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Short communication

Birth of piglets after OPS vitrification and transfer of compacted morula stage embryos with intact zona pellucida

Françoise BERTHELOT*, Françoise MARTINAT-BOTTÉ, Christine PERREAU, Michel TERQUI

Physiologie de la Reproduction et des Comportements,
UMR 6073 INRA-CNRS-Université F. Rabelais, 37380 Nouzilly, France

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Abstract — In swine, five to six days post-insemination, morulae and blastocysts are collected together after uterine flushing. The purpose of this study was to vitrify zona pellucida-intact morulae with Open Pulled Straw (OPS) technology and obtain piglets after transfer. Morulae (200) were vitrified after a two-step equilibration in ethylene glycol, dimethyl sulfoxide and sucrose in Hepes-buffered TCM199 + 20% NBCS medium (TCM). 2–6 morulae were loaded into OPS and plunged into liquid nitrogen. At embryo warming, a three-step dilution with decreasing concentrations of sucrose was applied. In each of 10 recipients, 20 morulae were transferred surgically. Day 25, gestation rate and the farrowing rate were 80% and 70%, respectively. The pregnant recipients farrowed from 1 to 8 piglets and the survival of total transferred embryos was 13%. Although survival rates are still compromised, OPS technology is therefore appropriate to cryopreserve porcine morulae with intact zona pellucida.

pig / embryo / morula / OPS vitrification / cryopreservation

Résumé — **Naissance de porcelets après transfert de morulae dans la zone pellucide intacte et vitrification par la méthode OPS.** Chez la truie, 5 à 6 jours après l'insémination, il est récolté des embryons aux stades morula et blastocyste associés. Le but de ce travail est de transférer des morulae vitrifiées et d'obtenir des porcelets. Des morulae (200) sont vitrifiées par la méthode OPS après 2 étapes d'équilibration dans un mélange égal d'éthylène glycol, de DMSO et de sucrose dilués dans du TCM199 Hépès + 20 % de sérum de veau nouveau-né (TCM). Pour le réchauffement, les paillettes sont sorties de l'azote et plongées immédiatement dans du TCM + sucrose, l'élimination des cryoprotecteurs se faisant en 3 étapes. Pour évaluer la viabilité des morulae vitrifiées/réchauffées, 10 receveuses reçoivent chacune 20 morulae après transfert chirurgical. Le pourcentage de mises-bas

* Correspondence and reprints
E-mail: berthelo@tours.inra.fr

est de 70 %, celui des porcelets nés par rapport au nombre d'embryons transférés est de 13 %. Cette technique qui doit être améliorée, permet de vitrifier des morulae et d'obtenir des porcelets après transfert.

porcin / embryon / morula / OPS vitrification / cryoconservation

1. INTRODUCTION

The vitrification of pig embryos with intact zona pellucida would be a major advantage to ensure minimal risk of disease transmission during embryo transfer of genetic material. To avoid the risk of collecting hatched or hatching blastocysts, it is recommended to collect the embryos at 5 to 6 days after insemination. However, even at this stage, morulae and blastocysts are often collected together after uterine flushing.

So, it is necessary to vitrify morula stage embryos as well as blastocysts, to be able to cryopreserve all the day 5–6 embryos.

Previously, the birth of piglets has been reported after vitrification and warming at the unhatched blastocyst stage [1, 2, 3, 11]. To our knowledge, there is no report of a successful transfer of vitrified/warmed morulae with intact zona pellucida. One study reported piglets after vitrification at the late morula stage, but zona pellucida was removed before transfer, which is not in accordance with general quality control practices for embryo transfer [5].

Only few studies have been performed at this stage in pigs and only in vitro development has been considered after vitrification and warming of morulae with intact zona pellucida. Three papers reported in vitro survival of cryopreserved morulae, using different vitrification methods:

– with Open Pulled Straw (OPS) technology, Vajta et al. [19] obtained 7/10 morulae hatching in vitro;

– with the OPS method, we obtained hatched blastocysts in vitro after morula vitrification [2];

– after cytochalasin treatment and vitrification of 17 morulae, Dobrinsky et al. [6] obtained one morula-to-blastocyst development in vitro, but no hatching.

The objective for the present experiment was to investigate in vivo survival rates using the OPS vitrification method for the cryopreservation of compacted porcine morulae with intact zona pellucida.

2. MATERIALS AND METHODS

Embryo donors (n = 39) and recipients (n = 10) were obtained from the INRA experimental pig herd in Nouzilly, France.

2.1. Recovery of embryos

Meishan cyclic gilts were inseminated twice at 24 and 48 h after a spontaneous oestrus. Embryos were recovered after slaughter and flushing of uterine horns with PBS medium + 2% newborn calf serum of gilts 5 to 6 days after the first insemination.

Embryos were evaluated under stereomicroscope and those at the compacted morula stage with intact zona pellucida were selected.

2.2. OPS vitrification method

As described in [2], all manipulations were performed in a room at 22–24 °C. Media and embryos were maintained at 39 °C on a heating plate. The vitrification procedure is summarised in Table I. The basic medium (TCM) used throughout experiment was TCM199 Hepes medium

Table I. Experimental outline of vitrification and warming.

Vitrification	Time (min)	Warming	Time (min)
TCM alone	1’	TCM + 0.13 M Suc	1’
TCM alone	1’	TCM + 0.13 M Suc	5’
TCM + 10% DMSO + 10% EG	3’	TCM + 0.075 M Suc	5’
TCM + 20% DMSO + 20% EG + 0.4 M Suc	1’	TCM alone	5’

Suc = Sucrose.
TCM = Hepes TCM199 + 20% NBCS.

