the apical epithelial surface (Hoffman et al. 1998). At this moment, a chorioallantoic placenta begins its development and finishes around twelve days of gestation (Adams, 1965).

In contrast to other species, when the rabbit embryo grows and reaches the blastocyst stage does not detach itself from its zona pellucida, due to another external cover, called mucin coat. This mucoprotein cover, acquired in the oviduct during tubal passage, is constituted by sulphated glycoproteins, and its function has been related to immunological response (Lopez, 1995). At 4.5 days post coitum during blastocyst expansion, the mucin coat and the zona pellucida naturally distend and thin out, being replaced by a new innermost layer called neozona. Finally, another layer derived from uterine secretion and called gliolemma is deposited outsite of neozona (Denker 2000). It has been suggested an important role to the coats in the positioning and local signalling in the context of implantation initiation (Denker 2000). Also, it has been observed that the absence of these covers in rabbit blastocysts hind pregnancy after transfer. Seven days post coitum antimesometrial expansions are observed, like small translucent bumps (decidualization), which are each of the embryos. Between 10 and 17 days post-coitum they will be established and will develop a hemicorial chorioallantoic

placenta (Hoffman et al. 1998)**.** The birth takes place 30-32 days post-coitum, depending on the number of fetuses gestated and the genetic lineage (Figure 4).

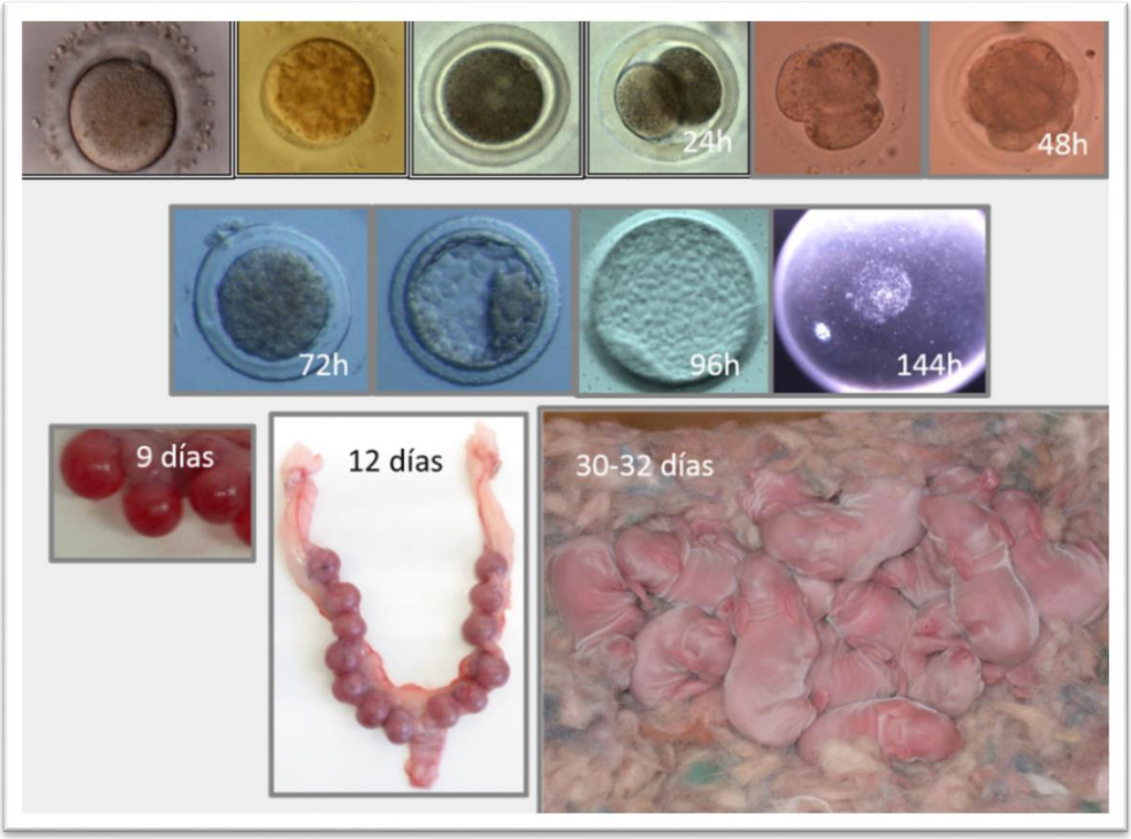

Figure 4. Embryo and foetal development in rabbit.

1.3. Manipulation of rabbit ovarian function and consequences

In rabbits, in order to ensure the maximum number of normal embryos recovered per donor, both eCG and pituitary derived FSH have been commonly used to induce superovulation (Kanayama et al. 1994, Kauffman et al. 1998, Hashimoto et al. 2004, Mehaisen et al. 2005, Mehaisen et al. 2006, and Salvetti et al. 2007). More recently, recombinant human LH and FSH have been used (Viudes-de-Castro et al. 2009 and 2015, and Cortell et al. 2010). The eCG prolongs plasma half-life and can negatively affect embryonic development (Taneja et al. 1990). The use of FSH has advantages over eCG, but is a more time-consuming protocol due to its shorter half-life. FSH requires to be administered twice daily over 3–4 days to stimulate the same amount of follicular growth that would result from a single injection of eCG (Renard et al 1982; Joly, 1997, and Kauffman et al. 1998).

As shown in the tables 1 and 2, results of superovulation treatments vary, and one of the reasons for this may be the variable LH : FSH ratio when preparations isolated from the pituitary gland are used. In rabbits, the effect of LH on superovulation has been studied using purified porcine FSH obtaining highly variable results (Hashimoto et al. 2004, and Salvetti et al. 2007). More recently, Viudes-de-Castro et al. (2015) using recombinant LH and FSH suggested that the window of LH in rabbits is FSH dose dependent. 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 it is insufficient to increase follicular recruitment when higher doses of FSH are used (Viudes-de-Castro et al. 2015).

On the other hand, several studies in different species have demonstrated an increase in anti-gonadotropin antibodies in animals repeatedly treated with gonadotropins (Swanson et al. 1996, Francois et al. 1999, Van and Davis 2001, combelles and Albertini 2003, Mehaisen et al. 2006, and Viudes-de-Castro et al. 2009).

Table 1. Results of superstimulation treatment induced by eCG in rabbits.

TREATMENT	AUTHORS	DOSES	OI	RR	OR	RE	RO
eCG	López- Bejar and López- Gatius 2000	80 UI	30 UI hCG (IV)	-	-	17.8	-
	Besenfelder et al. 2002	120 UI	180 UI hCG (IV)	-	36.3	24.3	3.2
	Tsiligianni et al. 2004	20 UI/Kg	120 UI hCG (IV)	-	28.7	14.2	4.31
	Mehaisen et al. 2005	50 UI	-	-	15.2	10.1	-
		200 UI	-	-	19.1	8.8	-
	Mehaisen et al. 2006	20 IU/kg	75 UI hCG (IV)	80.6	19.2	14.1	-
	Badawy et al. 2016	50 UI	-	-	20.54	-	-

OI: Ovulation induction, RR: Response rate, OR: Ovulation rate, RE: Recovered embryos, RO: Recovered oocytes.

IV: intravenously

Table 2. Results of superstimulation treatment induced by FSH in rabbits.

TREATMENT	AUTHORS	DOSES	OI	RR	OR	RE	RO
FSHo	Kauffman et al. 1998	6 x 0.5 mg/12h (SC)	150 UI hCG (IV)	-	25.6	19.4	2.25
		8 x 0.5 mg/12h (SC)		-	23.5	23.5	0.75
FSHp	Cheng et al. 1999	6 x 0.3 mg/12h	25 UI hCG/kg	-	28.3	15.5	2.1
FSHo	Mehaisen et al. 2006	3 x 0.6 mg/24h	75 IU hCG (IV)	87.5	25.2	17.7	-
FSHp	Salvetti et al. 2007	5 x 9 µg/12h (IM)	1.6 µg buserelin (IM)	77.8	26.7	21.2	-
FSHp +LHp(20%)				81.3	27.1	20.3	-
rhFSH	Viudes-de-Castro et al. 2009	3 x 25 UI/24h (IM)	2 µg buserelin (IM)	84.94	20.24	15.59	1.62
rhFSH+rhLH(5%)				88.42	18.46	15.93	0.96
rhFSH+rhLH(10%)				77.17	18.41	13.72	0.59
rhFSH	Viudes-de-Castro et al. 2015	18.75 UI/12h (IM)	1 µg buserelin (IM)	74.1	20.4	15.9	-
rhFSH+rhLH(10%)				73.5	20.6	12.2	-
rhFSH		37.50 UI /12h (IM)		79.0	28.2	25.0	-
rhFSH+rhLH(10%)				80.7	41.5	34.0	-

OI: Ovulation induction, RR: Response rate, OR: Ovulation rate, RE: Recovered embryos, RO: Recovered oocytes. FSHo: ovine FSH, FSHp: porcine FSH, rhFSH: recombinant human FSH, rhLH: recombinant human LH. IM: intramuscularly. IV: intravenously. SC: subcutaneously

The usual parameters to evaluate embryo quality are morphologic criteria according to International Embryo Transfer Society classification. Therefore, the evaluation of the expression profile of genes related to embryo development (Viudes-de-Castro et al. 2015) or some factors related to their metabolism (Cortell et al. 2015) may help to assess the quality of the embryos produced by superstimulation protocols.

