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IN VIVO RABBIT EMBRYO PRODUCTION AND CRYOPRESERVATION REVIEW. APPLICATION TO *EX SITU* CONSERVATION AND REDERIVATION

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Abstract: The development of reproductive technologies in this species is contributing decisively to the development of rabbit farming and the preservation of genetic resources. Obtaining embryos is an essential step to both genetic diffusion and the preservation of genetic resources from genetic erosion or natural disasters. In rabbits, it is common for embryos to be recovered post-mortem after ovarian hyperstimulation with gonadotrophins, although the quality and number of embryos are variable, affecting the embryo viability and offspring rate by the donor. *In vivo* embryo production within a conservation programme aims not only to obtain a large number of embryos, but also that they come from a greater number of male and female origins, in order to ensure an adequate representation of the original population. This is why both the quality and quantity of embryos obtained per donor rabbit and the rate of donors with offspring after embryo cryopreservation must be considered, as well as the response of the embryos to the chemical, physical and physiological stress to which they are subjected in the rederivation process and its postnatal repercussions on those that survive. Rederived rabbits from cryopreserved and transferred embryos showed phenotypic growth changes, which calls into question the neutrality of the technique and its usefulness in those cases in which a control population is required.

Key Words: embryo, superovulation, cryopreservation, rederivation, rabbit.

INTRODUCTION

The development of reproductive technologies in this species is making a decisive contribution to the development of rabbit farming and the preservation of genetic resources. Artificial insemination has facilitated genetic dissemination and transformed production systems, while obtaining and preserving gametes and embryos have contributed to the creation of germplasm banks of lines and breeds, both to favour the dissemination and evaluation of genetic progress and as a useful tool to reduce the risk of loss or erosion of the genetic resources available in this species.

Obtaining embryos is an essential step for *ex situ* conservation programmes of genetic resources. In rabbits, it is common for embryos to be recovered post-mortem. However, oocyte and embryo retrieval *in vivo* via laparotomy or laparoscopy is also technically possible, but perhaps the cost makes it unusual (Besenfelder *et al.*, 1998; Mehaisen *et al.*, 2005; Cortell *et al.*, 2010; García-Domínguez *et al.*, 2019). On the other hand, *in vitro* embryo production still faces important challenges in this species, as the maturation of oocytes, *in vitro* fertilisation, and the culture of zygotes for more than 48 h significantly reduce efficiency and viability *in vivo* compared to embryos obtained *in vivo* (Bracket

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and Oliphant, 1975; Seidel *et al.*, 1976; Kobayashi *et al.*, 1983; Carney and Foote, 1990, 1991; Murakami *et al.*, 1996; Zeng *et al.*, 1999; Jin *et al.*, 2000; Saenz-de-Juano *et al.*, 2012). Moreover, an additional issue that favours the loss of viability of embryos produced *in vitro* is the maternal contribution to formation of the mucin coat, which is essential for the subsequent transformation of the covers and blastocyst implantation (Murakami *et al.*, 1996; Denker, 2000, Marco-Jiménez and López-Béjar, 2013).

In vivo embryo production within a conservation programme aims not only to obtain a large number of embryos but also to ensure that they come from a greater number of male and female origins, in order to obtain an adequate representation of the original population. That is why both the quality and quantity of embryos obtained per donor rabbit and the rate of donors with offspring after embryo cryopreservation must be considered, as well as the response of the embryos to the chemical, physical and physiological stress to which they are subjected in the rederivation process and its postnatal repercussions on those that survive. This review aims to demonstrate the state of the art of techniques associated with an embryo cryopreservation programme, as well as to show some of the main effects on the development of rederived rabbits and the most relevant applications carried out.

Ovarian stimulation

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. Superovulation requires the administration of exogenous gonadotropin. However, stimulation treatments may generate a higher number of abnormal and immature follicles, either by increasing the cytogenetic defects or abnormal steroidogenesis patterns, affecting oocyte competence or future embryo development. As in other species, superovulation protocols have usually relied on the use of gonadotropins extracted from animal blood serum, pituitary extracts or human urine (equine chorionic gonadotropin [eCG], follicle-stimulating hormone [FSH], luteinising hormone, [LH] and human chorionic gonadotropin [hCG], Table 1). Nevertheless, an alternative to animal sources, which has

Table 1: Ovarian stimulation response to equine chorionic gonadotropin (eCG), pituitary or recombinant follicle-stimulating hormone (FSH).

