Cryobiology 67 (2013) 321–326

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Rabbit morula vitrification reduces early foetal growth and increases losses throughout gestation ☆

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ARTICLE INFO

Article history:
Received 13 August 2013
Accepted 17 September 2013
Available online 27 September 2013

Keywords:
Rabbit
Vitrification
Embryo survival
Foetal development
Gene expression

ABSTRACT

Several studies have extensively examined structural and biochemical damage induced by cryopreservation that may lead to loss of rabbit embryo viability, but very little information is available on alterations in growth during gestation and at gene expression level. We started our work by comparing the distribution of losses of embryo and foetal development between control and vitrified rabbit morulae. Furthermore, data on foetal sack, foetal and maternal placenta and foetus size for 10–14 days of gestation were evaluated by ultrasonography. We reported that vitrification procedure causes detrimental effects on rabbit embryo and foetal development, with two major peaks of losses: one before the implantation (at day 6) and the other during the second part of gestation (after day 14). However, foetal loss may occur during the implantation process and placenta development, as there was a reduction in development of foetus produced from vitrified-warmed embryos between day 10 and 14 of gestation. For these reasons, using a recent microarray study performed in frozen–thawed rabbit embryos as a point of reference, we analysed the effects of vitrification procedure on the expression of 10 candidate genes in 6-day-old blastocysts obtained after vitrification and transfer. We observed that the relative expressions of mRNA transcripts from SCGB1A1, EMP1, ANXA3 and EGFLAM genes were significantly altered. This could help explain why a large number (29%) of vitrified embryos were successfully implanted but subsequently failed to develop to term.

Further studies in subsequent embryo–foetal developmental stages, such as initiation of placenta formation, together with more sensitive high-throughput tools, should help us understand the deficiencies that hinder foetal development and identify the repairing mechanism employed by embryos to overcome vitrification effects.

Introduction

Cryopreservation of embryos is considered an important tool in reproductive biotechnology to prevent loss of biodiversity or preserve the genetic resources of domestic animals through the establishment of embryo banks [19]. Vitrification was introduced in 1985 as a simple and cheaper way to cryopreserve mammalian embryos in the absence of ice by using fast cooling rates and a highly concentrated cryoprotectant solution [27]. Since then, embryos of many mammalian species have been vitrified, but with inconsistent results among species or embryo developmental stage [30,33]. In rabbits, between 25% and 65% successful offspring have been obtained after vitrification procedures [14,34,35,17,24]. Moreover, it has been shown that there is a marked difference in the timing and incidence of mortality throughout gestation between different cryopreservation procedures. For example, Mocé et al. [25] and Marco-Jiménez et al. [24] observed high mortality rate from 15 days of pregnancy of vitrified embryos.

In general, a complete cryopreservation procedure involves several embryo manipulations such as in vitro handling, exposure to toxic concentrations of cryoprotectants or transfer to another maternal tract. It appears that tolerance to cryoprotectant toxicity is an important barrier to successful preservation of living systems, but the toxicity mechanism of cryopreservation solutions still remains unknown [7]. Previous studies have demonstrated that the cryopreservation process influences gene expression of preimplantatory embryos, and alterations due to cryopreservation

☆ This work was supported by the Valencian Regional Government research programme (Prometeo 2009/125) and the Spanish Research Projects (CICYT AGL2011-29831-C03-01). M. D. Saenz-de-Juano was supported by a research grant from Generalitat Valenciana (Programa VALI+d, ACIF/2011/254).

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0011-2240/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cryobiol.2013.09.165

conditions have been observed in a relative abundance of specific transcripts related to development [15], metabolism [32] oxidative stress and apoptosis [5]. In mice, Mamo et al. [22] identified 183 altered genes in vitrified 8-cell embryos, related to cell metabolism, regulatory and stress. However, a microarray analysis performed by Larman et al. [16] revealed no significant differences between the transcriptomic pattern of vitrified and fresh mouse blastocyst after 6 h of in vitro culture. It has been shown that in vitro culture systems do not mimic the uterine environment, and in vitro developed embryos differ from their *in vivo* counterparts [4,21]. In fact, a transcriptomic experiment performed by Aksu et al. [1] in bovine embryos revealed that the number of genes altered after vitrification method depended on the embryo origin, and in vitro produced embryos showed major gene expression differences (962 vs. 17 genes). These discrepancies highlight the current dearth of knowledge of direct effects and remaining effects of cryopreservation on embryo gene expression, and which repairing mechanisms are employed by those embryos that overcome vitrification damage and continue with their normal development. In rabbit, little is known about alterations in gene expression due to cryopreservation procedures. A recent microarray study in our laboratory of rabbit late blastocysts, previously frozen and transferred, identified 70 differentially expressed genes just at the time of implantation onset [28]. These altered genes were mainly related to regulation of lipid metabolic and catabolic processes, response to chemical stimulus and mitochondrial structure. These deficiencies might hinder implantation and foetal development and provoke the high mortality rate observed in frozen embryos between implantation and placenta formation phase (from day 7 to 15). The study of target genes suggested by the microarray, as well as other genes associated with embryo development and implantation, could be useful in understanding the differences in developmental potential observed and the molecular deficiencies that might hinder implantation and foetal development.

