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Eventual re-vitrification or storage in liquid nitrogen vapor does not jeopardize the practical handling and transport of vitrified pig embryos

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ABSTRACT

This study aimed (1) to evaluate the in vitro post-warming survival of porcine embryos after re-vitrification and (2) to assess the efficacy of transport of embryos in dry shipper (DS) in maintaining the viability and quality of vitrified embryos for a 3-day period. Embryos at the compacted or cavitating morula (CCM) and unhatched blastocyst (UBL) stages were surgically obtained from weaned, crossbred sows. In the first experiment, more than 85% of the embryos survived an initial vitrification and warming and achieved comparable survival rates to those of their fresh counterparts. In contrast, those embryos subjected to a second vitrification and warming had clearly lower survival rates (60% and 64% for re-vitrified embryos from the CCM and UBL groups, respectively) compared to the survival rates of the initial vitrification and fresh control groups ($P < 0.01$). Hatching rates were similar in re-vitrified blastocysts derived from vitrified CCMs and fresh control groups (50.8% and 55.3%, respectively). However, differences ($P < 0.01$) in hatching rates were recorded in re-vitrified blastocysts derived from vitrified UBLs and fresh control blastocysts (14.7% and 90.0%, respectively). In the second experiment, vitrified embryos were stored in a liquid nitrogen tank for one month. Then, the straws containing the embryos were transferred to a DS (DS group) or to another liquid nitrogen tank (control group) for an additional three days. Embryos from the DS and control groups had similar survival and hatching rates, regardless of the embryonic stage considered. The DS storage of CCMs and UBLs did not affect their development after culturing, including total cell numbers, compared to the control, although their apoptotic index was slightly higher ($P < 0.05$), regardless of the developmental stage. In conclusion, although re-vitrification negatively affects embryo survival, this study demonstrated that >60% of vitrified embryos could be successfully re-vitrified and re-warmed. The present study also showed the effectiveness of the DS for the storage of vitrified porcine CCMs and UBLs for at least three 3 days.

1. Introduction

The competitiveness of the swine industry is under pressure globally. In this regard, ample use of reproductive technologies is of vital importance, particularly considering embryo transfer (ET). ET technology has been a demand of the pig industry for more than 60 years due to its numerous applications, particularly for the safe exchange of high-value genetic material with reduced transportation costs. Moreover, this technique substantially diminishes animal welfare problems. Despite these advantages, the commercial use of ET in pigs is still very limited when compared with other species [1]. The need to use surgical procedures for embryo collection and the difficulties encountered in embryo cryopreservation in pigs have led to a loss of interest in this technology for

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decades. However, in recent years, the situation has changed thanks to the technological development of better vitrification procedures [2] and of a safe, non-surgical ET [1].

In ET programs, the embryos must be stored from the time of their collection until they can be transferred to the recipients. Today, the only effective method for long-term preservation of porcine embryos is vitrification, which avoids the formation of intracellular ice crystals [3]. The improvement of vitrification procedures, specifically by using the open pulled straw (OPS) method [4], has provided high in vitro post-warming embryo survival rates and hopefully higher pregnancy and farrowing rates after surgical ET of in vivo-derived porcine embryos vitrified without any previous treatment [5]. Moreover, recent studies clearly showed similar survival rates after 24 h in culture between vitrified and fresh embryos [6].

Although major progress has occurred in porcine embryo vitrification, many factors still remain unsolved. