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LONG-TERM AND TRANSGENERATIONAL EFFECTS OF CRYOPRESERVATION ON RABBIT EMBRYOS

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ABSTRACT

The short term effects of cryopreservation and embryo transfer are well documented (reduced embryo viability, changes in pattern expression), but little is known about their long-term effects. We examined the possibility that embryo vitrification and transfer in rabbit could have an impact on the long-term reproductive physiology of the offspring and whether these phenotypes could be transferred to the progeny. Vitrified rabbit embryos were warmed and transferred to recipient females (F0). The offspring of the F0 generation were the F1 generation (cryopreserved animals). Females from F1 generation offspring were bred to F1 males to generate an F2 generation. In addition, two counterpart groups of non-cryopreserved animals were bred and housed simultaneously to F1 and F2 generations (CF1 and CF2, respectively). The reproductive traits studied in all studied groups were litter size (LS), number born alive at birth (BA) and postnatal survival at 28^{th} day (PS, number of weaned/number born alive expressed as percentage). The reproductive traits were analysed using Bayesian methodology. Features of the estimated marginal posterior distributions of the differences between F1 and their counterparts (F1-CF1) and between F2 and their counterparts (F2-CF2) in reproductive characters showed that vitrification and transfer procedures cause a consistent increase in LS and BA between F1 and CF1 females (more than 1.4 kits in LS and more than 1.3 BA), and also between F2 and CF2 females (0.96 kits in LS and 0.94 BA). We concluded that embryo cryopreservation and transfer procedures have long-term effects on derived female reproduction (F1 females) and transgenerational effects on female F1 offspring (F2 females).

1. INTRODUCTION

Embryo cryopreservation and transfer procedures are widely used as assisted reproductive technologies (ART) in both laboratory and domestic animals. These techniques induce environmental changes that influence the relationship between genotype and phenotype by modifying the gene expression of the embryo [1, 2, 3], and may not be neutral concerning behavioural features of the individuals due to changes in maternal effects [4, 5]. Some of these environmental changes have an impact on the phenotypic appearance and, perhaps, on the phenotype of their progeny (transgenerational phenotypic changes) [6]. The interaction between organisms and their environment could induce epigenetic modification that may result in the appearance of a new phenotype, and could represent heritable changes in gene expression that do not involve changes in the genetic code [7].

In mammals, mothers and offspring have an extended association during gestation and lactation. For this reason, maternal effects can contribute to individual differences within a population with alternative phenotypes [8, 9]. Uterine maternal effects are heritable and non-heritable maternal attributes, separate from the direct transmission of nuclear genes that influence offspring development [10]. Postnatal maternal performance is also a significant epigenetic factor in development [11] and includes components such as litter size, milk quality and quantity, and various aspects of maternal behaviour. Maternal effects can condition the expression of the progeny genome [12], and in this sense, clearly fit as epigenetic factors.

In rabbit embryos it is known that cryopreservation causes environmental changes inducing altered gene expression patterns [13, 14] resulting in reduced early foetal development and increase foetal losses [15, 14], but little is known regarding long term outcomes.

The aim of our present study was to investigate whether cryopreservation and transfer procedures of rabbit embryos could have an impact on the long-term reproductive physiology of the offspring, and if these phenotypes could be transferred to the progeny.

2. MATERIALS AND METHODS

2.1 Animals

All experimental procedures involving animals were approved by the Research Ethics Committee of the Universidad Politécnica de Valencia (UPV).

All animals came from line V, a maternal rabbit line selected on a number of young weaned per litter [16]. Animals were housed at the experimental farm of UPV. At 63 days of age, animals were kept individually under the same environmental conditions. Animals were kept under a controlled 16-h light:8-h dark photoperiod and fed a commercial diet.

2.2 Experimental design

Vitrified rabbit embryos were warmed and transferred to recipient females and the resulting pregnant females were designated the F0 generation. The offspring of the F0 generation were the F1 generation (cryopreserved animals; females and males). Females (n=65) from F1 generation offspring were bred to

other F1 males to generate an F2 generation. Females (n=50) from F2 generation were bred similarly.

In addition, two counterpart groups of animals from the same genotype and generation obtained by natural mating (non-cryopreserved and non transferred animals) were bred and housed simultaneously in the same experimental farm as F1 and F2 generations (CF1 and CF2, respectively). Each of the groups consisted of 50 females.