Several authors have observed that superovulation treatments with high doses of eCG or FSH may generate a higher number of abnormal and immature follicles by increasing the cystic and or

hemorrhagic follicles (García Ximénez and Vicente 1990, Schmidt et al. 1992, Kim et al. 1988, Cheng et al. 1999, Hashimoto et al. 2004). Additionally, these treatments may decrease the embryo recovery rates (Kennelly and Foote 1965, Hafez 1969, and Schmidt et al. 1992), produce cytogenetic defects and chromosomal alterations in embryos (Fechheimer and Beatty 1974, and Paufler et al. 1975), making them more sensitive to cryopreservation protocols and decreasing their potential ability to produce live pups (Mehaisen et al. 2006) and affecting the oocyte metabolism and consequently the future embryo development (Cortell et al. 2015).

Other authors have shown no differences between superovulated and non-treated females on embryo quality, developmental capacity and the expression profile of OCT4, NANOG, and SOX2 genes for both fresh and frozen-thawed embryo (Salvetti et al. 2007, Viudes-de-Castro et al. 2009 and 2015).

2. OBJECTIVES

The current study was performed to evaluate the effect of long-acting FSH-CTP and rh FSH alone or supplemented with rhLH on ovarian stimulation in rabbit does, and to determine the impact of this stimulation on *in vitro* and *in vivo* development of fresh and cryopreserved embryos.

The specific objectives were:

- Comparison of the recombinant human gonadotropins (rhFSH, rhLH and FSH-CTP, Corifollitropin alfa) on the ovarian response, fecundation and embryo recovery rate.
- Evaluation of *in vitro* embryo development and the expression of development pluripotency genes: octamer-binding transcription factor (OCT4), NANOG homeobox (NANOG) and sex determining region Y-box 2 (SOX2).
- Assessment of the *in vivo* viability of embryos derived from the different superovulation treatments.

3. MATERIALS AND METHODS

3.1. Animals an 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 of the Polytechnic University of Valencia (procedure 2015/VSC/PEA/00061).

One hundred sixty-two nulliparous does of 18 – 20 weeks old were used (Figure 5). Does belonged to a New Zealand White line selected for litter size at weaning (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.

Figure 5. Line A rabbit.

3.2. Experimental design: the following scheme shows the experimental work.

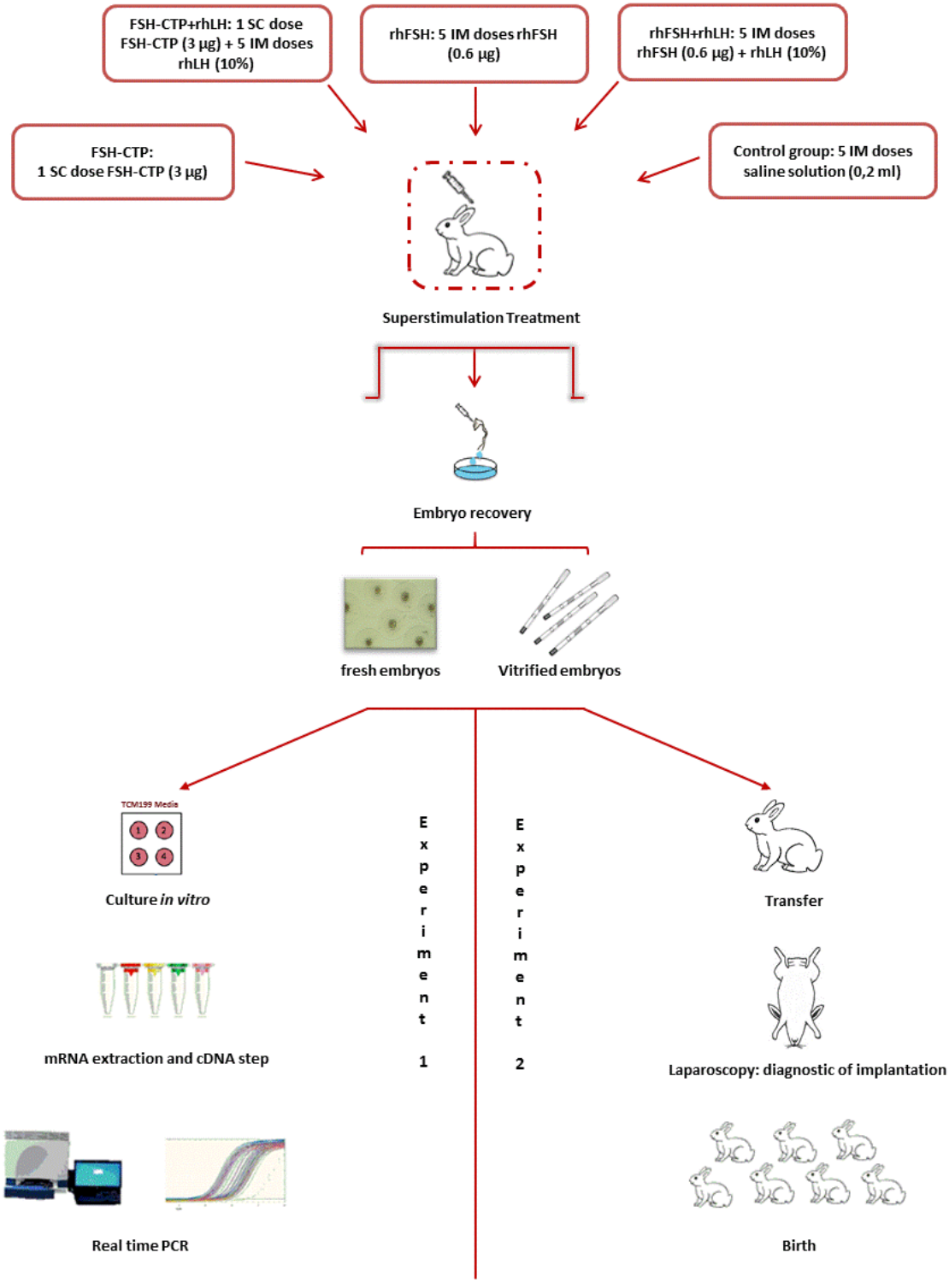

Figure 6. Experimental scheme.

3.3. Hormonal treatment

Ovarian stimulation was induced using Corifoltropin alfa (FSH-CTP; Elonva, 150 microgramos/0.5 ml; Merck Sharp & Dohme S.A.; Spain) alone or in combination with recombinant human LH (rhLH; Luveris 75; Serono Europe Ltd., London, United Kingdom); and recombinant human FSH (rhFSH; Gonal-F 75; Serono Europe Ltd., London, United Kingdom) alone or in combination with rhLH. Rabbit does, weighing 3.9 to 4.2 kg, were assigned randomly to five experimental groups:

1. Group FCT-CTP: 20 rabbit does were subcutaneously treated once with 3 µg of FSH-CTP.
2. Group FSH-CTP + rhLH: 20 rabbit does were subcutaneously treated once with 3 µg of FSH-CTP and intramuscularly treated with a 10% of rhLH distributed in five equal doses at 12-hours interval.
3. Group rhFSH: 20 rabbit does were intramuscularly treated with 3 µg of rhFSH distributed in five equal doses at 12-hours intervals.
4. Group rhFSH + rhLH: 20 rabbit does were intramuscularly treated with 3 µg of rhFSH in combination with a 10% of rhLH distributed in five equal doses at 12-hours intervals.
5. 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 (Line A) 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.

3.4. Embryo recovery

Embryo recovery was carried out in 12 batches (Figure 7). 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, Boivine, (BSA); Solon, USA), 0.133 g/L $CaCl_2$, 0.100 g/L $MgCl_2$, antibiotics (penicillin G sodium 300.000 IU/L, penicillin G procaine 700.000 UI, and dihydrostreptomycin sulfate 1250 mg/L; Penivet 1; Divasa Farmavic, Barelona, Spain) 10 ml/L.

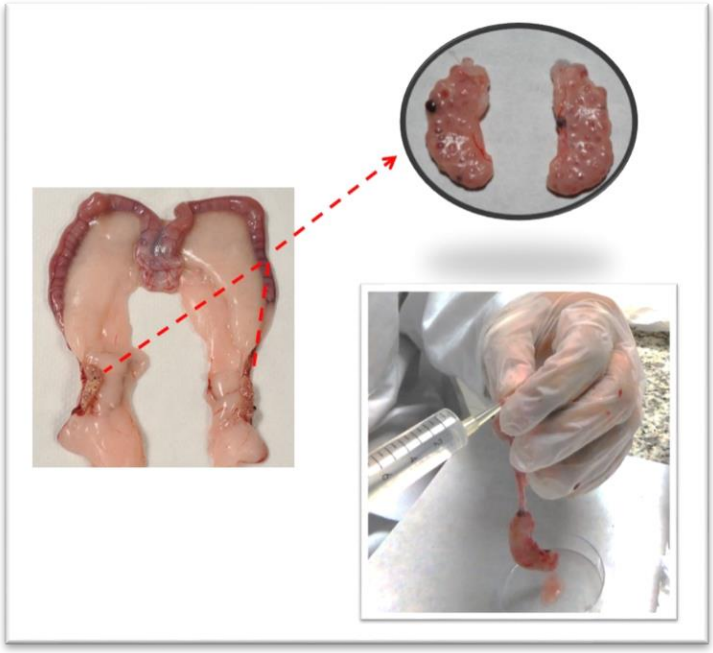

Figure 7. Embryo recovery.

