EFFECT OF VITRIFICATION AND CRYOSTORAGE LENGTH ON VIABILITY OF RABBIT EMBRYOS AFTER THAWING

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ABSTRACT

The effects of vitrification and cryostorage length in liquid nitrogen (LN2) on *in vitro* survival of rabbit embryos were examined. A total of 374 morphological normal embryos collected from 20 superovulated multiparous New Zealand White rabbit were divided into two groups. The first group (n=94) was non-vitrified used as a fresh control group and the second group (n=280) was vitrified and storage in liquid nitrogen at three different times (2 days, 6 months or one year). Regardless the cryostorage length in LN2, post thawing morphological normal appearance rates of cryopreserved rabbit embryos were significantly affected by vitrification procedure. Only 62.1-67.4% from embryo vitrified-thawed appears undamaged. Blastocyst and hatching rates were decreased ($P<0.05$) in vitrified group compared to the control group, whilst no difference was observed among different storage time in liquid nitrogen. Blastocyst and hatching rates ranged between 51.7-58.9 and 39.8-47.4% in vitrified-thawed embryos groups compared to 94.7 and 90.4% in control group. Significance difference was observed in embryo diameter between vitrified groups and control group (123-124 *vs.* 129 μm). Blastocyst and hatching rates did not vary according to the cryostorage length from 2 days until 1 year in LN2. In a same way, no significant differences were found in diameter of warmed embryos when the cryostorage increases. Our results indicate that compared to the control group, the vitrification process decreases the *in vitro* embryo development. In contrast, the cryostorage length in liquid nitrogen did not affect the *in vitro* development of rabbit embryos (≥16-cells stage).

Key words: Vitrification, storage time, rabbit embryos.

INTRODUCTION

Vitrification is one of the most widely used techniques for embryo cryopreservation and preserve genetic resources. Vitrification, defined as the solidification of a solution at low temperature by extreme elevation in viscosity during cooling without ice crystallization (Massip, 2001), has been increasingly used to replace slow freezing in the past decade (Dobrinsky, 2002). The glass state formed during vitrification has the same ionic and molecular distribution as the liquid phase, thus avoiding both chemical and mechanical damage to embryos. Several protocols have been developed for the vitrification of rabbit embryos, obtaining successful results after conducting *in vivo* viability tests (Kasai *et al*., 1992; Lopez-Bejar and Lopez-Gatius, 2002; Vicente *et al*., 2003; Moce *et al.*, 2010; Chrenek and Makarevich, 2011). Structurally intact embryos and high morphological quality grade may be essential components for successful rabbit embryo cryopreservation.

Storage of embryos is important and useful for creation of gene banks of rare animals, but also for clinical practice in the sphere of human reproduction. Survival of cryopreserved rabbit embryos may be affected by another important factor such as the length of storage in liquid nitrogen (LN2). In mouse embryos, increasing the storage duration reduces the viability and increases the chromosome aberrations of embryos (such as aneuploidy and polyploidy, Mozdarani and Moradi, 2007). The aim of our work was to investigate the effect of vitrification procedure, and the length of rabbit embryo storage in liquid nitrogen on their *in vitro* post-thawing survival rate and embryo diameter.

MATERIALS AND METHODS

This experimental work was carried out at the laboratory of Reproduction and Biotechnology, Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt. A total of 374 morphological normal embryos collected from 20 superovulated multiparous New Zealand White rabbit were used in the present work. Embryo donors were administered intramuscularly 20 IU PMSG/kg live weight (Folligon, Intervet, International B.V. Boxineer-Holland), 68 h before artificially insemination. Immediately prior to insemination, females were intravenously injected with 40 IU/kg live weight of hCG (Pregnyl, Organon, Nile Co., Egypt). Donors were artificially inseminated with semen collected from a male of proven fertility from the same breed. The embryos were recovered by flushing oviducts (48-50 h post insemination) twice with 5 mL of Dulbecco´s Phosphate Buffered Saline (®DPBS, (PBS: Gibco, Cat. No 21300-017, UK) supplemented with $CaCl_2$ (0.132 g/L), 0.2% (w/v) of bovine serum albumin (®BSA, Sigma Chemical Co., St. Louis, Mo, USA) and antibiotics (10,000 IU Penicillin G potassium+ 10 mg streptomycin sulfate/ml, Sigma) at room temperature (20-25 ºC). After recovery, embryos were washed twice in fresh DPBS supplemented with 10% FCS and antibiotics, counted and morphologically evaluated under stereoscopic microscope. Embryos with no abnormalities in mucin coat, *zona pellucida* and with homogenous blastomeres were scored as excellent and good embryos (grade 1 or 2) according to International Embryo Transfer Society classification. The other embryos were considered as grade 3 embryos (poor quality). Morphologically normal embryos (grade 1 or 2) from each donor doe were pooled and divided into two groups. The first group of embryos (n=94) was non-vitrified used as a fresh control group and the second group (n=280) was vitrified using procedure described by Vicente *et al*. (1999). Vitrified embryos were storage in liquid nitrogen at three different times (2 days, 6 month or one year).

