IN VITRO VIABILITY OF SPLIT RABBIT EMBRYOS BEFORE AND AFTER VITRIFICATION

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ABSTRACT

Of 125 embryos (category I and II) recovered from 18 does, 25 were maintained intact as control, 25 bisected using a simplified splitting protocol, 25 frozen thawed according to a vitrification method, 25 bisected-vitrified, and 25 vitrified-bisected. The survivability of intact embryos and demiembryos were evaluated after 24 hours of *in vitro* culture by development ability, morphological assessment, and cell count with double staining method. The development rate after *in vitro* culture of intact control embryos (96%) was higher ($P<0.001$) than vitrified embryos (36%). The development rate of demiembryos was higher (74%) than bisected-vitrified embryos (34%), and vitrified-bisected embryos (10%) as well ($P<0.001$). A higher number of cells and percentage of surviving cells was observed in control embryos (91%), followed by fresh demiembrios (84%), vitrified embryos (74%) ($P<0.01$), bisected-vitrified demiembryos (67%), and vitrified-bisected demiembryos (61%). Embryos and demiembryos originated from category I embryos showed higher development rate than those obtained from category II. The reduced survivability for the vitrified-bisected embryos might be due to the combined effects of loss of cohesion and reduction of cells as a result of the bisection and to the crioprotectant toxicity during hydratation. In conclusion, bisection and vitrification can be used together without micromanipulation and freezing complex systems, but the embryo quality is the main factor in the success of these procedures.

Key words: Vitrification, Cryopreservation, Embryos.

INTRODUCTION

Production of normal offspring, including monozigotic multiplets from microsurgically split embryos, has been reported in many mammalian species (Celestinos and Gatica, 2002; Tanaka *et al*., 1997). These techniques are expected to provide the investigators with monozygotic multiplets, enabling them to increase the accuracy and efficiency of the experiments. Production of multiple offspring from a single embryo implies that the reproductive efficiency of domestic animals may be increased, especially in the superior and scare breeding stocks (Romo, 2000).

Many of the modern techniques such as identical twins, genotyping and sex determination by PCR, require a biopsy or a half embryo (Herr and Reed, 1991; Sparks *et al.,* 1994; Bredbacka *et al*., 1995). Since some of these techniques are rather time consuming, it is often needed to store the half embryo frozen (Schmidt *et al*., 1992).

Vitrification of embryos is a useful cryopreservation method since it does not necessitate expensive freezing equipment such as a programming freezer. Besides, the method can eliminate the extra and intracellular ice formation which will be lethal to embryos. Since the first successful report on mouse embryos by Rall and Fahy (1985), several methods have been proposed (Sommerfeld and Niemann, 1999; Saito and Imai, 1997). These methods include the combination of permeating and non permeating cryoprotectants (Arav *et al*., 2000) in a solution that requires high concentration of these cryoprotectants to achieve vitreous state.

The success of vitrification of embryos depends on avoiding both cryoprotectant toxicity and osmotic injury. This could be controlled by the selection and concentration of appropriate crioprotectants, time and temperature during the exposure (Vajta *et al*., 1997). Because embryos are sensitive to split and cryopreservation, these technologies must be carefully selected, performed, and coordinated. Literature is available on conventional freezing demi-embryos, vitrification and embryo splitting (Schmidt *et al.,* 1992; Gustafsson *et al.,* 1994; Bredbacka *et al.,* 1995; Vajta *et al*., 1997; Rho *et al.,* 1998). However, the overall efficiency of the split-cryopreservation technology and surviving of split embryos after vitrification, has not been studied so far.

The aim of the present study was to evaluate the degree to which the split process may influence the freezability of embryos (early blastocyst or compact morulae).

MATERIALS AND METHODS

Embryo collection

A total of 157 embryos were recovered 96 hours after mating, from 18 superovulated does (8.7 embryos for doe). Of these, 125 blastocysts presented the morphologic quality (Category I and II) and stage of development expected for the moment of recovery, while 32 embryos degenerate or classified as category III (Linder and Wright, 1983) were rejected from the experiment. Of 125, 25 were cultivated intact as control, 25 bisected, 25 vitrified, 25 bisected-vitrified, and 25 vitrified-bisected.

Microsurgical splitting of embryos

Microsurgical splitting of embryos was performed according to the method developed by Britton (1988) by using a Narishige micromanipulators and Nikon inverted microscope with the aid of phase-contrast illumination at 100X. A microblade prepared from a razor blade attached to a micromanipulator was used to split the embryos. The zonae pellucidae of embryo was softened by incubation in Pronase solution (0.2% in D-PBS) for about 30 sec at room temperature, followed by thorough washing in culture medium. Each embryo was placed in a 60 µl drop of medium (D-PBS 20% FCS) on a microscope slide at room temperature and then splitted into halves using the "scratched technique". This technique involves production of scratches parallel to the orientation of the microblade on the bottom of a plastic Petri dish to avoid slipping of the embryo during splitting. The embryo was then bisected by a vertical movement of the microblade. Morulae were splitted through the central axis of the embryo, while blastocysts were splitted symmetrically so that the inner cell mass (ICM) and trophoblast was divided into two approximately equal fragments. Demi-embryos kept zona pellucida free.

Vitrification of embryos

The vitrification of embryos was performed following the method developed by Saito and Imai (1997). The basic constituents of the vitrification solutions were 20% (v/v) glycerol, 20% (v/v) ethylene glycol (M.W=8,000), 3M sucrose, 3% (w/v) polyetilene glycol and xylose in D-PBS. In all the vitrifications solutions (VS), the equilibration to each solution was done in three steps as follows: (VS1) 10% glycerol + 0.1 M sucrose + 1% polyethylene glycol + 0.1 xylose, VS2 10% glycerol + 10% ethylene glycol + 0.2 M sucrose + 2% polyethylene glycol + 0.2 M xylose, and VS3 20% glycerol + 20% ethylene glycol + 0.3 M sucrose + 3% polyethylene glycol + 0.3 M xylose. In all three types of solutions, exposure times to each step was 5, 5, and 1 min for VS1, VS2, and VS 3, respectively. Whole equilibration procedures was carried out at room temperature.

Immediately after exposition to the final VS each embryo was loaded into a 0.25 ml straw (L Aigle, France) as follows: 5 mm of D-PBS + 0.5 M sucrose + 20% FC (S-PBS), air bubble (AB), 50 mm of S-PBS, AB, 5 mm of SV3, AB, 10 mm of VS3 with the embryo, AB and 5 mm of VS3 in length. After loading, both sides of the straw were heat-sealed; then the slanted straws were slowly immersed into

liquid nitrogen from the cotton plug side (the embryos in the solution remain near the upper end of the straw). This process was completed within 1 min from the exposure to the last solution until the plunging into liquid nitrogen as the final step of the equilibration. After storage in liquid nitrogen for more than one week, the straws were warmed up in 20ºC water until the crystallized S-PBS began to melt (about 10 sec). Then the content of the straw was expelled into an empty Petri dish and mixed by gently shaking the dish. The embryos were transferred to 0.5 M sucrose solution (S-PBS) and then to 0.25 M sucrose solution (0.5 S-PBS) for 5 min each and washed twice in D-PBS + 20% FCS. Whole dilution procedures after warming were carried out at room temperature.

In vitro viability assay

After treatment, the embryos and hemiembryos were placed into a small droplet of medium D-PBS with 10% FCS covered with liquid paraffin in plastic Petri dishes and cultured in an atmosphere of 5% CO_2 in air at 38.5ºC. After 24 h of culture, development, morphology and quality of embryos were evaluated. The survival of both treated and control embryos was based on general appearance. Embryos and hemiembryos were considered viable if they advanced to the next developmental stage.

Staining

The procedure of double staining (DT) (Iwasaki *et al.,* 1990) was carried out in 24 intact embryos, 37 fresh hemiembryos, 9 vitrified embryos and 17 hemiembryos bisected-vitrified after 24 hours of *in vitro* culture. DT procedure allowed the cellular count and the distinction of the alive (blue) nucleus and the dead (red) nucleus. The embryos and hemiembryos were evaluated with Sigma Scan Pro, a professional scientific image analysis to count nuclei from digitized images of embryos.

Statistical analysis

The viability of rabbit embryos after 24 h of culture were analyzed by χ^2 test. The cell number, percentage of alive cells, and percentage of cellular loss after treatment of embryos were analyzed by analysis of variance (ANOVA). Differences at a probability value (P) of 0.05 or less were considered significant.