2.3 Embryo collection

Non superovulated does were used as embryo donors. Does were slaughtered at 70-72 h postcoitum. Embryos were collected at room temperature by flushing the oviducts and the first one-third of the uterine horns with 5 mL of embryo recovery media consisting of Dulbecco's Phosphate-Buffered Saline (DPBS; Sigma, Alcobendas, Madrid, Spain) supplemented with $CaCl_2$ (0.132 g/L), 0.2% (w/v) bovine serum albumin (BSA; Sigma), and antibiotics (penicillin G sodium 300 000 IU, penicillin G procaine 700 000 IU, and dihydrostreptomycin sulphate 1250 mg; Penivet 1; Divasa Farmavic, Barcelona, Spain). After recovery, morphologically normal embryos (morulae and early blastocysts) were vitrified. Embryos were classified as normal when they presented homogenous cellular mass and intact *zona pellucida* [17].

2.4 Cryopreservation and warming procedures

Collected embryos were vitrified and warmed using the methodology described by Vicente *et al.* [18]. Embryos were vitrified in two step addition procedure. The vitrification media contained: embryo recovery media without antibiotics

supplemented with 20% (v/v) dimethyl sulphoxide (DMSO, Sigma) and 20% (v/v) ethylene glycol (EG, Sigma) as cryoprotectants.

After storage in LN_2 (less than 6 months) embryos were warmed by submerging the straws into a water bath at 20ºC for 10s. To remove the vitrification media, the two-step procedure was used. Briefly, warmed embryos were introduced into a culture dish containing 0.7 mL of 0.33 M sucrose and 0.2% BSA in DPBS, and after 5 min embryos were washed in 0.2% BSA in DPBS before transfer.

2.5 Embryo transfers

After warming, embryos were evaluated morphologically and only those without damage in mucin coat or zona pellucida were transferred. Multiparous non-lactating females were used as recipients. Between 60 and 64 hours before transfer, recipient does were synchronised by intramuscular administration of 1 µg buserelin acetate (Hoechst, Marion Roussel, Madrid, Spain). Only females that presented vulva colour associated with receptive status were induced to ovulate. Asynchronous transfers were carried out by endoscopy as described by Besenfelder and Brem [19], the mean number of transferred embryos per doe was 8.6)

2.6 Traits measured in experimental groups

Transfer results were assessed on the basis of pregnancy rate (PR, proportion of pregnant females at 12^{th} days after transfer), fertility at birth (BR, birth rate, proportion of females that gave birth after transfer), embryo survival in pregnant females (ES, number of total born/total transferred embryos expressed as percentage) and number of born alive at birth (BA).

In females F1, F2 and their counterparts (CF1 and CF2) the reproductive traits studied were litter size (LS, number of total born at birth), number born alive at birth (BA) and postnatal survival at day 28th (PS, number of weaned/number born alive expressed as percentage). The reproductive traits were controlled from the 1st until the 4th parity order. Hence records of 640 parities from 839 matings were controlled (180, 157, 137 and 166 parities from F1, CF1, F2 and CF2 females respectively)

2.7 Statistical analyses

The reproductive traits were analysed using Bayesian methodology. The mixed model used for the variables was:

$$y_{ijklm} = m + T_i + OP_j + YS_k + d_l + e_{ijklm}$$

where y_{ijklm} is the trait to analyse; m is the general mean; T_i is the systematic effect of type of animal (F1, CF1, F2, CF2); OP_j is the systematic effect parity order (4 levels); YS_k is the systematic effect year-season with 9 levels; d_l random effect of the doe (it was assumed that the doe effects were uncorrelated); and e_{ijklm} is the residual.

Bounded flat priors were used for all unknowns. Data were assumed to be normally distributed. Marginal posterior distributions of all unknowns were estimated using Gibbs Sampling. The Rabbit program developed by Institute of Animal Science and Technology (Valencia, Spain) was used for all procedures. After some exploratory analyses, we used one chain for 1,000,000 samples, with a burning period of 200,000 and saving every 100 thereafter to avoid high correlation between samples. Convergence was tested using Geweke's Z

criterion, and Monte Carlo sampling errors (MCse) were computed using time-series procedures described by Geyer [20].

3. RESULTS

3.1 Transfer data

A total of 553 cryopreserved embryos were transferred to 60 females and resulted in 43 pregnancies. Pregnancy losses before the birth were 3. The 40 remaining pregnancies resulted in a total of 196 born, representing 35% global efficiency. The mean number of born alive per birth was 4.09.

