IN VIVO DEVELOPMENT OF VITRIFIED RABBIT EMBRYOS: EFFECTS ON PRENATAL SURVIVAL. PRELIMINARY RESULTS

MOCE M.L.[1], SANTACREU M.A., CLIMENT A., PEIRO R., BLASCO A.

Departamento de Ciencia Animal, Universidad Politécnica de Valencia,
46071 Valencia. Spain.

ABSTRACT

The aim of this work was to study the distribution of the prenatal mortality in vitrified and fresh embryos. Animals came from an experiment of divergent selection on uterine capacity. The embryos were obtained *post-mortem* from 181 donor does 72 hours after mating. Embryos were kept at room temperature or vitrified before the transference. Vitrified embryos were transferred into a total of 22 does and fresh embryos were transferred into a total of 45 does. The number of embryos transferred per Fallopian tube was standardized to 8, so each recipient doe received 16 embryos. Recipient does were slaughtered on day 28 of gestation. Vitrification procedure did not affect pregnancy rate (85% *vs* 80% for vitrified and fresh embryos respectively). Number of implanted embryos, live fetuses at d 14, 17 and 28 was similar for vitrified and fresh embryos. Vitrification procedure seems to affect the early fetal survival but not the late fetal survival. Fresh embryos had a higher early fetal survival than vitrified embryos (difference of 1.25 fetuses per uterine horn), but late fetal survival was high and similar in both. The vitrification procedure used in our experiment seems to damage embryos in such a way that the early fetal development is compromised**.**

Key words: rabbit, embryo, vitrification, prenatal survival, cryopreservation.

INTRODUCTION

Embryo cryopreservation is widely used to preserve genetic resources. Embryo vitrification is a technique that permits the rapid cooling of liquid medium in the absence of ice crystal formation by using high levels of cryoprotectans (DOBRINSKY, 2001). Vitrification is a technique more simple and cheaper than embryo freezing, but embryo exposure to high levels of cryoprotectant additives (CPAs) can have deleterious effects on the development of the embryo after devitrification (LEONI *et al.,* 2003).

Several protocols have been developed for the vitrification of rabbit embryos obtaining successful results after conducting *in vivo* viability tests (for example VICENTE *et al.,* 1999 and LÓPEZ-BÉJAR and LÓPEZ-GATIUS 2000).

[1] Present address: Departamento de Producción Animal y Ciencia y Tecnología de los Alimentos. Universidad Cardenal Herrera-CEU. Edificio Seminario. 46113 Moncada, Valencia. Spain.

The aim of this work was to compare the prenatal survival of embryos vitrified using the procedure described by VICENTE *et al.* (1999) with the prenatal survival of fresh embryos.

MATERIAL AND METHODS

Animals

Animals used as donors and recipients came from an experiment of divergent selection on uterine capacity. The two lines were generated from a synthetic population bred at the experimental farm of the Universidad Politécnica de Valencia. Both were divergently selected for 10 generations, and selection was relaxed from the 11th generation to the 15th generation. Animals were housed at the experimental farm of the Universidad Politécnica de Valencia in individual metal cages. Animals were kept under controlled 16h light: 8h dark photoperiod and fed a commercial diet.

Embryo Recovery

Contemporary does from the lines selected for High (H) and Low (L) uterine capacity were used. Does came from the 13th, 14th and 15th generations: 89 does from H line and 92 does from the L line. Unilateral ovariectomies were performed on females from the 13th generation at 14 to 16 wk of age (ULO females). Females from the 14th and 15th generations were not unilaterally ovariectomized (INTACT females). Natural matings were carried out with males from the same line of the donor females. Does were slaughtered by stunning and exsanguination at 72 to 75 h post-coitum. The oviducts and the first one third of the uterine horns were excised and flushed once with 5 mL of Dulbecco´s Phosphate Buffered Saline (®DPBS, Sigma) supplemented with $CaCl_2$ (0.132 g/L), 0.2% of bovine serum albumin (®BSA, Sigma, Alcobendas, Madrid, Spain) and antibiotics (penicillin G sodium 300.000 IU, penicillin G procaine 700.000 IU and dihydrostreptomycin sulfate 1250 mg; ®Penivet 1, Divasa Farmavic, Barcelona, Spain) at room temperature. After recovery, morphologically normal embryos were kept at room temperature until transfer or vitrification. A total of 712 fresh and 320 vitrified embryos were transferred.