The recovered fluid was collected into sterile Petri dishes (P60) 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 (Figure 8).

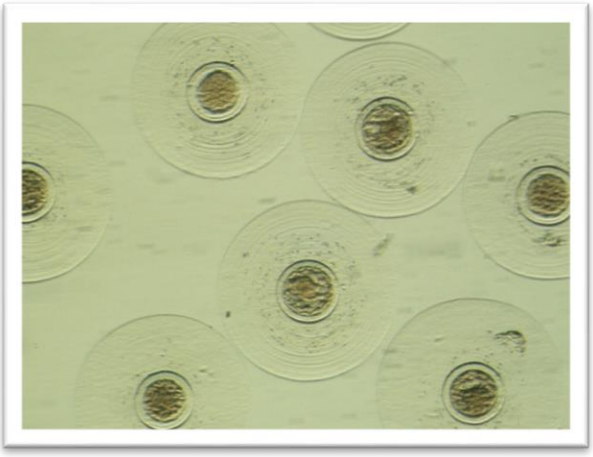

Figure 8. Normal (transferable) embryos.

The ovulation rate, which was estimated counting the ovarian follicles with scar under the microscope stereoscope (Figure 9), the number of oocytes and the normal and abnormal embryos were recorded.

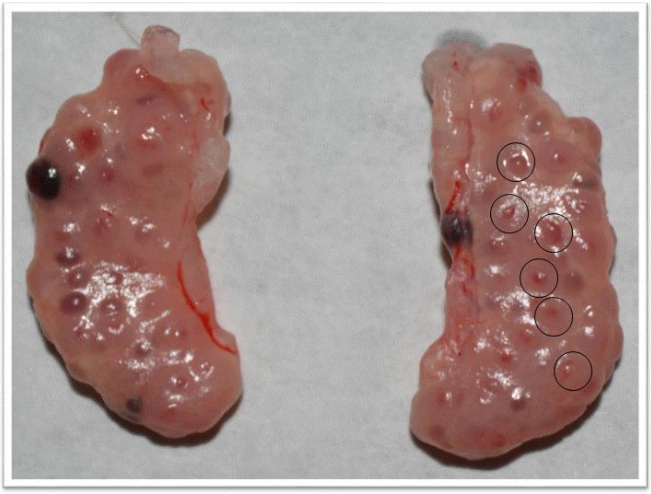

Figure 9. Ovarian follicles with scars.

The recovery rate was calculated as the percentage of relation between number of embryos plus oocytes and the ovulation rate. The fertilization rate was calculated as the percentage of relation between the number of normal embryos and the number of embryos and oocytes recovered.

$$\textbf{recovery rate} = \left({}^{(total\ embryos + oocytes)}/_{ovulation\ rate}\right)$$

$$\textbf{fertilization} = \left({}^{normal\ embryos}/_{(total\ embryos + oocytes)}\right)$$

3.5. Embryo vitrification procedure

Vitrification was carried out in 12 batches. A total of 810 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) and 10% (v/v) ethylene glycol (2.23 M EG, Sigma) 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 0.25 ml plastic straws (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-vitification procedure was performed by placing the French ministraws 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.

3.6. Experiment 1: *In vitro* development and quality of embryos

3.6.1. *In vitro* culture

In vitro culture was performed in 12 batches. A total of 530 fresh and 450 de-vitrified embryos were cultured for 48 h in Tissue Culture Medium 199 (TCM199) + 10% Fetal Bovine Serum (FBS, Sigma-Aldrich Quimica S.A., Spain) and antibiotic (penicillin G sodium 300.000 IU/L, penicillin G procaine 700.000 UI, and dihydrostreptomycin sulfate 1250 mg/L; Penivet 1; Divasa Farmavic, Barelona, Spain) at 38.5 °C, 5% CO2 and saturated humidity.

Both devitrified and fresh embryos were assessed according to their developmental stage at 24 and 48 hours (non-developed, blastocyst, expanding blastocyst and expanded blastocyst) (Figure 10).

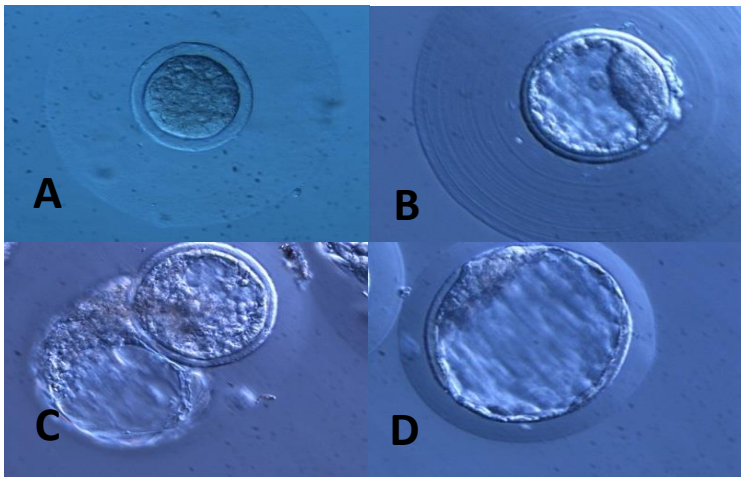

Figure 10. Embryo development.

A. Morula, B. Blastocyst stage, C. Expanding blastocyst and

D. Expanded blastocyst.

3.6.2. 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 instructions. Four independent embryo pools were used for each experimental group. Then, reverse transcription was carried out using qScript^{TM}cDNA Synthesis kit (Quantabio, Beverly, MA, USA), 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 3), and 15 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. The specificity was confirmed by melting curve analysis.

A ΔΔCt method adjusted for PCR efficiency was used, using the geometric average of H2AFZ (H2A histone family member Z (Mamo et al. 2008) and GAPDH (glyceraldehyde- 3-phosphate dehydrogenase (Santos et al. 2004) as a housekeeping normalization factor (Weltzien et al. 2005).

Relative expression of cDNA pool from all samples was used as the calibrator to normalize all samples within one PCR run or between several runs.

Table 3. List of primers used for quantitative real-time polymerase chain reaction.

Gene symbol	Forward primer	Reverse primer	Fragment (bp)
H2AFZ	AGAGCCGGCTGCCAGTTCC	CAGTCGCGCCCACACGTCC	85
GAPDH	GCCGCTTCTTCTCGTGCAG	ATGGATCATTGATGGCGACAACAT	144
OCT4	CGAGTGAGAGGCAACTTGG	CGGTTACAGAACCACACACG	125
NANOG	CCAGGTGCCTCTTACAGACA	TCACTACTCTGGGACTGGGA	104
SOX4	AGCATGATGCAGGAGCAG	GGAGTGGGAGGAAGAGGT	270

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

3.7. Experiment 2: *In vivo* survival

3.7.1. Embryo transfer

Embryo transfer was carried out in 14 batches. A total of 721 embryos (324 non-vitrified and 397 vitrified embryos) were transferred into 56 recipient females. Ovulation was induced in the receptive females (according to the turgidity and colour 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 (Figure 11) 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, and fed a standard pellet diet *ad libitum*, having free access to water.

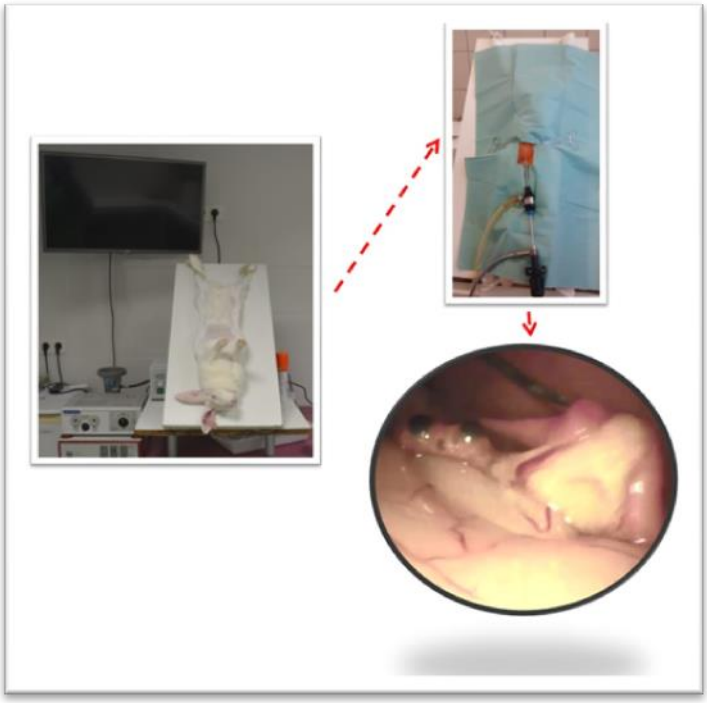

Figure 11. Transfer steps on the recipient rabbit doe.

3.7.2. Embryo survival rate and fetal losses

Eleven days after ovulation induction, recipient does were laparoscopized and the number of corpora lutea and implanted embryos per female were recorded. Animals were anesthetized as described above.

In vivo implantation rate was calculated as the successful implantation of the total transferred embryos per each recipient. The fetal losses were calculated as the proportion of implanted embryos that did not finish gestation.