Treatment	No. Doses[1]	Total Dose[2]	Ovulation Induction[3]	OR	Embryos[4]	Reference
eCG	6	75 IU	2.5 mg eLH	16.6	14.9	Kennelly and Foote, 1965
	1	120 IU	60 IU hCG	-	21.3	Illera *et al.*, 1990
	1	100 IU	0.8µg aGnRH	15.7	14.3	Stradaioli *et al.*, 1997
	1	80 IU	30 IU hCG	-	17.8	López-Béjar and López-Gatius, 2002
	1	80 IU	180 IU hCG	32.9	18.5	Saratsi *et al.*, 2002
	1	80 IU	2µg aGnRH	19.2	15.4	Mehaisen *et al.*, 2006
Pituitary FSH	6	1.50 mg pFSH	2.5 mg eLH	53.7	43.5	Kennelly y Foote, 1965
	6	6.0 mg pFSH	Mate	50.5	25.8	Kanayama *et al.*, 1994
	1	6.0 mg pFSH in PVP	Mate	57.4	49.2	Kanayama *et al.*, 1994
	3	1.8 mg oFSH	2 µg aGnRH	25.2	20.3	Mehaisen *et al.*, 2006
	5	2.25 mg pFSH+Sal. 20% LH	1.6 µg aGnRH	27.1	20.3	Salvetti *et al.*, 2007a
	6	3mg pFSH	75IU hCG		28.6	Hashimoto *et al.*, 2007
	1	3 mg pFSH in Al-gel	75IU hCG	-	26.3	Hashimoto *et al.*, 2007
	6	5.4 µg reFSHh+10% reLHh	2 µg aGnRH	41.5	34.0	Viudes-de-Castro *et al.,* 2015
Recombinant FSH	1	3µg CTPα	2µg aGnRH	52.9	37.9	Viudes-de-Castro *et al.*, 2017
	1	3 µg CTPα+7.5 I.U hCG	2µg aGnRH	51.1	41.4	Viudes-de-Castro *et al.*, 2019

[1]Number of doses received in the 3 d before ovulation induction.

[2]Total dose and gonadotropin type. eCG, pFSH (porcine pituitary FSH), oFSH (ovine pituitary FSH), reFSHh (human recombinant FSH), CTPα (corifollitropin alpha), eLH (equine pituitary LH) and reLHh (human recombinant LH).

[3]Ovulation induction treatment: dose and gonadotropin used (eLH -equine pituitary- LH, hCG-human chorionic gonadotrophin-, aGnRH –GnRH analogue synthetic, buserelin acetate-). OR: average number of ovulated follicles per doe.

[4]Average number of embryo recovered per doe.

been made available by biotechnology, is recombinant FSH and LH. The use of these hormones might reduce the variation of pituitary-derived FSH and when the exogenous recombinant FSH is from the same species, it may prevent the humoral immune response and transmission of diseases across species (Hesser *et al.*, 2011; Carvalho *et al.*, 2014). FSH from pituitary extracts or recombinants could have advantages in superovulatory response and embryo quality over eCG, but due to its relatively short elimination half-life and rapid metabolic clearance is a more time-consuming protocol, requiring two daily injections to maintain the threshold level during ovarian hyperstimulation. However, to reduce the number of doses in FSH treatments, different protocols have been used, both in rabbits and other species, introducing aluminium hydroxide gel or PVP to increase the effective stimulation period (Kanayama *et al.*, 1994; Hashimoto *et al.*, 2007). Treatments with eCG and FSH manage to double and sometimes triple the average ovulation rate of the population that is treated (20 to 40 compared to 12-14 of the controls, Table 1). However, it can be observed that 20-30% of donors failed to recover normal embryos (Joly *et al.*, 1998; Mehaisen *et al.*, 2006, Salvetti *et al.*, 2007a; Joly *et al.*, 2012, Viudes-de-Castro *et al.*, 2017), even when donors were not overstimulated, depending on their reproductive history and line or breed (Vicente *et al.*, 2003; Salvetti *et al.*, 2007a).