Embryo Vitrification

Embryos from unilaterally ovariectomized does and embryos from intact does were vitrified and devitrified using the method describe by VICENTE *et al.* (1999). The vitrification media had the following composition: 1:1:2 (v/v/v) of dimethyl-sulphoxide (®3.5 M DMSO, Sigma), ethylene glycol (®4.4 M EG, Sigma) in DPBSCa (DPBS supplemented with 0.132 g $CaCl_2$/L), and supplemented with BSA (2 g/L).

Embryo Transfers

Recipient females came from the 15th generation: 35 does from the High line and 32 does from the Low line were used. Vitrified embryos were transferred into a total of 22 does and fresh embryos were transferred into a total of 45 does. Nulliparous females 19 to 20 wk of age were used as recipients. Twenty one days before the transfer, recipient does were synchronized by i.m. administration of 1 µg busereline acetate (®Hoechst,

Marion Roussel, Madrid, Spain). Only females that presented vulva color associated with receptive status were induced to ovulate with a second injection of busereline acetate. The second injection was administered 72 h before transfer when fresh embryos were transferred, and 60-63 hours before transfer when vitrified embryos were transferred. To perform the transfers, rabbits were anaesthetized with an i.m. injection of Xylazine (®Rompun 2%, Bayer AG, Leverkusen, Germany) at a rate of 0.2 mL/kg body weight; 5 min later this injection was followed by a i.v. dose of 2-3 mL of Ketamine HCL and clorbutol (®Imalgène 500, Merial-Lyon, France) in the marginal ear vein. Embryo transfers were performed by using the laparoscopic technique described by BESENFELDER and BREM (1993). The number of embryos transferred per Fallopian tube was standardized to 8, so each recipient doe received 16 embryos (eight embryos from High line into one oviduct and eight embryos from Low line into the other one). Transfers to right or left uterine horn were randomized. Transferred vitrified embryos came from a mixture of embryos from unilaterally ovariectomized and intact does (31 uterine hors) or only from intact does (9 uterine horns). Fresh embryos came always from intact females (89 uterine horns).

Traits

For each uterine horn, number of live fetuses (LF14) was recorded by laparoscopy at d 14 of gestation. Number of implanted embryos (IE) was estimated as total number of embryos in the uterus (LF14 + dead embryos). Recipient females were slaughtered at d 28 of gestation and, for each uterine horn, number of live fetuses (LF28) and number of dead fetuses (ND28) were recorded. Number of live fetuses at d 17 of gestation (LF17) was estimated as LF28 + ND28. Pregnancy rate was defined as the total number of uterine horns having at least one live fetus at d 28 of gestation divided by the total number of uterine horns.

Statistical Analysis

A chi-squared test was performed to determine the effect of the embryo treatment (vitrified or fresh embryos) on pregnancy rate. Only pregnant uterine horns that had at least one fetus alive at 28 d of gestation were included in the other analyses. Least–squares analyses were performed on the variables IE, LF14, LF17 and LF28 with the effects of embryo treatment (vitrified or fresh), recipient by donor line (HRHD: high recipient with high donor, HRLD: high recipient with low donor, LRHD: low recipient with high donor, LRLD: low recipient with low donor), origin of embryo (mixture of embryos from unilaterally ovariectomized and intact females or only embryos from intact females) and female as random effect. In order to study fetal survival, additional analyses of LF17 and LF28 included IE as a covariate. Number of live fetuses at d 28 of gestation was also analyzed including LF17 as a covariate. The MIXED procedure of the SAS statistical package was used for these analyses.

RESULTS AND DISCUSSION

Vitrification procedure did not affect pregnancy rate (85% *vs* 80% for vitrified and fresh embryos, respectively). Number of implanted embryos was similar for vitrified and fresh embryos (Figure 1). As the uterine horns received equal number of embryos, embryo survival was the same for vitrified and fresh embryos. VICENTE *et al.* (1999) using the same vitrification procedure observed an embryo survival of 70% which is similar to the embryo survival obtained in this experiment (71%). We did not find any significant effect of the embryo treatment on number of live fetuses at d 14, 17 and 28 (Figure 1).