The cryoprotective solution was a 1:1:2 solution (v/v/v) of dimethyl-sulfoxide (3.5 M DMSO, Sigma), ethylene glycol (4.4 M EG, Sigma), in DPBS+0.132 g $CaCl_2$/L+0.2 (w/v) BSA/L of cryoprotective solution. Vitrification was carried out in two steps. First, normal embryos were pipetted into 0.2 ml of PBS medium and placed in a culture dish and then 0.2 ml of the cryoprotective solution was added and agitated. Embryos were kept in this medium for 2 minutes. In the 2nd step, 0.6 ml of the cryoprotective solution was added and quickly agitated. Then, embryos suspended in the final vitrification solution were loaded into 0.25 ml plastic straws (IMV, L'Aigle, France), sealed and plunged directly into liquid nitrogen. The exposure time of embryos to the final vitrification solution did not exceed 1 minute. The two vitrification steps were carried out at 20 ºC. The straws contained three sections separated by air bubbles. The first consisted of PBS in the cotton plug, the second section contained the embryos suspended in vitrification medium (0.1 ml) and the third section consisted of PBS. The straws were sealed and identified. Each straw held between 5 to 8 normal embryos. Thawing was performed by immersing the straws in a water bath at 20 ºC for 10-15 sec. The cryoprotective solution was removed from the embryos in a two step dilution procedure at room temperature (20-25 ºC). Embryos suspended in the final vitrification solution were released into a culture dish containing 1 ml of 0.33 M sucrose in PBS medium. After 5 minutes, embryos were washed twice in fresh PBS medium and morphologically scored before culture. Only embryos with homogenous cell mass and intact zona pellucida were *in vitro* cultured.

The non-vitrified (fresh) and warmed embryos were cultured in a standard *in vitro* culture condition for 72 h in 50 µl microdrops of Ham's F10 medium+ 20% FBS (Sigma) under mineral oil (Sigma) at 38.5 °C in an incubator containing 5% CO2 and humidified air. The *in vitro* cleaved and development ability of non-vitrified and devitrified embryo at the different cryostorage length and quality were assessed and recorded for analysis.

Other non-invasive parameter for testing embryo viability seems to be the embryo diameter (Makarevich *et al.*, 2006). After warming, embryo diameters excepting zona pellucida, were measured from the same images on the screen of the monitor using scale bar micrometer, which was previously calibrated on a ×40 objective and ×10 eyepieces.

Chi-squared test was used with data in percentage. Data for embryo diameters were expressed as mean values±S.E. and assessed by analysis of variance using GLM procedure of SAS® Program (1998). Differences between groups at P<0.05 were considered as significant.

RESULTS AND DISCUSSION

Vitrification procedure

Regardless the cryostorage length in LN2, post thawing morphological normal appearance rates of cryopreserved rabbit embryos were significantly affected by vitrification procedure (Table 1). Only 62.1-67.4% of vitrified-thawed embryos appears undamaged. In addition, *in vitro* embryo development (percentage of blastocysts and hatching rates) significantly differed (P□0.05) between vitrified embryos and fresh ones regardless the duration of cryostorage in liquid nitrogen. *In vitro* blastocyst and hatching rates ranged between 51.7-58.9 and 39.8-47.4% in vitrified-thawed embryos groups compared to respectively, 94.7 and 90.4% in the control group. Results indicated that after thawing, the percentage of blastocysts and hatching blastocysts were significantly affected by vitrification process. In a same way, significance difference was observed in embryo diameter between vitrified groups and fresh control group (123-124 *vs*. 129 µm). These differences could be related to the composition of the vitrification solution (permeating cryoprotectants and non-permeating macromolecules or saccharides) influencing the embryo cryosurvival. These results are comparable with those obtained by Popelkova *et al.* (2009) who found that rabbit blastocysts and hatching rates in the vitrified EG+Ficoll (63 and 63%, respectively) group were significantly lower compared to a control group without vitrification (97 and 97%, respectively). Popelkova *et al.* (2005) also observed high significant differences in hatching rates between open-pulled straw vitrified rabbit embryos (56%) and non-frozen ones (94%). In recent studies, Moce *et al.* (2010) observed that embryo vitrification in rabbits affects fetal survival, increasing mortality soon after implantation. Chrenek and Makarevich (2011) observed that vitrification procedure caused decrease in total cell numbers of vitrified hFVIII- positive rabbit embryos compared to the control group (117.00 ± 36.00 *vs*. 141.00 ± 34.80).