Short FSH half-life is now resolved by a new generation of recombinant gonadotrophins, for example, corifollitropin alpha (CTPα); this recombinant hormone comprises an alpha-subunit that is identical to that of FSH and a hybrid beta subunit which is produced by fusion of the carboxy terminal peptide from the beta subunit of hCG to the beta subunit of FSH (Fares *et al.*, 1992; Fauser *et al.*, 2009, Leao *et al.*, 2014). Corifollitropin alpha (CTPα) has an approximately two-fold longer half-life (65-h plasma half-life) and an almost four-fold extended time to peak serum levels (Dujikers *et al.*, 2002; Devroey *et al.*, 2004). Rabbit females treated with 0.75 µg per kg 60-72 h before ovulation induction multiply their ovulation rate by 4 or 5 if they are nulliparous and by 3 if they are multiparous (Viudes-de-Castro *et al.*, 2017, 2019; Vicente *et al.*, 2022). Moreover, ovulation rate and the number and quality of recovered embryos are significantly increased when hCG was used with CTPα, so a single injection of long-acting CTPα supplemented with hCG can be used in rabbits effectively (Table 1). As in the previous superovulation treatments, nulliparous does respond better than multiparous dams and some negative effects on the quality of the oocytes and on the chronology and embryonic viability have also been detected. Recently, Vicente *et al.* (2022) demonstrated that treatments and, also, parity could affect the expression of MATTER gene in oocytes and, therefore, negatively influence oocyte quality and subsequent fertilisation rate and delivered kits. However, the impact on the viability of embryo cryopreservation is not as pronounced as in the previous studies (Kauffman *et al.*, 1998; Mehaisen *et al.*, 2006; Vicente *et al.*, 2018).

In vivo retrieval by laparotomy or laparoscopy is technically possible but uncommon; nevertheless, it offers the possibility of optimising the number of embryos recovered per female, especially when populations with a small number of individuals or animals of great genetic value are used (Maurer *et al.*, 1968; Besenfelder *et al*, 1998; Forcada *et al.*, 2000; Mehaisen *et al.*, 2005; Cortell *et al.*, 2010). It has been possible to observe a loss of superovulation response after the second or third treatment of both FSH and eCG (Maurer *et al.*, 1968, Mehaisen *et al.*, 2006, Cortell *et al.*, 2010); despite a variable number of rabbits, it is possible to carry out three or four successful superovulation treatments (from 25 to 50% of the rabbits, reaching more than 100 transferable embryos per doe).

Embryo cryopreservation

Since the 1970s, it has been possible to successfully freeze rabbit embryos (Bank and Maurer, 1974) and in the 1990s the first vitrification procedures for this species were established (Smorag *et al.*, 1989; Kasai *et al.* 1992). Most studies related to embryo cryopreservation in rabbits have focused on technical factors that affect the efficiency of the process, addition-dilution protocols and types of cryoprotectants, vitrification devices and embryonic stages (Kojima *et al.*, 1990; López-Bejar *et al.*, 2002; Hochi *et al.*, 2004; Lin *et al.*, 2011; Viudes-de-Castro *et al.*, 2010, Marco-Jiménez *et al.*, 2013, 2016; Kulíková *et al.*, 2016), but few comparative studies have examined the effect of donor genotype (Maurer and Haseman, 1976; Joly *et al.*, 1998; Vicente *et al.*, 2003; Mehaisen *et al.*, 2005; Joly *et al.*, 2012). In general, the best *in vivo* survival results are obtained when morulae and early blastocyst stages are frozen or vitrified in dimethyl sulfoxide and/or ethylene glycol (Table 2) and transferred to females with an asynchrony of 12 to 17 hours in relation to the age of the embryos (Tsunoda *et al.*, 1982; Marco-Jiménez *et al.*, 2013). It is also necessary to point out that, as with the transfer of fresh embryos, the lineage and reproductive history of the females hosting the cryopreserved embryos will affect the procedure success rate (Vicente *et al.*, 1993a, 2003, Marco-Jiménez *et al.*, 2013). Thus, it is preferable to use females from maternal lines with excellent reproductive efficiencies

Table 2: Survival rate at birth of cryopreserved rabbit embryos.

Procedure	Donor line or breed	Stage of embryos	Survival rate at birth (%)	Reference
Freezing	Dutch-Belted	8-cell	10	Bank and Maurer, 1974
	Dutch-Belted	Morula	26	Maurer and Haseman, 1976
	New Zealand White	2-cell	27	Tsunoda *et al.*, 1977
	New Zealand White	16-cell	50	Rao *et al.*, 1984
	New Zealand White	2-cell	26	Renard *et al.*, 1984
	New Zealand White	Morula	48	Tsunoda *et al.*, 1982
	Crossbred	Pronuclear	14	Al-Hasani *et al.*, 1992
	A line	Morula	25	Vicente and García-Ximénez, 1993b.
	V line	Morula	42	Vicente and García-Ximénez, 1994.
	1077 line	Morula	38	Joly *et al.*, 1998
	1029 line	Morula	51	Joly *et al.*, 1998
	PS 19 line	Morula	48	Salvetti *et al.*, 2007b
	Diverse	Morula	21-41	Joly *et al.*, 2012
Vitrification	Japanese breed	Morula	65	Kasai *et al.*, 1992
	New Zealand White	Morula	26	Smorag *et al.*, 1989
	V line	Morula	43-59	Vicente and García-Ximénez, 1994; Vicente *et al.*, 2003; Marco-Jiménez *et al.*, 2013b
	Chinchilla breed (paralytic tremor)	Morula	23.5	Papis *et al*, 2005
	New Zealand White	Morula	52	López-Bejar and Lopez-Gatius, 2002
	R line	Morula	13-32	Vicente *et al.*, 2003; Mehaisen *et al.*, 2006; Marco-Jiménez *et al.*, 2018
	A line	Morula	44-65	Marco-Jiménez *et al.*, 2013, Vicente *et al.*, 2018, García-Domínguez *et al.*, 2020a

rather than rabbits from lines or breeds with low reproductive performance. The ultimate goal is to obtain offspring from the maximum number of parents in order to conserve biodiversity or establish a sufficiently representative control group to evaluate or export a genetic line, as we will see later in this review.

Two main European rabbit cryopreservation programmes have been developed: the National French bank (NFB), with the aim of preserving both commercial lines and rabbit breeds (Joly *et al.*, 1998, 2012, Salvetti *et al.*, 2007b), and the rabbit embryo bank of the Universitat Politècnica de València (UPV) to preserve, evaluate and disseminate synthetic lines selected to improve the productivity of this sector (Vicente and García-Ximénez, 1993a,b; Vicente *et al.*, 2003; Marco-Jiménez *et al.*, 2018). Until now, more than 60 000 embryos have been frozen or vitrified, and more than 40 000 of them remain stored in banks. The NFB has around 26 000 embryos and roughly 2000 donors and sixty genetic breeds or lines (https://www.cryobanque.org/), while the UPV bank stores around 20 000 embryos from 8 rabbit selected lines and about 1900 donors.

Both programmes use rabbit embryos at the morula or blastocyst early stages, but differ in the cryopreservation technique. The NFB uses slow freezing, and the embryos are exposed in 3 stages of 5 min to a freezing medium of increasing concentration of dimethyl sulfoxide (0.5; 1 and 1.5 M DMSO) in phosphate buffered saline (PBS) supplemented with 0.4% (weight/volume) of bovine serum albumin (BSA). After packaging in 0.125 mL straws, the temperature drops from –1°C/min to –7°C, in which the freezing of the medium containing the embryos is induced. Subsequently, the temperature drops from -0.5°C/min to -35°C, a temperature at which they are immersed in liquid nitrogen (Renard *et al.*, 1982, Salvetti *et al.*, 2007b). Thawing is performed by exposing the straws to room temperature for 30 seconds, then thawing is completed in a water bath at 20°C, removing the freezing medium in three stages of decreasing the concentration of the cryoprotectant in PBS supplemented with BSA (1; 0.5 M DMSO and PBS). Meanwhile, the UPV bank uses vitrification as a cryopreservation technique (Vicente and García-Ximénez, 1994, Vicente *et al.*, 1999). The embryos are exposed in two stages to the vitrification solution composed of 20%