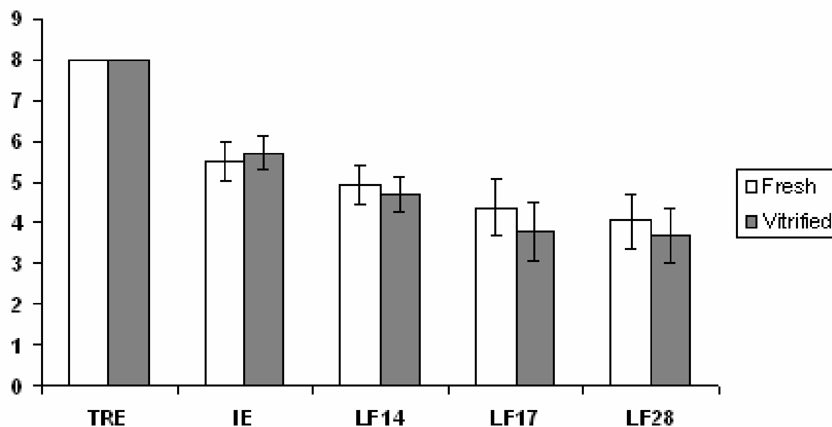

Figure 1. Least-squares means with their standard errors for number of transferred embryos (TRE), number of implanted embryos (IE), number of live fetuses at d 14 of gestation (LF14), at d 17 of gestation (LF17) and at d 28 of gestation (LF28) for fresh and vitrified embryos. Results are reported per uterine horn.

Number of live fetuses at 28 day of gestation was studied including IE as a covariate in order to clarify whether the vitrification procedure affected fetal survival (Table 1). We did not find any effect of the vitrification procedure on fetal survival, but differences between fresh and vitrified embryos were relevant (0.89 fetuses by uterine horn). Fetal survival was divided into early fetal survival (from d 7 to d 17 of gestation) and late fetal survival (from d 17 to d 28 of gestation). To study early fetal survival, number of live fetuses at d 17 of gestation was studied including IE as a covariate, and to study late fetal survival LF28 was analyzed including LF17 as a covariate. Fresh and vitrified embryos differed for early fetal survival but not for late fetal survival (Table 1). Fresh embryos had a higher early fetal survival than vitrified embryos (difference of 1.25 fetuses per uterine horn), but late fetal survival was high and similar in both (Table 1).

Rabbits develop two kinds of placentas. The yolk sac placentation regresses around 10 day of gestation and serves as the primary placental organ prior to establishment of the chorioallantoic placenta. The chorioallantoic placenta begins its development at implantation time and finishes it around 12 day of gestation (AMOROS 1952 cited by ADAMS, 1960). Thus, time between day 10 and 12 is a critical period for fetal survival. As

indicated before, high levels of CPAs are used in vitrification solutions and they can affect the embryo development, thus the greater early fetal mortality showed by vitrified embryos could be due to vitrification procedure affecting trophoblast cells and consequently the formation of the placenta. In cattle, VAJTA *et al.* (1997) observed that from 0 to 4 hours after warming a large numbers of injuries can be observed in both trophoblastic and embryonic cells, however at 24 hours after warming most of the injuries disappeared. Furthermore, in humans, THIRKILL AND DOUGLAS (1997) observed that dimethyl sulfoxide (which is one of the components of the vitrification solution used in this experiment) inhibited the differentiation of cytotrophoblast to syncytiotrophoblast in vitro, although the effects were reversible when DMSO was eliminated.

Table 1. Least-squares means for fresh embryos and differences between fresh and vitrified embryos per uterine horn

Trait	Covariate	Fresh ± SE	(Fresh - Vitrified) ± SE	Sig
LF17	IE	4.93 ± 0.32	1.25 ± 0.54	*
LF28	LF17	4.21 ± 0.15	-0.25 ± 0.27	ns
LF28	IE	4.55 ± 0.32	0.89 ± 0.56	ns

IE: number of implanted embryos, LF17: number of live fetuses at d17 of gestation, LF28: number of live fetuses at d 28 of gestation.
Sig: Significance level for the difference (Fresh – Vitrified);* $P < 0.05$

CONCLUSION

The vitrification procedure used in our experiment seems to damage the embryos in such a way that the early fetal development is compromised. The study of the causes which induce a greater early fetal mortality in cryopreserved embryos would be helpful to optimize the results of vitrification procedures.

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