RESULTS AND DISCUSSION

Morphological appearance and post culture development

The percentage of development after 24 h (Table 1) of culture was higher in control intact embryos (96%), than in vitrified intact embryos (36%). Among the bisected embryos the fresh one had greater percentage of development (74%) than those submitted to vitrification (34%) and those bisected after vitrification (10%). All the observed differences were (P<0.001) significant.

Table 1: Percentage of development post culture (24 hours) of every experimental group of 25 embryos

Blastocyst	Group	No. embryos (% of development)	Development on the basis of original embryos
Fresh	Intact	24 (96)^a	96%
	Bisected	37 (74)^b	148%
Vitrified-Defrosted	Intact	9 (36)^c	36%
	Bisected –vitrified	17 (34)^c	68%
	Vitrified –bisected	5 (10)^d	20%

a, b, c, d: Different letters in the same column indicate significant difference (P <0.001)

Number of total cells and percentage of alive cells post culture

The intact embryos presented greater (P<0.001) percentage of alive cells than cultivated hemiembryos and vitrified embryos. The cultivated hemiembryos had greater proportion of alive cells than embryos submitted to both procedures. Only the blastocysts and hemiblastocysts from embryos qualified as category I presented development. Table 2 shows the cellular number, percentage of cellular loss after treatment and percentage of alive cells.

Table 2: Cellular number, percentage of cellular loss and percentage of alive cells in embryos and hemiembryos after 24 hours of *in vitro* culture

Blastocyst	Group	No.	Cellular Number (% of cellular loss)	% of alive cells
Fresh	Intact	24	955^{a}	91.7^{a}
	Bisected	37	780^{b} (18.4)	84.8^{b}
Vitrified-Defrosted	Intact	9	921^{a} (3.6)	74.1^{c}
	Bisected –vitrified	17	750^{b} (21.5)	67.1^{d}
	Vitrified –bisected	5	814^{b} (14.5)	61.8^{d}

a, b, c, d: Different letters in the same column indicate significant difference (P <0.001)

The present study shows that acceptable results can be achieved following *in vitro* culture of embryos that have been bisected but that the survivability of such embryos seems to be reduced following vitrified-thawing. There were no difference in developmental capacity between biopsied embryos and intact embryos following *in vitro* culture. However the level of survival was overestimated using *in vitro* culture systems compared to survival after embryo transfer in agreement with other reports (Gustafsson *et al.,* 1994).

The percentage of development obtained with fresh bisected embryos (74%) is in the range reported following *in vitro* culture of demi-embryos (Schmidt *et al.,* 1992, Bredbacka *et al.,* 1995). There was a significant reduction of the survival rate of bisected embryos following vitrified and thawing based developmental capacity *in vitro*. It is generally accepted that freezability of embryos is reduced following splitting. Schmidt *et al.* (1992) reported survival rates of 52% for frozen-thawed hemiembryos compared to 60% for fresh embryos. Vajta *et al.* (1997) report a survival rate of 86% for biopsied vitrificated-thawed embryos versus 69% for vitrificated-thawed biopsied embryos.
A number of cells (around 10%) are shown to be damaged due to the splitting procedure (Iwasaki *et al.,* 1990). A further proportion of cells will die during freezing and thawing. The fluorescence test has proved to be useful to assess cell viability after freezing and thawing (Rho *et al.,* 1998).

CONCLUSIONS

The method of vitrification was adopted for criocopreservation of blastocysts after splitting them. The bisection before the vitrification is a valuable alternative of freezing embryos that have a breakable constitution (hemiembryos, sexed embryos following biopsy and cloned embryos). Both the procedures of bisection and vitrification can be used without complex systems for micromanipulation and freezing. The quality of the embryo subjected to bisection and vitrification affects the *in vitro* survival, observed loss and cellular death, that is reflected in the number of viable cells after the culture. It will be needed of major studies before determining the maximum efficiency, in terms of production of alive embryos.

ACKNOWLEDGEMENTS

This study was supported by the Instituto de Reproducción Animal, Universidad Austral de Chile, and the author wish to thank Agribrands Purina México and my wife Dolores for the time.

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