3.8. Statistical analysis

Embryo donor rate, ovulation rate, number of recovered embryos, recovery and fertilization rate were analyzed by ANOVA using a general linear model procedure, included the treatment as a fixed effect. In order to analyse the litter size and pup weight at birth and at weaning, the stimulation treatment, the type of embryos (vitrified and fresh) and their interaction were included as fixed effects. Moreover, the following covariates were introduced in the models:

- Embryos transferred to analyse litter size at birth.
- Litter size at birth to analyse birth weight.
- Litter size at birth and weaning and age at weaning to analyse weaning weight.

For developmental rate at blastocyst and expanded blastocyst, implantation, survival rate at birth and foetal losses, a probit link with binomial error distribution was used, including as fixed effect the treatment and the type of embryos and their interaction.

Data of relative mRNA abundance were analysed using a general linear model including as fixed effect the treatment, the type of embryos and their interaction. NANOG data were normalized by an Arctangent transformation for its subsequent analysis.

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 $P < 0.05$.

4. RESULTS

4.1. Evaluation of ovarian stimulation treatments on the ovarian response and recovery variables

The effects of ovarian stimulation treatment on the recovery variables are shown in Table 4. The stimulation treatment did not significantly affect ovulation induction, donor does neither recovery rates. All groups subjected to ovarian stimulation treatments showed a significant increase in ovulation rate and number of transferable embryos related to control group (Table 4 and Figure 12). There was not statistical difference among the ovarian stimulation groups with respect to the ovulation and fertilization rate. However, fertilization rate of donor does treated with FSH-CTP+rhLH and rhFSH had lower percentage of embryos than control group.

4.2. Experiment 1. *In vitro* development and quality of recovered embryos

4.2.1. Evaluation of *in vitro* embryo development at 24 and 48 hours

The rate of embryos reaching blastocyst or expanded blastocyst stage at 24 and 48 hours was affected by both ovarian stimulation treatments and embryo type (vitrified and fresh) (Figure 13, 14 and Table 5). The embryo development at any stage was significantly lower for rhFSH+rhLH group. Embryos from FSH-CTP group showed significantly higher expanded blastocyst rate at 24 and 48 hours than control and rhFSH+rhLH embryos, but similar than FST-CTP+rhLH and rhFSH groups (Figure 13 and 14).

Embryo development at any stage was significantly lower for vitrified group at 24 and 48 hours (Table 5). In addition, the interaction between ovarian stimulation treatment and type of embryo was observed to blastocyst rate at 48 hours. While in others stimulation treatments fresh embryos had the best rate, FSH-CTP+rhLH treatment showed similar blastocyst rate between fresh and vitrified embryos (Figure 15).

Table 4. Effect of ovarian stimulation treatments on recovery variables.

(least square mean ± standard error)

Groups	N° does	Ovulation induction rate (n)	Donor does rate (n)	Ovulation rate	Recovery rate	Fertilization rate
FSH-CTP	20	0.80 ± 0.089 (16)	0.75 ± 0.097 (15)	52.9 ± 4.62[a]	81.0 ± 6.0	91.3 ± 5.8[ab]
FSH-CTP+rhLH	20	0.90 ± 0.067 (18)	0.85 ± 0.080 (17)	59.8 ± 4.35[a]	82.1 ± 5.7	84.5 ± 5.5[b]
rhFSH	20	0.85 ± 0.080 (17)	0.70 ± 0.102 (14)	47.8 ± 4.49[a]	71.3 ± 5.8	77.8 ± 5.8[b]
rhFSH+rhLH	20	0.85 ± 0.080 (17)	0.75 ± 0.097 (15)	55.7 ± 4.49[a]	64.6 ± 5.8	93.0 ± 6.0[ab]
Control	26	1.00 (26)	1.00 (26)	12.4 ± 3.63[b]	80.1 ± 4.7	99.7 ± 4.6[a]

(n): number of does.

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

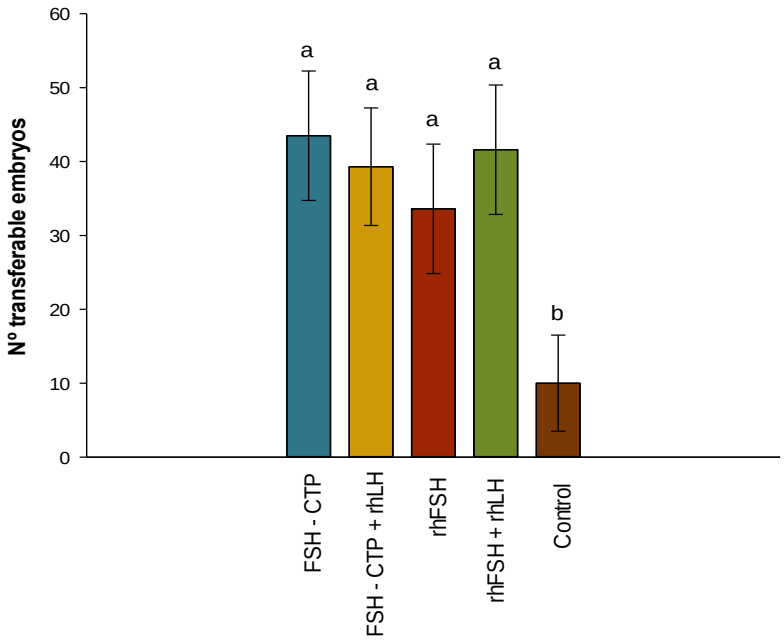

Figure 12. Transferable embryos recovered by donor doe.

[a,b]Values on the bars with different superscripts are statistically different (P<0.05).
(least square mean ± standard error).

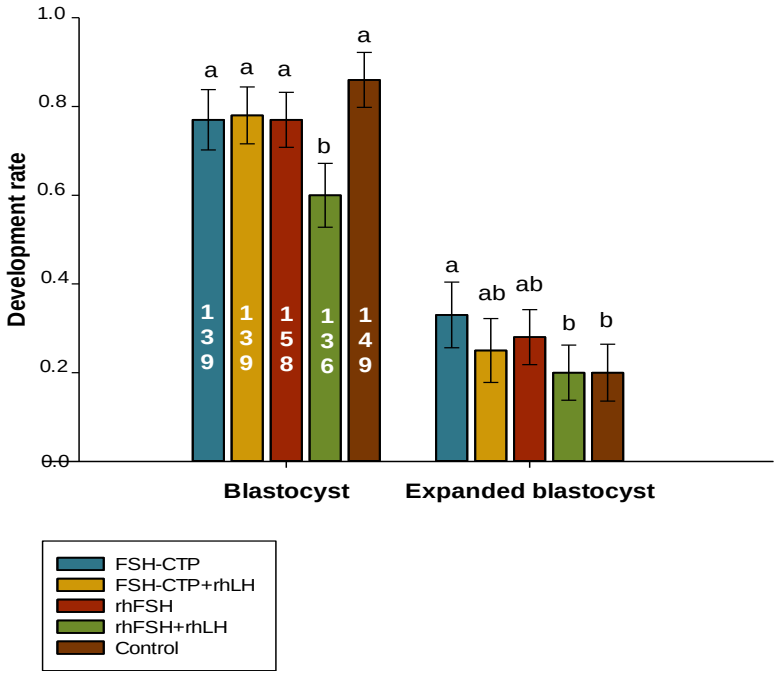

Figure 13. In vitro development embryos at 24 hours.

[a,b]Values at the same embryo stage with different superscripts are statistically different (P<0.05).
number into the column: number of blastocyst at 24 h.
(least square mean ± standard error)

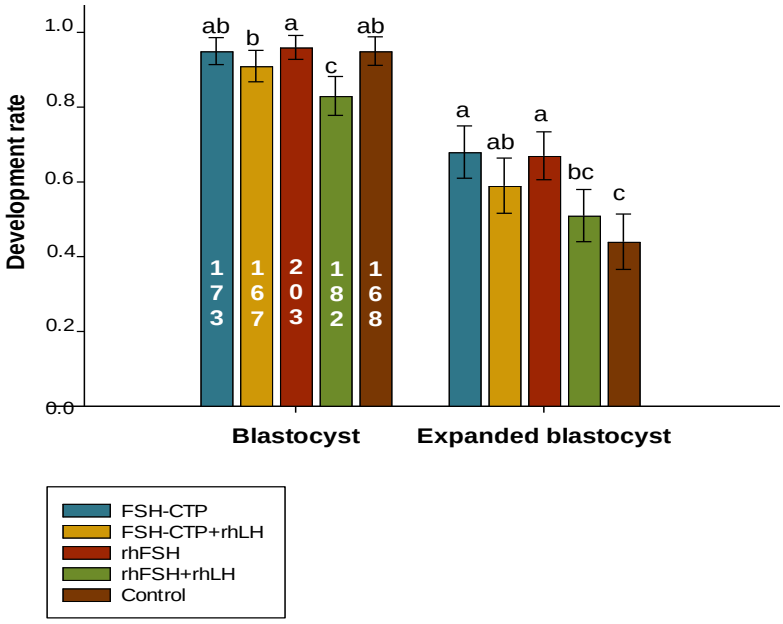

Figure 14. In vitro development embryos at 48 hours.

[a,b,c]Values at the same embryo stage with different superscripts are statistically different (P<0.05).
number into the column: number of blastocyst at 48 h.
(least square mean ± standard error)

Table 5. Effect of Embryo type on in vitro development.