(Sigma, France) supplemented with 20% newborn calf serum (NBCS, Bio Whittaker, France). Cryoprotectants were dimethylsulfoxide (DMSO, Sigma, France) and ethylen glycol (EG, Sigma, France). Groups of 2 to 6 compacted morulae were equilibrated in TCM + 10% DMSO + 10% EG and in TCM + 20% DMSO + 20% EG + 0.4 M sucrose for 3min and 1 min, respectively (Tab. I).

During the last step, morulae were gathered in a 2 µL droplet of vitrification medium in the bottom of a petri dish, loaded into the narrow end of the OPS by capillary effect and plunged into liquid nitrogen [17]. At warming, straws were immediately plunged into TCM containing 0.13 M sucrose. After 1 min, the embryos were transferred into another well with the same medium for 5 min and then for another 5 min in TCM containing 0.075 M sucrose. Finally, 20 vitrified/warmed zona pellucida-intact morulae (without selection) were placed into a culture dish with TCM into a 5% CO2 5% O2 and 90% N2 incubator at 39 °C up until transfer (30 min to 2 hours later).

2.3. Surgical transfers

Meishan gilts were used as recipients, since it has been shown that gestation and embryo survival were better in this breed [13]. The transfer was performed surgically in asynchronous (–24 h gilts compared to donors). Twenty morulae were loaded into a catheter connected to a 1 mL syringe and transferred into one uterine horn after a mid-ventral laparotomy [2].

Pregnancy was assessed by ultrasonography at around 25 days post-oestrus [14].

Pregnant gilts were allowed to go to term and the number of piglets was recorded at farrowing. Survival rate at farrowing was the ratio of the number of live-born piglets to the number of vitrified/warmed morulae transferred and is expressed as a percentage.

3. RESULTS

A total of 39 gilts were slaughtered at day 5–6 after the first insemination. The total number of collected embryos was 420 and 218 (52%) were at the compacted morula stage. Eighteen morulae were lost before vitrification. Out of the remaining embryos 202 (34%) were at the blastocyst stage, 4% were at the hatched blastocyst stage and 10% were unfertilised or blocked at the 2- to 16-cell stage.

Among 10 recipients, two were non-pregnant and returned to oestrus at day 20 post-oestrus. Eight recipients became pregnant, one of them returned to oestrus at day 31 post-oestrus, and the 7 other recipients farrowed 1 to 8 live-born healthy piglets (Tab. III). Gestation rate at day 25 of gestation was 80% and farrowing rate was 70% (Tab. II).

The total number of live born piglets was 26 and the total number of transferred

Table II. Results of gestations and farrowings obtained after transfer of porcine morulae (20 vitrified/warmed compacted morulae per recipient).

Number of recipient gilts	Number of gestations at day 30 (gestation rate)	Number of farrowed gilts (farrowing rate)
10	8 (80%)	7 (70%)

Table III. Litter size and survival rate after transfer of porcine morulae (20 vitrified/warmed compacted morulae per recipient).

Total number of transferred morulae	Number of live-born piglets per farrowing	Survival rate
200 (10 × 20)	4 – 1 – 4 – 8 – 4 – 3 – 2	13% (26/200)

compacted morulae was 200 (20 × 10). Consequently, the survival rate was 13% (Tab. III).

4. DISCUSSION

Porcine embryos as well as embryos of Drosophila species [15] are difficult to cryopreserve. Increased survival rates, and better results were obtained in these species by increasing cooling rate during vitrification. OPS technology provides elements that increase the cooling rate approximately tenfold compared to what is achieved with standard straw (0.25 mL, IMV France). Factors involved are:

(1) a smaller volume of cryoprotectant (2 μL);

(2) reduced thickness of the straw wall, decreasing thermoinsulation;

(3) open end of the straw permitting direct contact with liquid nitrogen.

The purpose of our study was to verify whether cooling rate was also a key factor in porcine morula vitrification as described for blastocysts [2]. After 10 transfers of OPS vitrified compacted morulae, a pregnancy rate of 80% was obtained, which is similar to that observed after transfer of fresh embryos in this breed (83%) [13]. This method has so far resulted in the birth of 26 live piglets.

In different species, a number of vitrification studies have been undertaken in morula stage embryos. In cattle, two in vitro studies were performed. Using traditional straw, 67% of morulae developed into blastocysts [8]. With OPS vitrification, survival of IVP bovine morulae was not compromised [18]. In rabbit, vitrified morulae were transferred and the survival rate (foetus or kits) observed in four studies were 41%, 24%, 65% and 31% respectively [8, 9, 10, 20]. In mice, only in vitro development after vitrification was tested [4, 16] and a high development rate was obtained up to 86% and 82%, respectively. In pigs, like in mice, in vitro development was studied [6, 7, 12] but few morulae developed into blastocysts, 6%, 33%, 0% respectively. The porcine morula stage, like the blastocyst stage, seems more difficult to vitrify than in rabbits and mice.

Nevertheless, our study demonstrated the possibility of obtaining the birth of piglets after OPS vitrification. The farrowing (70%)

and survival rates of the embryos (13%) are encouraging. However, at this embryo stage, the OPS vitrification method should be improved before being used in practice.

5. CONCLUSION

This report describes the first production of piglets from vitrified zona pellucida-intact compacted morulae with the OPS method without micro-manipulation or chemical treatment.

We have shown that very fast cooling and/or warming rates are primordial keys to cryopreserve porcine compacted morula stage embryos with intact zona pellucida. The Open Pulled Straw technology is, in the conditions described, the most efficient method to cryopreserve compacted morula stage porcine embryos, as it is for blastocyst stage embryos [2].

This simple and inexpensive method, suitable for both compacted morula and blastocyst stages will be a new tool for preserving and exchanging porcine genetic resources.

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