3.2 Generation (F1 and F2) data

In all Bayesian analyses, Monte Carlo standard errors were small and lack of convergence was not detected by the Geweke test.

Features of the estimated marginal posterior distributions of litter size (LS), born alive (BA) and postnatal survival (PS) for the different groups studied were shown in Table 1.

Features of the estimated marginal posterior distributions of the differences between F1 and their counterparts (F1-CF1) and between F2 and their counterparts (F2-CF2) in reproductive characters are presented in Table 2 and Table 2, respectively. Marginal posterior distributions were approximately normal and only the posterior mean of the difference between groups is given.

Results show that the probability of the difference between females F1 and their counterparts being greater than zero ($P_{F1\text{-}CF1>0}$) is 1 for LS and BA characters. Notice that these groups at least differed in 1.11 kits and 0.94 born alive, with a

probability of 80% (k80%; Table 2). The same trend in LS and BA was observed for F2-CF2 difference, showing a probability of being greater than zero ($P_{F2-CF2>0}$) equal to 0.98 and 0.96 for LS and BA respectively. The differences between these groups in terms of LS and BA showed a guaranteed value at 80% (k80%; Table 3) of 0.55 kits and 0.50 born alive, respectively.

Regarding PS, the results showed that F1 females presented a lower PS than their counterparts, with a probability of being lower than zero ($P_{F1-CF1<0}$) equal to 0.97 (Table 2). On the other hand, this tendency is not observed in the case of F2 females, where the F2-CF2 difference observed is favourable for F2 females ($P_{F2-CF2>0}$ equal to 0.81; Table 3). However, the zero is included inside the highest posterior density at 95% of probability ($HPD_{95\%}$) in both cases, so further assumptions must be taken with caution.

4. DISCUSSION

In this study, our principal finding is that vitrification and transfer procedures of rabbit embryos have long-term and transgenerational consequences on female reproductive traits.

Effects on F1

The results in F1 females are unequivocal in showing that vitrification and transfer procedures cause a consistent increase in LS and BA. The differential phenotypes for reproductive traits found between contemporary female groups (F1-CF1) could be a result of direct action on the embryo due to manipulation prior to implantation (3 days old embryo vitrification and transfer procedures) and/or as an indirect action due to changes in maternal effects.

Regarding the environmental changes due to the direct action of vitrification and transfer procedures, previous data involving similar protocols reported short-term consequences such as altered gene expression patterns [13, 14] and reduced viability [15, 21] compared with *in vivo* contemporary embryos that were not cryopreserved and transferred. The results in our experiment show that vitrification and transfer procedures cause a decrease in embryo viability (global efficiency 35%) and these results are in agreement with those previously published [21, 22]. However, beyond the short-term effects we observed long-term effects in adult female reproduction (more than 1.4 kits per birth and more than 1.3 live born kits per birth, expressed as posterior mean of the difference between F1 females and their counterparts). These long-term effects could be provoked in part by epigenetic marks probably induced by the cryopreservation and transfer procedure and/or during the gestation period. It is known that events occurring at preimplantation stages might alter later processes in development because during this period the embryo must undergo different events, including embryonic genome activation, compaction, lineage differentiation and blastocoels formation [23]. In this sense, our findings agree with previous observations from the different ART procedures in humans and mice, where for instance the medium used for culturing IVF embryo in humans affects the birth weight of the resulting newborns [24]; or in mice, where authors observe that ART procedures can lead to morphological and behavioural features in adult mice derived from frozen embryos [25]. Nevertheless, Auroux *et al.* [26] also found a beneficial effect on longevity in adults. The same trend was also observed for embryos cloned by nuclear transfer or cultured *in vitro* in cattle and sheep, where studies revealed a disturbing “large offspring”

phenotype (for review see [27]) probably caused by the impact of these techniques on imprinting, as occurs with the Beckwith-Wiedemann syndrome (BWS) in humans [28].

In the case of indirect actions of ART techniques, when a cryopreserved population of a prolific species is rederived in order to estimate the genetic improvement, authors always observe a positive maternal effect due to the low number of implanted embryos compared with normal gestation, and usually employ animals from the second generation in order to avoid it [29, 30]. In our case, F1 females came from small litters (mean LS: 5.11). This low number of implanted embryos provides a better uterine environment for foetuses, probably causing different epigenetic marks than those provided to foetuses gestating in a control population with normal litter size [11], and this better uterine environment finally provides better reproduction fitness in these animals [31].