(least square mean ± standard error)

Embryo type	cultured embryos	24 Hours		48 Hours	
		Blastocyst	Expanded blastocyst	Blastocyst	Expanded blastocyst
Vitrified	450	0.56 ± 0.024[b]	0.12 ± 0.016[b]	0.87 ± 0.016[b]	0.50 ± 0.024[b]
Fresh	530	0.90 ± 0.014[a]	0.42 ± 0.022[a]	0.96 ± 0.009[a]	0.65 ± 0.021[a]

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

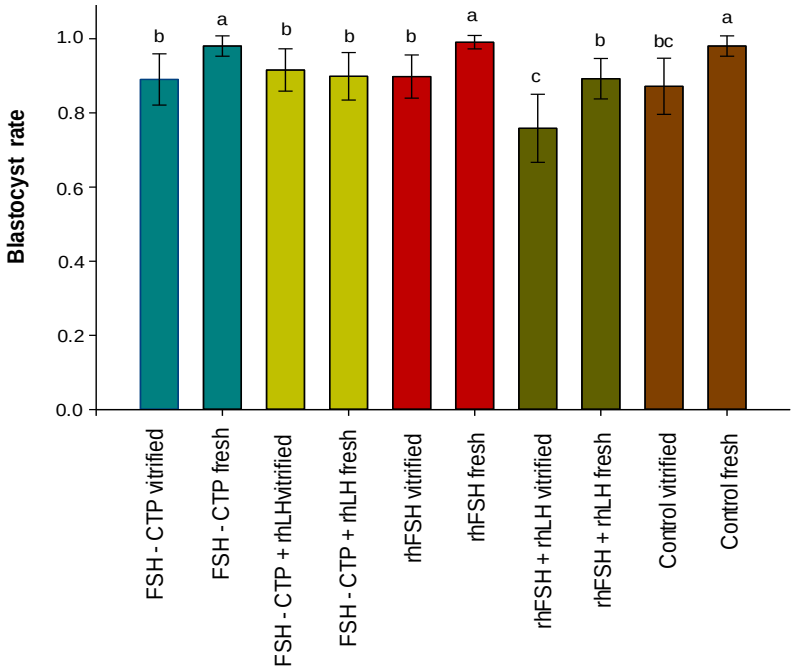

Figure 15. Blastocyst rate at 48 hours of different ovarian treatment by type of embryos.

[a,b,c]Values on the bars with different superscripts are statistically different (P<0.05).
number into the column: number of blastocyst at 48 h.
(least square mean ± standard error)

4.2.2. Evaluation of relative gene expression of blastocyst after *in vitro* culture

Results of ovarian stimulation treatment on SOX2, OCT4 and NANOG gene expression of embryos are shown in Figure 16. No significant difference among relative values of SOX2 and OCT4 gene expression from different experimental groups was detected. While ovarian stimulation groups showed similar relative value of NANOG gene expression, FSH-CTP, FSH-CTP+rhLH and rhFSH groups showed significantly higher NANOG gene expression than the control group (Figure 16).

Expression of genes maintaining the pluripotent embryonic stem cell phenotype were affected by embryo type, being the relative abundance of SOX2, OCT4 and NANOG higher for vitrified group (Table 6). In addition, interaction between ovarian stimulation treatment and embryo type was significant for SOX2 gene expression. Vitrified and fresh embryos from rhFSH groups had similar gene expression (0.67 ± 0.121 and 0.68 ± 0.121, data not shown in tables).

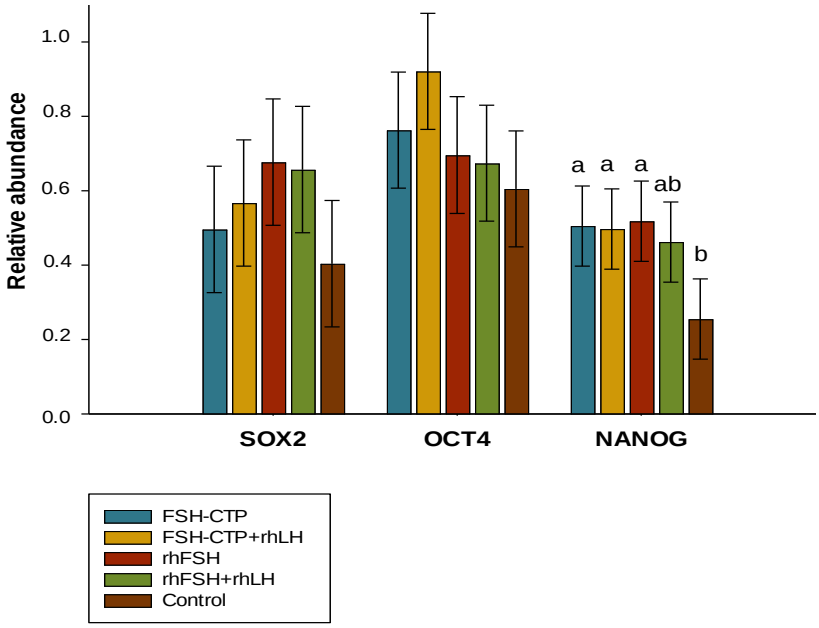

Figure 16. Relative gene expression by stimulation treatment.

[a,b]Values at the same block of columns with different superscripts are statistically different (P<0.05).
(least square mean ± standard error)

Table 6. Effect of Embryo type on genes expression in blastocyst.

(least square mean ± standard error)

Embryo type	Number of samples	SOX2	OCT4	NANOG
Vitrified	20	0.70 ± 0.056[a]	0.80 ± 0.051[a]	0.51 ± 0.036[a]
Fresh	20	0.42 ± 0.053[b]	0.66 ± 0.048[b]	0.38 ± 0.034[b]

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

4.3. Experiment 2: *In vivo* survival of recovered embryos

4.3.1. Evaluation of embryo viability after transfer

Embryos from ovarian stimulated does with rhFSH+rhLH showed the lowest embryo survival rates to implantation and birth (0.53 ± 0.042 and 0.33 ± 0.039, respectively) (Figure 17). Foetal losses in this treatment group was also the highest (0.34 ± 0056), while FSH-CTP and FSH-CTP +rhLH ovarian stimulation treatment did not increase statistically the foetal losses with respect to the Control group (0.22 ± 0.042, 0.20 ± 0.044 and 0.13 ± 0.037, for FSH-CTP, FSH-CTP+rhLH and Control group,

respectively; data not shown in tables). Moreover, the embryos from does treated only with rhFSH showed lower viability at birth related to control group.

From a total of 56 recipients does, 86.2% (25/29) for vitrified group and 85.2% (23/27) for fresh group gave birth (Table 7). In spite of the number of recipient given birth was similar between fresh and vitrified embryos, the implantation rate and the birth rate were affected by the vitrification process. No difference in foetal losses was observed between embryo type groups (Table 7).

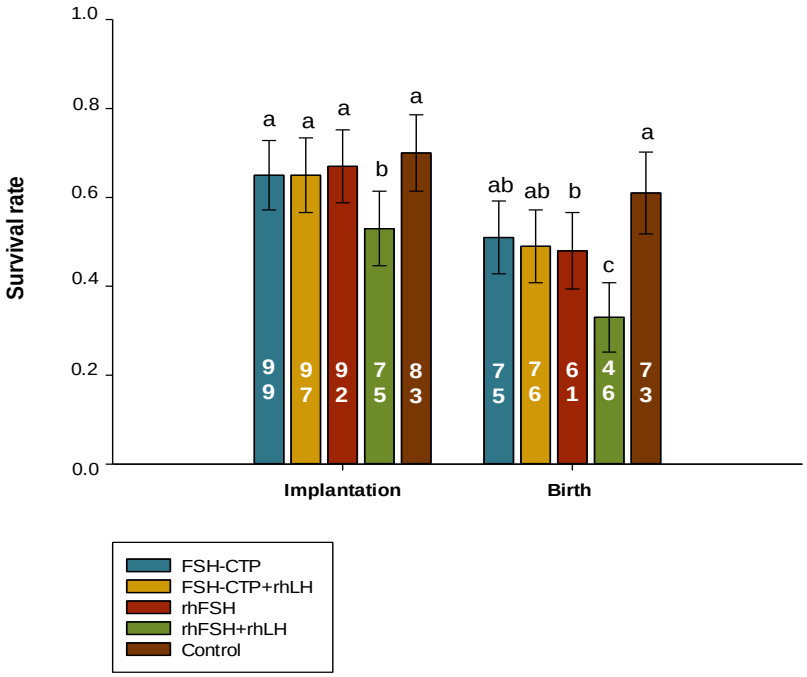

Figure 17. Survival rate of embryos at implantation and birth.

[a,b,c]Values at the same block of columns with different superscripts are statistically different (P<0.05).
Number into the "implantation" column: N° implanted embryos at 11 days of pregnancy.
Number into the "birth" column: N° born pup
(least square mean ± standard error)

Table 7. Effect of Embryo type on implantation and birth rate.