(V/V) dimethyl sulfoxide and 20% (V/V) ethylene glycol in PBS supplemented with 0.2% (w/v) BSA. In the former, the embryos remain for 2 min in a solution at 50% of the final concentration of cryoprotectants, and in the latter, the embryos are exposed for less than 1 min to the vitrification solution, during which they are loaded into 0.125 mL straws and submerged in liquid nitrogen. Devitrification is carried out first in nitrogen vapour for 30 s and later in a water bath at 20°C. Washing of the cryoprotective solution is carried out in two stages: the embryos in the vitrification solution are poured into a 0.33 M sucrose medium in PBS supplemented with BSA in which they remain for 5 min before being transferred to the transfer medium (PBS supplemented with BSA). In addition, rabbit morulae have also been successfully vitrified in media containing ethylene glycol, ficoll and sucrose as cryoprotectants (Kasai *et al.*, 1992) or glycerol and ethylene glycol (López-Bejar and Lopez-Gatius, 2002). Recently, we reported the feasibility of using commercially available minimum essential volume devices (cryotop and loop) that allow slight improvements in survival rates (Marco-Jiménez *et al.*, 2013 and 2016, García-Domínguez *et al.*, 2020a). Moreover, we have developed a new simple and practical vitrification device based on the minimum volume cooling principle for the simultaneous vitrification of many oocytes or embryos named CryoEyelet® (patent: ES2713800, 2020). CryoEyelet® is a suitable system for the simultaneous vitrification of 25 rabbit embryos at the late morula- early blastocyst stages, with efficiency in terms of *in vitro* and *in vivo* development similar to that of the Cryotop® device.

Results after freezing and vitrification can be different; whereas vitrification procedure allows a higher proportion of transferable embryos after thawing (95-100% *vs.* 70-80%), *in vivo* survival is more affected by the superovulation, embryo stage and genetic origin of the donors than freezing procedure. So, for example: morula frozen and transferred reach an average survival rate of around 40% (21-51%), while survival of vitrified morulae is around 40%, but with a more variable range (13-65%).

Rederivation applications

Rederivation from cryopreserved embryos has been extensively employed in rabbit to establish new lines applying phenotypic criteria to export lines, to assess genetic programmes or to evaluate the impact of cryopreservation at omic and phenotypic level. Some examples are as follows:

Establishing new lines

A maternal line (H) was founded applying hyperprolific criteria and embryo cryopreservation techniques (Cifre *et al.*, 1996, 1998). Briefly, in the first step, 20 hyperprolific females were mated with the best bucks from a maternal line selected by litter size (V, Estany *et al.*, 1989) to obtain male VH. In a second step, batches of 6-10 hyperprolific females were mated with VH males and embryos were vitrified to avoid health problems and to make contemporary individuals of founder generation. A total of 103 hyperprolific does and 47 VH males were used to obtain 1068 vitrified embryos, 550 young live rabbits after transfer and subsequent establishment of the first generation with 156 adult rabbits from 87 donor does. The results after the three first generations demonstrated that the hyperprolific scheme was effective. For instance, the prolificacy of the new line was superior to high performances of the V line or to those of crossbred females performed with the V line and a second maternal line (A, Estany *et al.*, 1989).

Genetic lines exportation

Embryos belonging to selected lines V and R of the Animal Science Department (Universitat Politècnica de València) were exported at "Las Brujas" Experimental Station (INIA-Uruguay). A total of 562 vitrified embryos were transferred to 64 nulliparous does from undefined crosses belonging to the "Las Brujas" Experimental Station, 340 from 38 donors of line V and 222 from 26 donors of line R. Lines and selection methodologies applied were described by Estany *et al.* (1989 and 1992). The 71 rabbits weaned after transfer from line V allowed us to establish a first V population in Uruguay to contribute to rabbitry development in this country. On the contrary, the overall survival rate of line R was low and insufficient to create a population of this line (García *et al.*, 2000). Four years later, a second batch of embryos from the R line and a second maternal line (A line) were exported and transferred to complete the programme established to promote the development of Uruguayan rabbit farming.