Effects on F2

Our data also indicate that effects of ART can be observed in the F2 generation. In this work, females from F2 generation (in contrast to females from F1) came from larger litters (LS: 10.69) than the contemporary ones (F2; LS: 9.20), so we expected to observe a reduced or zero difference in litter size due to maternal effect (more foetuses in uterus), but surprisingly the LS in F2 females was higher than C2, supporting the idea that heritable transgenerational effects could be possible. Evidence for transgenerational impacts have previously been confirmed in rodents, where the prenatal protein restriction on F0 can exert effects on growth and metabolism of F1 and F2 generation through changes in methylation status of glucocorticoid receptor [32]. However, since in mammals

247 gametes are formed during foetal development, if the environmental effect has
248 occurred during pregnancy, then F1 (an embryo) and F2 (its future gametes)
249 progenies have a chance to experience this environmental effect [33], so we
250 could not conclude that vitrification and transfer provokes heritable
251 transgenerational effects. Further studies on F3 generation should address this
252 possibility.

253 In conclusion, we report that the females derived from cryopreserved and
254 transferred embryos (F1 females) have evidence of increased reproductive
255 traits compared to contemporary ones. We have also shown that these ART
256 procedures influence the future litter size of female F1 offspring (F2 females).

257 Future studies on F1 and F2 female tissues will have to be designed to provide
258 insights into epigenetic control regions related with reproductive traits in rabbits.

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356

Table 1.- Features of the estimated marginal posterior distributions of litter size (LS), born alive (BA) and postnatal survival (PS, %) for the different groups studied.

	F1 (PM) [$HPD_{95\%}$]	**CF1 (PM) [$HPD_{95\%}$]**	**F2 (PM) [$HPD_{95\%}$]**	**CF2 (PM) [$HPD_{95\%}$]**
LS	10.69 [9.50, 11.88]	9.20 [7.86, 10.59]	11.81 [10.46, 13.09]	10.87 [9.79, 12.05]
BA	9.71 [9.50, 11.88]	8.33 [7.86, 10.59]	11.03 [10.46, 13.09]	10.07 [9.79, 12.05]
SP (%)	75.65 [65.53, 85.56]	82.16 [70.93, 94.03]	85.74 [74.50, 96.78]	82.54 [72.64, 91.95]

F1: cryopreserved does; CF1: Contemporary does to F1 does;

F2: females offspring from F1; CF2: Contemporary does to F2 does

PM = posterior mean;

$HPD_{95\%}$ = highest posterior density interval at 95%

365

366 **Table 2:** Descriptive statistics of the posterior marginal distributions of the
367 estimable functions between contemporary types of does (F1and CF1), for litter
368 size at birth (LS), born alive (BA) and postnatal survival (PS, %).

	LS_{F1-CF1}	BA_{F1-CF1}	PS_{F1-CF1}, %
PM	1.48	1.38	-6.51
$HPD_{95\%}$	0.66, 2.35	0.40, 2.37	-13.0, 0.38
$P(_{F1-CF1}>0)$,%	100	100	3
k80%	1.11	0.94	-3.62

369 PM = posterior mean of the difference between F1 and CF1 females.

370 $HPD_{95\%}$ = highest posterior density interval of the difference at 95%

371 $P(_{F1-CF1}>0)$= Probability of PM being higher than zero

372 k80% = guaranteed value at 80% of probability.

373

374

375 **Table 3:** Descriptive statistics of the posterior marginal distributions of the
376 estimable functions between contemporary types of does (F2 and CF2), for litter
377 size at birth (LS), born alive (BA) and postnatal survival (PS, %).

	LS_{F2-CF2}	BA_{F2-CF2}	PS_{F2-CF2}, %
PM	0.94	0.96	3.19
$HPD_{95\%}$	0.01,1.81	-0.07, 2.04	-3.92, 10.57
$P(_{F2-CF2}>0)$,%	98	96	81
k80%	0.55	0.50	0.07

378 PM = posterior mean of the difference between F2 and CF2 females.

379 $HPD_{95\%}$ = highest posterior density interval of the difference at 95%

380 $P(_{F2-CF2}>0)$= Probability of PM being higher than zero

381 k80% = guaranteed value at 80% of probability.

382

383