The aim of our study was to examine the effect of vitrification procedure on embryo and foetal growth and losses during gestation and on the mRNA expression before the beginning of implantation in 10 candidate genes selected by their role in embryo development and implantation (*OCT4*, *VEGF*, *HBA*, *LAMA 4*, *SCGB1A1*, *EMP1*, *C1qTNF1*, *ANXA3*, *EGFLAM*, *TNFAIP6*).

Material and methods

Unless stated otherwise, all chemicals in this study were purchased from Sigma–Aldrich Química S.A (Madrid, Spain).

Animals

Rabbit does belonging to the New Zealand White line from the ICTA (Instituto de Ciencia y Tecnología Animal) at the Universidad Politécnica de Valencia (UPV) were used as donors and recipients. All experimental procedures involving animals were approved by the Research Ethics Committee of the UPV and licensed by European Community Directive 86/609/EC.

Experiment 1. In vivo development ability of vitrified rabbit embryos

Embryo recovery

Donor does were artificially inseminated with pooled sperm from fertile males and euthanised at 72 h post-insemination. A total of 612 morulae were recovered by perfusion of each oviduct and uterine horn with 10 mL pre-warmed Dulbecco Phosphate Buffered Saline (DPBS) supplemented with 0.2% of Bovine Serum Albumin (BSA). Morphologically normal embryos were distributed in pools about 15 embryos for vitrification procedure or fresh transfer.

Embryo vitrification and warming procedure

Morulae were vitrified using the methodology described by Vicente et al. [35]. Briefly, the vitrification procedure was carried out in two steps at 20 °C. In the first step, embryos were placed for 2 min in a vitrification solution consisting of 12.5% dimethyl sulphoxide (DMSO) and 12.5% ethylene glycol (EG) in DPBS supplemented with 0.2% of BSA. In the second step, embryos were suspended for 30 s in a solution of 20% DMSO and 20% EG in DPBS supplemented with 0.2% of BSA. Then, embryos suspended in vitrification medium were loaded into 0.25 ml plastic straws and two sections of DPBS were added at either end of each straw, separated by air bubbles. Finally, straws were sealed and plunged directly into liquid nitrogen. Warming was performed by horizontally placing the straw 10 cm from liquid nitrogen for 20–30 s and when the crystallisation process began, the straws were immersed in a water bath at 20 °C for 10–15 s. The vitrification medium was removed while loading the embryos into a solution containing DPBS and 0.33 M sucrose for 5 min, followed by one bath in a solution of DPBS for another 5 min.

Embryo transfer by laparoscopy

A total of 345 vitrified and 267 fresh morphologically normal embryos were transferred into oviducts by laparoscopy to 44 recipient does (12–15 embryos per recipient) following the procedure described by Besenfelder and Brem [3]. Ovulation was induced in recipient does with an intramuscular dose of 1 mg of Buserelin Acetate (Suprefact, Hoechst Marion Roussel S.A, Madrid, Spain) 68–72 h before transfer. To sedate the does during laparoscopy, anaesthesia was administered by an intramuscular injection of 16 mg xylazine (Bayer AG, Leverkusen, Germany), followed 5 min later by an intravenous injection of 16–20 mg ketamine hydrochloride (Imalgène, Merial SA, Lyon, France).

Embryonic development, implantation, and delivery rates

To evaluate the late blastocyst development rate, 22 recipient does (12 recipient does transferred with vitrified embryos and 10 with fresh embryos) were euthanised 72 h after transfer and 6-day-old embryos were recovered by perfusion of each uterine horn with 20 mL of DPBS supplemented with 0.2% of BSA.

Survival rates of vitrified and fresh embryos were assessed by laparoscopy in the remaining recipient does (22) on the basis of implantation rate (number of implanted embryos at day 14 from total embryos transferred) and birth rate (kits born/total embryos transferred).

Ultrasound examination

Six recipient does from each experimental group were examined on day 10 and 14 post-ovulation induction by a portable colour Doppler ultrasound device (Esaote, Spain) with a 7.5 MHz linear probe (4–12 MHz range). Prior to examination, does were anaesthetised with ketamine 35 and xylazine 16 mg/kg intramuscularly and the abdomen of the doe was clipped. Does were placed in a polystyrene cage where they were prevented from moving. The ultrasound examination was performed from right to left with the probe in sagittal orientation and, after localisation of different foetal sacks, 5–7 whole foetal sack examinations per doe were performed. The identifiable structures (foetal sack, foetus and foetal and maternal placenta) were measured from frozen frame pictures on the monitor, using the Esaote 16 ultrasound software.

Measurements are illustrated in Fig. 1. For the foetal sack (FS, Fig. 1B), the measurement was taken when the largest surface area appeared on the screen. For whole foetus measurements, crown-rump length (CRL) was determined as the maximum distance from