(least square mean ± standard error)

Embryo type	N° recipients	N° pregnant rabbits (%)	Implanted embryos rate	Delivery rate (%)	Foetal losses rate	Birth rate
Vitrified	29	27 (93.1)	0.55 ± 0.026[b]	25 (86.2)	0.23 ± 0.030	0.42 ± 0.025
Fresh	27	27 (100)	0.72 ± 0.026[a]	23 (85.2)	0.22 ± 0.029	0.55 ± 0.029

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

In addition, interaction between ovarian stimulation treatment and embryo type (vitrified or fresh) was found. FSH-CTP, FSH-CTP+rhLH and rhFSH groups had similar implantation rate for fresh and vitrified embryos, in the same way, FSH-CTP, rhFSH and rhFSH+rhLH groups showed similar birth rate for fresh and vitrified embryos (Figure 18 and 19).

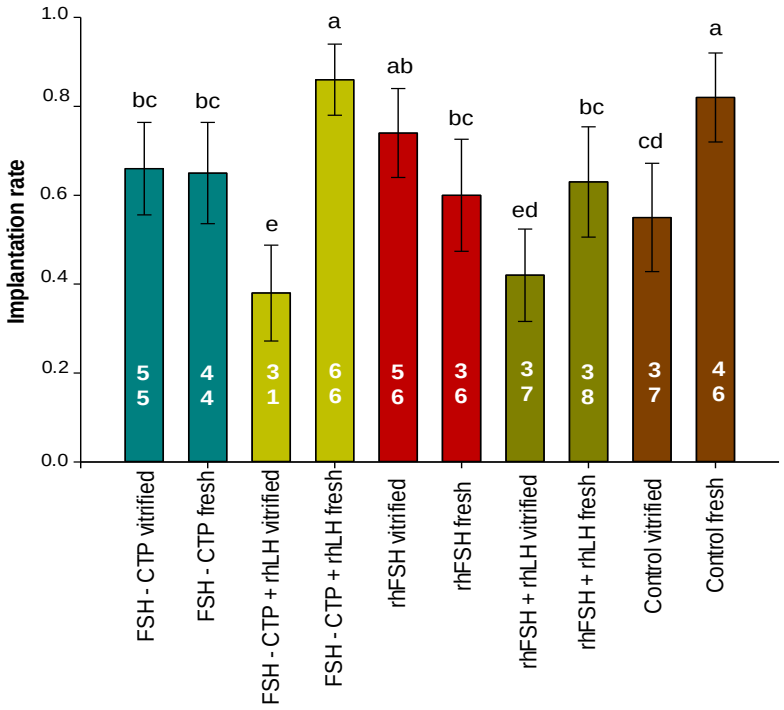

Figure 18. Implantation rate of different ovarian treatment by type of embryos.

[a-e]Values on the bars with different superscripts are statistically different (P<0.05).
Number into the columns: N° implanted embryos at 11 days of pregnancy.
(least square mean ± standard error)

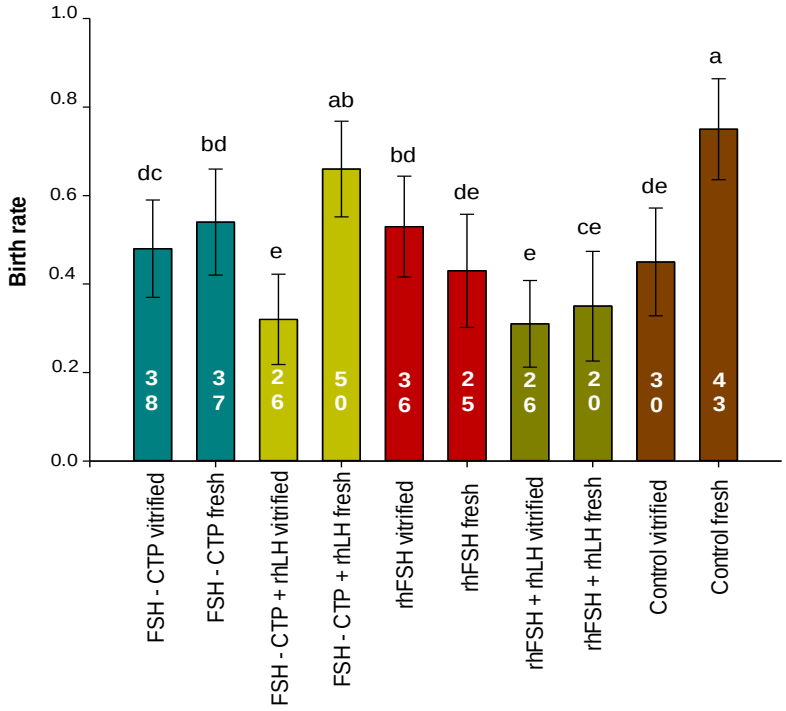

Figure 19. Birth rate of different ovarian treatment by type of embryos.

[a-e]Values on the bars with different superscripts are statistically different (P<0.05).
Number into the columns: N° born pup
(least square mean ± standard error)

4.3.2. Litter size at birth and weaning in experimental groups

The litter size at birth and weaning are shown in the Figure 20. The ovarian stimulation treatment did not significantly affect the litter size at birth and weaning (Figure 20 and Table 8). Transferable embryos as covariable did not show statistical difference (12.9 and 0.4 ± 0.31 for estimated mean and its coefficient of transferable embryos, respectively).

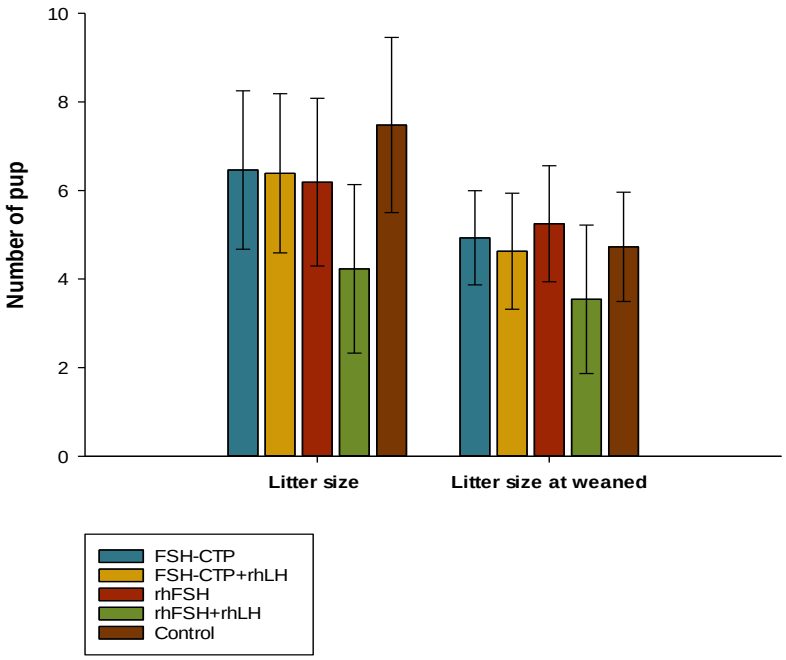

Figure 20. litter size per recipient doe at birth and weaning.

(least square mean ± standard error)

Table 8. Effect of Embryo type on litter size at birth and weaning.

(least square mean ± standard error)

Embryo type	Number of recipient	Transferred embryos	Litter size at birth	Litter size at weaning
Vitrified	29	397	5.4 ± 0.63	4.4 ± 0.46
Fresh	27	324	6.9 ± 0.66	4.9 ± 0.46

4.3.3. Pup weight at birth and weaning

The weight at birth and weaning were not significantly affected by the ovarian stimulation treatment (Figure 21 and 22).

The vitrification process affected significantly the pup weight at birth and weaning. The higher weight observed for the vitrified embryos group at birth (3.1 g. more than fresh embryos group) was compensated by the fresh embryos during the lactation, showing higher weight at weaning (Table 9).

The use of covariables such as the litter size at birth and weaning and the age at weaning did not affect the analysis results. The estimated means of covariables and their coefficient are shown in Table 10.

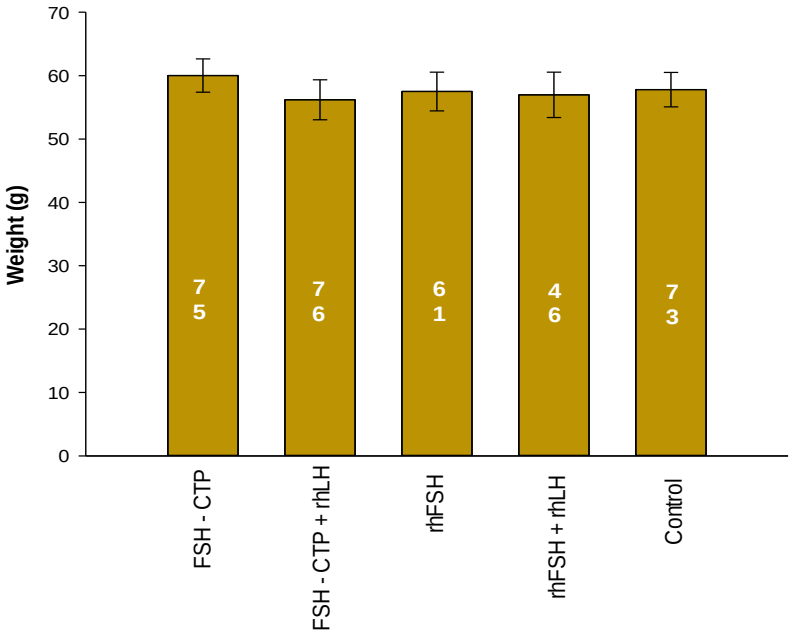

Figure 21. Weight at birth.

Number into the columns: N° born pup

(least square mean ± standard error)

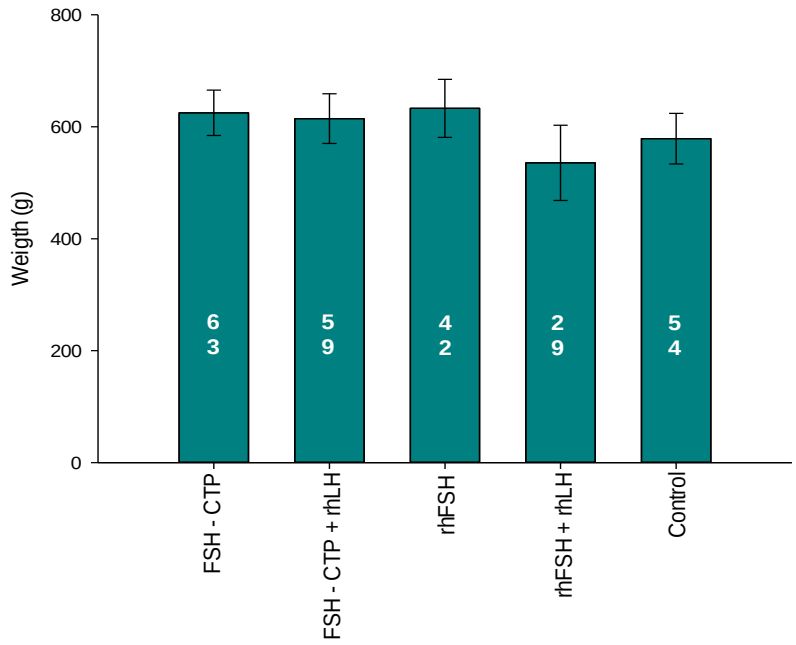

Figure 22. Weight at weaning.

Number into the columns: N° live young rabbits at weaning
(least square mean ± standard error)

Table 9. Effect of Embryo type on pup weight at birth and weaning.

(least square mean ± standard error)

Embryo type	Number of born pup	Number of weaned pup	Birth weight	Pups weight at weaning
Vitrified	156	113	59.2 ± 1.04[a]	544,9 ± 17,27[b]
Fresh	175	134	56.1 ± 0.92[b]	649,8 ± 15,98[a]

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

Table 10. The estimated means of covariables and their coefficient.

Character	Estimated mean	Covariable coefficient
Litter size at birth	7.8	-2.2 ± 0.37
Days at weaning	31.6	11.2 ± 4.07
Litter size at birth	7.6	-38.1 ± 8.08
Litter size at weaning	6.3	-28.4 ± 7.51

5. 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. Thus, the selection process was carried out for a limited set of commercial characteristics to produce animals with high genetic resources. Progress in the knowledge of reproduction technology such as artificial insemination, oocyte and embryo recovery, cryopreservation, nuclear transplantation, control of sex ratio and embryo transfer have beneficial impacts on scientific and economic development in the field of animal industry (Besenfelder et al. 2000). One of these domestic species is the rabbit; there is an increasing interest in using rabbits for research as a laboratory model as well as for industrial production of meat, wool and fur. Since its generation time is short and the heritability of growth and carcass traits are moderate, rapid improvement of these traits through selection can be accomplished.

Embryo cryopreservation provides an important tool for animal breeding, enabling the establishment of genome cryobanking, saving stocks for an unlimited time, savings in space and money, and protecting against loss through diseases or hazards. Inbred strains, mutations, and special genetic combinations can be preserved; thereby genetic pedigree standards can be established and checked for genetic drift in subsequent generations (Hafez 2000). The establishment of a rabbit embryo bank has been possible since 1974, when Bank and Maurer (1974) demonstrated that these embryos could be frozen and stored. Joly et al. (1996) reported that a total of 400 seminal doses and 500 embryos must be frozen to supply enough fertile offspring for re-establishment of populations. In 1992, the Animal Science Department of the Polytechnic University of Valencia (UPV, Spain) constituted an embryo bank from three selected rabbit lines (A and V maternal lines and R paternal line). Since 1992, the evaluation of the cryopreservation procedures to re-establish populations of these valuable lines has indicated a rapid efficiency improvement of cryopreservation results (survival rate at birth: 26% *vs* 56%, Vicente and García-Ximénez 1993 *vs.* Vicente et al. 1999). Furthermore, García et al. (2000a) and Lavara et al. (2011 and 2015) reported that cryopreservation programmes guaranteed the re-establishment of maternal rabbit line populations (22% to 36% overall survival rate at birth of vitrified embryos) and the evaluation of the genetic progress in these lines. On the other hand, García et al. (2000b) tried to export two selected lines (V and R lines) from Spain to Uruguay. These authors were able to re-establish V line in Uruguay with a sufficient number of young rabbits at weaning, although R line proved more difficult to cryopreserve (71 *vs* 21 weaned kits for V line *vs* R line). Nowadays, this bank stores embryos from four maternal lines (A, V, B and H lines) and one growth line (R). The recovery and cryopreservation of embryos at morulae stage is carried out at the

end of the selection process (after third parity); currently, in each generation of selection, about 300 embryos by line from 35 to 45 donor females mated with 15 to 20 males are preserved.

The cryopreservation programme includes three main steps: embryo recovery, cryopreservation and transfer. The efficiency of an embryo cryopreservation programme depends on provision of optimal conditions for each step. The use of superovulation and /or *in vivo* recovery techniques could be an excellent way to improve the genetic resources stored in each generation but factors such as hormones used in the reproductive control of females, or genotype of donors and recipients, affect the efficacy of the programme (Renard et al. 1982, Vicente and García-Ximénez 1993, Lavara et al. 2011 and 2015, and Marco-Jiménez et al. 2013). This study focused on both the improvement of stimulation treatment and the evaluation of the embryo cryotolerance.

Contradictory results have been observed on the effect of LH supplementation. Some authors have observed that LH could alter the oviductal and/or uterine environment affecting the transport of gametes and/or embryos, fertilization rate, embryo recovery rate. On the other hand, others authors have shown beneficial effects of LH on ovarian response, oocyte maturation and oocyte/embryo quality (Sirard et al. 2006, Ruvolo et al. 2007, Durnerin et al. 2008, Franco et al. 2009, and Viudes-de-Castro et al. 2015), so this controversy could be due to FSH and LH origin. In the present study, we evaluated two types of recombinant human FSH supplemented with or without a recombinant human LH and no effects on the ovulation rates results were observed in agreement with previous studies (Viudes-de-Castro et al. 2009). This hypothesis supports that only very small amounts of LH activity are sufficient to increase estrogen secretion up to measurable plasma levels (Mannaerts et al. 1991). In addition, these results also disagree with a study in women (Ruvolo et al. 2007, Durnerin et al. 2008, and Franco et al. 2009) and rabbit (Viudes-de-Castro et al. 2015), with rhFSH supplemented with rhLH, which suggests that LH prepares follicles to FSH, and thereby encourages follicular development as well as hormone secretion.

It is necessary to highlight that ovarian stimulation treatments provoke anovulatory process and donors´ ovulation without normal embryos. In our study, 19 ovarian stimulated does (23.75%), failed to produce embryos, being similar results as the ones observed by Mehaisen et al. (2006) with eCG and ovine FSH, and Salvetti et al. (2007) with purified porcine FSH alone or with LH, and higher than Viudes-de-Castro et al. (2009) results with rhFSH alone or in combinantion with rhLH. The lack of ovulation also appears in spontaneous ovulation species treated with gonadotropins (Herrler et al. 1991). These findings support that the use of exogenous gonadotropins in superovulation could alter the endogen LH release due to the increase of estradiol and progesterone plasmatic concentration (Gosselin et al. 2000) and interfere on

positive feedback of the estradiol on the LH secretion, blocking the ovulation process. In addition, Holmes et al. (1985) reported, in rabbit does, that high exogenous progesterone values markedly reduced the number of ovaries with ovulated follicles after LH stimulation. Similarly, Salvetti et al. (2007) observed that the mean of preovualtory 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. This may be partly due to the schedule of FSH-CTP, rhFSH and rhLH, which has not yet been set properly in rabbits.

In the present work, the ovulation rate and number of embryos recovered by donor are similar or greater than the others works previously published. When superovulation was induced with eCG, different authors observed a response interval of 16 to 40 for the ovulation rate, and 11 to 24 for the number of normal embryos (Renard et al. 1982, Besenfelder et al. 2000, Mehaisen et al. 2005 and 2006, and Badawy et al. 2016), while FSH treatments showed 19 to 56 for ovulation rate and 12 to 34 for number of embryo recovered (Kauffman et al. 1998, Cheng et al. 1999, Besenfelder et al. 2000, Mehaisen et al. 2005 and 2006, Salvetti et al. 2007, Viudes-de-Castro et al. 2009 and 2015).