Assessment of genetic progress

Estimation of response to selection in rabbit breeding programmes has been extensively described, applying mixed model methods but also using the comparison between two different generations by rederivation of a control population cryopreserved generations ago (García and Baselga, 2002a, Khalil and Al-Saef, 2008, Marco-Jiménez *et al*, 2018). Cryopreservation offers the advantage of rederiving a control population that facilitates the evaluation of genetic progress, as populations are raised under the same environmental, handling and feeding conditions. Moreover, it is a valuable tool to preserve genetic variability and breeds from pathogens or catastrophes. Rederivation of a control population has been used to estimate the response to selection in both maternal (García and Baselga, 2002a; García and Baselga, 2002b, Laborda *et al*., 2012, Peiró *et al.,* 2021) and paternal lines (Piles and Blasco, 2003, Juárez *et al*., 2020). Two examples and three studies are briefly described below.

The first example is the evaluation of response to selection for litter size at weaning (García and Baselga, 2002a). Briefly, embryos from generation 15 of a maternal line (V) were vitrified and kept in a bank of liquid nitrogen for around 5 yr. It was intended that each buck would contribute at least two embryo straws from different females to the embryo bank. Then, the embryos were thawed and transferred to foster mothers of line V and, after birth, became contemporary to generation 21 of line V. A total of 73 does of generation 15 were obtained and, in order to avoid the effect of embryo cryopreservation and transfer, unselected offspring from both generations (15 and 21) were obtained from natural mating. The results of this study showed that the responses to selection evaluated with either a control group or by applying mixed model methods agreed (0.085 weaned per generation and 0.09 weaned per generation, respectively), which could be considered as an argument in favour of the statistical models and genetic parameters used in the analysis of the selection process.

A second example was the evaluation of response to selection for daily weight gain (ADG). A first study was carried out on a paternal line (R) after 7 generations of selection, using frozen embryos stored from generations 3 and 4 (Piles and Blasco, 2003). As in the previous study, those born from cryopreserved embryos were bred to avoid the possible effects of the techniques used and to expand the number of animals in the trial. Unfortunately, the very low survival rate of frozen embryos from this line limited the number of founders in the control population and reduced the unselected offspring obtained from natural mating that were used as the control population. Both methods of estimating the selection response, mixed model and estimating the genetic effects of the animals in each generation and comparing the last generation of selection with a control population, yielded similar results (heritability of daily gain 0.11, Piles and Blasco, 2003). At the commercial slaughtering age, selected animals had a higher growth rate and slaughter weight (49.8 *vs.* 45.9 g/d, 2350 *vs.* 2180 g, respectively). Another consequence of selection was that animals from the 10th generation were heavier than the control animals throughout the Gompertz growth curve, while the other parameters of the Gompertz curve were scarcely affected by selection (Blasco *et al*., 2003). A second study was recently carried out using vitrified embryos from two generations of this line (18th and 36th, Juárez *et al*., 2020). This experimental approach not only reduced the influence of environmental effects, management and nutrition, but also the rederivation procedure. A total of 516 vitrified embryos were transferred to 54 recipients using the same protocol as before, with 301 embryos belonging to 36th generation and 259 embryos to 18th generation. Viability at delivery was 22-23% of the transferred embryos for both rederived generation and constituted the 19th and 37th generation. Over approximately two years, animals within each population were crossed to produce the next generations and re-establish the original population size (Marco-Jiménez *et al*., 2018). After two generations of both rederived populations (21th *vs.* 39th generations), weaning and end fattening weights and ADG trait showed some progress because of the selection, with ADG being 0.113 g/d by generation. Nevertheless, it was the lowest compared to the results obtained in the same line between the 3rd-4th *vs.* the 11th generation (Piles and Blasco, 2003). In general, results demonstrated that the selection programme had improved ADG without variations in adult body weight, but after 37 generations of selection, this trait seemed exhausted (Juárez *et al*., 2020). Moreover, a sexual dimorphism favouring females was observed in this paternal line.

Direct effects and transgenerational effects of embryo cryopreservation. Biomedical model and livestock consequences.

Embryos are subjected to extreme environments in the recovery, cryopreservation and transfer processes, which can irreversibly damage the embryos or generate an adaptive response that allows them to survive the procedure. This

adaptive response not only modifies gene expression and prenatal and postnatal development of those born from cryopreserved embryos, but could also have consequences on the subsequent generations through heritable and non-heritable epigenetic changes. Direct or short-term effects of the rederivation process have been reported on embryo gene expression, placenta proteome, growth of the foetus and newborn weight (Mocé *et al*., 2010; Saenz-de-Juano *et al*., 2012; Vicente *et al*., 2013b; Saenz-de-Juano *et al*., 2014; García-Domínguez *et al*.,2020b), and in the postnatal growth and weight of organs, such as the liver or adrenal gland (Lavara *et al*., 2015; García-Domínguez *et al*., 2020b,c; Juárez *et al*., 2022). Reproductive traits such as sperm production, fertility and litter size at birth seem not to be influenced by the effects of cryopreservation (Cifre *et al*., 1999, García-Domínguez *et al*., 2020d, Juárez *et al*., 2022). However, Lavara *et al.* (2014) showed that cryopreservation and embryo transfer increased litter size, live births and postnatal survival in cryopreservation-born females. These discordant results could be due to the genotype of the line studied.

We have demonstrated that the embryo vitrification procedure affected the long-term phenotype of the derived offspring, identifying profound differences in the molecular physiology of the liver in the vitrified progeny. There is consistent evidence regarding an overall modification of the whole hepatic metabolism after embryo cryopreservation. The most evident change found in omics studies was related to the metabolism of some polyunsaturated fatty acids, particularly of the linoleic and arachidonic acids in hepatic tissue (García-Domínguez *et al*., 2021). Dysregulations in the levels of both acids were found in adult livers after assisted reproduction technologies (Wang *et al*., 2013) and their depletion can result in growth retardation (Hadley *et al*., 2016). Concordantly, direct and transgenerational effects of embryo vitrification and transfer could result in a lower growth rate and reduced body weight in adulthood. Moreover, we were able to find phenotypic changes in the offspring of vitrified embryos after three generations (García-Domínguez *et al*., 2021; Juárez *et al*., 2022). However, despite these phenotypic changes, this progeny was apparently healthy and fertile (García-Domínguez *et al*., 2021).

This situation could be of special importance in the livestock sector, where genomic selection in combination with reproductive technologies is revolutionising the design and implementation of breeding programmes. Thus, characterisation and implementation of the assisted reproduction technologies effects in the selection programme can lead to adjusting the accuracy of the model and better estimating the genetic gains, including ART animals in the analyses.

CONCLUSION

It is important to point out that in this species there are multiple ovulation, recovery, cryopreservation and transfer (MOET) protocols that are as efficient and useful for conserving, exporting or establishing control populations as in other livestock species. However, as occurs in other species, it must be considered that these procedures have been fine-tuned in a few lines or breeds and that when used on other breeds, the results obtained are not what was expected. The genotype of the donors, the sensitivity of the embryos to the physiological stress conditions by driving the ovulatory response, their collection and handling outside the oviduct, exposure to cryoprotectants, the drop in temperature and the adaptive and conditioned response of the embryos are determining factors in the final survival. In addition, the skill and experience of the team applying the techniques must be taken into account.

Unlike other species, very interesting tests have been carried out in the rabbit to evaluate the effects of the cryopreservation programme on the genome and phenotype of the rederived rabbits. This effect is not new, and was already considered in the design of the studies previously cited in the evaluation of the selection programmes, comparing the populations obtained from the next generation to that obtained by transfer of cryopreserved embryos. However, the results of recent studies at the omic and phenotypic level show that the effects are transgenerational, affecting lipid metabolism and growth, but being apparently innocuous on reproductive efficiency. In addition, these results should be taken into consideration in other species and should allow the development of new, less invasive MOET protocols on the physiology of follicular and embryonic development, thus reducing their transgenerational impact.

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