Table 1: Effect of vitrification and cryostorage length on morphologically normal embryos, embryos developed *in vitro* and diameter (µm) of rabbit embryos post-thawing.

Items	Fresh† (control, n=94)	Storage time in LN2††		
		Two days	Six months	One year
No. embryos vitrified	0	95	98	87
Morphologically normal embryos post-thawing, n (%)	-	64 (67.4)	61 (62.2)	54 (62.1)
Blastocysts, n (%)	89 (94.7)^{a}	56 (58.9)^{b}	51 (52.0)^{b}	45 (51.7)^{b}
Hatching blastocysts, n (%)	85 (90.4)^{a}	45 (47.4)^{b}	39 (39.8)^{b}	36 (41.4)^{b}
Embryo diameter (±SE)	129^{a}±1.1	124^{b}±1.3	123^{b}±1.3	124^{b}±1.4

† Percentage based on the number of morphologically normal embryos recovered and cultured *in vitro* (94 embryos).
†† Percentage based on the number of vitrified embryos.
^{a,b} Values with different superscripts in the same column differ significantly (P<0.05).

A large number of studies in other species have reported that the vitrification procedures cause several structural and biochemical changes that may lead to loss of embryo viability. Vitrification caused morphologic changes and DNA fragmentation in pig embryos (Fabian *et al*., 2005) and a negative effect on the ultrastructure of cattle embryos (Fair *et al*., 2001). Moreover, Zhao *et al*. (2009) observed that vitrification altered mitochondrial distribution and decreased the mitochondrial membrane potential in mouse two pronuclear embryos. Concerning biochemical changes, ovine embryos needed 9 to 12 h of culture to complete resumption of DNA synthesis and 29 to 35 h to reacquire the capacity of protein secretion after warming. Moreover, the secretion pattern was qualitatively different, although the secretory activity was quantitatively completely restored (Leoni *et al*., 2003). In addition,

Dhali *et al.* (2007) found that vitrification and warming procedures alter the transcriptional activity of some apoptosis-related genes in mouse.

Cryostorage length in LN2

Results in Table 1 showed that morphological normal appearance and hatching rates of cryopreserved rabbit embryos were not affected by the duration storage in liquid nitrogen (2 days, 6 months and one year). Only 64 from 95 (67.4%) vitrified embryo stored during 2 days in LN2 appears morphologically normal compared to 61/98 (62.2%) vitrified embryo stored during 6 months and 54/87 (62.1%) embryo stored during one year. The percentage of blastocysts and hatching rates were not influenced by the cryopreservation length. In a same way, no significance differences were observed in embryo diameter among vitrified groups according to different storage time in LN2. Our results indicate that the duration of embryo storage in liquid nitrogen did not affect the *in vitro* development of rabbit (≥16-cell stage) up to advanced hatching blastocyst stage and their diameter. These results are in accordance with those reported by Koprdova *et al*. (2009) who did not notice significant differences in the survival rate of rabbit embryos vitrified at the morula stage after different cryostorage length in liquid nitrogen (1-5 hours or 1 week-1 year). Salvetti *et al*. (2007) and Lavara *et al.* (2011) demonstrated that frozen and vitrified rabbit embryos can be stored in liquid nitrogen for 15 years without effects on survival rate at birth. In contrast, in mouse, Mozdarani and Moradi (2007) reported that increasing the storage duration in liquid nitrogen (24 hours, 1-2 weeks, 1-6 months) reduces the viability and increases the chromosome aberrations of embryos (such as aneuploidy and polyploidy).

CONCLUSIONS

This study evidenced that compared to a control group (fresh embryos), the *in vitro* rabbit embryos development was negatively affected by vitrification. In contrast, the cryostorage length (2 days, 6 months and one year) of embryos in liquid nitrogen did not affect the *in vitro* post-thawing development of rabbit embryos (≥16-cell stage). Further studies are needed to improve the cryopreservation techniques in rabbit species.

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