When ovarian stimulation treatments are applied, the *in vitro* and *in vivo* viability of embryos could be compromised. Exogenous gonadotropins can induce changes in oocyte maturation and metabolism (Ramalho-Santos et al., 2009) and negative adjustments to environments of fertilization and early embryo development. This last as a consequence of steroidonegic alterations from anaovulatory and haemorragic follicles usually associated with these treatments (Guthrie et al. 1997, Mehaisen et al. 2005, Salvetti et al. 2007, and Cocero et al. 2011). The usual *in vitro* parameters to evaluate the oocyte or embryo quality are morphological appearance at the recovery moment, development rate, metabolites and messenger RNA expression, such as the transcription factor octamer-binging 4 (*OCT4*), NANOG homeobox (*NANOG*) and Sex determining region Y-box 2 (*SOX2*), which have essential roles in early development and are considered as key regulators of the pluripotency maintenance system (Boyer et al. 2005), and consequently, changes in their expression might trigger failures in the development and implantation of the embryos.

In the present work, a negative effect of combination rhFSH with rhLH was observed on expanded blastocyst rate at 24 and 48h hours. Surprisingly, control group (untreated ovarian stimulated does) had the worst rate of expanded blastocyst at 48h of culture. While relative expression levels to OCT4 and SOX were similar among the experimental groups, the relative expression of NANOG was lower for control group in accordance with a minor rate of development of expanded blastocyst. NANOG expression started

at very low levels during cleavage stages. It increase stochastically during the morula stage and it is upregulated mainly at inner cell mass, which might be significant for priming differentiation during epiblast maturation (Komatsu and Fujimori 2015). These results might suggest modifications of development patterns and disturbances in the necessary synchrony between uterine environment and embryos. Viudes-de-Castro et al. (2015) did not observe changes in the expression patterns of this gene neither development rates in blastocyst embryos cultured since 8-16 cell stage when they evaluated the application of rhFSH treatment on rabbit does. In the present study, 40-60% of blastocyst analysed were in expanded blastocyst stage, step prior to remodel of neozona and onset the gastrulation process, moment of embryo development characterized by a reduction of OCT4 expression and an increment of NANOG (Saenz-de-Juano et al. 2010). In cows, it was shown that hormonal superstimulation with porcine or ovine FSH with high and low LH percentage respectively, affect the mRNA profile of some genes (Mundim et al. 2009, and Chu et al. 2012). Mundim et al. (2009), showing differences in gene expression profile of a genes group related to embryo development between *in vivo* superovulated embryos and control embryos, but no differences were observed respect to *in vitro* produced embryos. Chu et al. (2012) observed that mRNA expression profile of genes related to developmental oocyte competence was affected by follicular stimulation in bovine.

In vivo results showed that implantation and survival rate at birth were affected by ovarian stimulation treatment. Implantation rate was negatively affected by rhFSH supplemented with rhLH suggesting a minor embryo competence to implant; moreover, this unfavorable effect induced more fetal losses and consequently, the minor survival rate at birth. rhFSH group showed a survival rate at birth lower than control group. Data cannot be compared with the study of Viudes-de-Castro et al. (2015) because the quality of embryos was measured *in vitro*. The *in vivo* results can be contradictories; Renard et al. (1982) observed a negative effect for eCG or FSH superovulatory treatment on the live born rate (56.3% *vs* 38.5%). Mehaisen et al. (2006) observed that embryos from eCG and ovine FSH showed similar *in vivo* survival rates than non-superovulated embryos (44 to 49). Salvetti et al. (2007) did not observe differences in birth rate from superovulated with pFSH and pFSH+pLH and non-superovulated embryos (100, 100 and 80%, respectively), neither in embryo survival rate (49.0, 43.9 and 52.3, respectively).

On the other hand, the effect of vitrification on development and viability of rabbit embryos has been extensively documented (García-Ximénez and Vicente 1992, Vicente et al. 1999, Mocé et al. 2010, Vicente et al. 2013, Marco-Jiménez et al. 2013, Saenz-De-Juano et al. 2014 and 2015). Vitrification procedure linked to transfer alters embryo development and gene expression of pre-implantatory rabbit embryos compared to their *in vivo* counterparts. Previous studies have demonstrated that vitrified embryos at morulae stage

that were able to reach late blastocyst stage (6 day old embryos, preimplantation stage) were also able to implant (day 14 of gestation), but not all implanted embryos had the ability to continue their gestation. So, these results manifested that alterations caused by vitrification are not completely resolved in viable implanted embryos. Previous observations in vitrified rabbit embryos detected problems in the formation of the placenta (Mocé et al. 2010, and Marco-Jiménez et al. 2013), suggesting that the causes of this mortality observed during the second part of gestation were probably orchestrated in the period comprised between the initiation of implantation and placental development (6 to 14 days of gestation in rabbit). Focussing in these days, Vicente et al. (1999) detected deficiencies in the foetus and placental growth and Saenz-De-Juano et al. (2014 and 2015) identified 60 upregulated genes and 89 differentially expressed protein spots (59 upregulated and 30 downregulated) in the foetal placenta of vitrified group. Identified genes were involved in the response to organic substance and wounding, chemical homeostasis, macromolecular complex subunit organisation, and lipid transport, localisation and metabolism. These authors observed that one of them was the serotransferrin gene (TRF), which encodes a circulating serum protein responsible for iron transport. It has been observed that increased expression of TRF in human placenta from pregnancies of selected abnormalities may indicate an increased need of foetal iron supplies, which could lead in a foetal stress and cause serious effects such as foetal growth delay, others such as alfa-enolase can be related to placental dysfunction (preeclampsia and pre-term labour). Then, the vitrification procedure introduces gene expression changes that can be manifested through foetal development, might be during postnatal life. In the present study, we observed that OCT4, SOX2 and NANONG were overexpressed in vitrified blastocyst, as reported in mouse (Zhao et al. 2012). Implantation and survival rate at birth were lower for vitrified embryos than fresh embryos, but there was not greater foetal losses between implantation and birth. If the losses had occurred before 12^{th} days of gestation in this study, it might be due to a disturbance of the pluripotential expression patterns determining both an abnormal cell proliferation and differentiation and asynchronic dialogue between endometrium and embryo. Despite the use of the covariables litter size at birth or at weaning in the respective analysis, alive born weights from vitrified group were bigger than those of fresh group but at the end of the lactation period showed a lower weight. Birth weight was in agree with Vicente et al. (2013) and Saenz de Juano et al. (2014), who reported that alive foetuses from vitrified group had a lower growth in foetal length between 10-12 days and then seemed to be offset during gestation to finish with a favourable difference of 8% in the birth weight. On the other hand, Saenz de Juano et al. (2014 and 2015) demonstrated that vitrification is not neutral, and induce a substantial alteration of placental protein expression, even at the end of gestation and suggested that vitrification entail long-term consequences. Cifre et al (1999) and

Lavara et al. (2015) indicated that vitrification and transfer procedures modify the growth velocity without modifying the adult body weight in males and females. It is important to consider that if the phenotypes observed in this study are due to epigenetic marks, a special attention should be taken when vitrification and embryo transfer procedures are used as tools to evaluate the response to selection in rabbit lines, because the estimated selection response could be biased.

An important aspect concerning the negative interaction between superovulation and cryopreservation are the effects on viability of embryos (Maurer et al. 1968, Renard et al. 1982, Carney and Foote 1990, Kauffman et al. 1998, and Mehaisen et al. 2006). For example, Renard et al. (1982) reported the decrease of live born rate from 38.5% in non-frozen superovulated embryos to 27.7% in frozen superovulated embryos, while Kauffman et al. (1998) obtained a percentage of 31% live born of non-superovulated vitrified embryos *vs* 20% live born of vitrified embryos from FSH group. Mehaisen et al. (2006) observed that born rates of vitrified embryos from eCG or FSH groups, were also decreased (16.8% *vs* 44.9% in eCG group; 12.8% *vs* 49.4% in FSH group for vitrified *versus* non-vitrified embryos, respectively). Superovulated embryos seem to have a higher sensitivity to low temperatures and this leads to a decrease in their subsequent potential capacity for development after vitrification based on a reduced number of trophoblastic cells and inner cell mass per embryo (Vajta 2000, and Leoni et al. 2001). The surviving blastomeres after vitrification procedures may be insufficient in number to permit the re-expansion of the blastocoelic cavity and continue the physiological development. In the present study, a significant interaction was observed between stimulation treatment and type of embryo (fresh and vitrified), vitrified and fresh embryos from FSH-CTP and rhFSH groups had the same implantation, but only vitrified and fresh embryos from rhLH had the same survival rate at birth.

6. CONCLUSION

- Does treatment with FSH-CTP and rhFSH alone or supplemented with rhLH achieved ovarian superstimulation. Superovulation treatments with rhFSH or FSH-CTP alone did not affect embryos *in vitro* development, while rhFH administration supplemented with rhLH compromised the *in vitro* viability at 48 hours. On the other hand, the expression of pluripotent genes (SOX2 and OCT4) was not affected and an overexpression of NANOG gene was observed, in concordance with the higher *in vitro* development of superstimulated embryos.
- The litter size and the weight at birth and weaning were non-affected by superstimulation treatments.
- Vitrification caused a decrease in the *in vitro* development at 48 hours in all groups, except in FSH-CTP supplemented with rhLH. Nevertheless, vitrification had not influence on implantation rate of rhFSH and FSH-CTP groups.
- Vitrification process did not affect the litter size, but had an important effect on the weight at birth, being higher than the fresh group. By contrast, weaning weight was lower than in the control group.
- The results of this study suggest that the use of FSH-CTP (3 µg/doe) is enough to superovulate rabbit does without compromising the quantity and quality of embryos, either fresh or vitrified. In addition, the use of this hormone reduces the frequency of animal handling, improving the animal welfare.

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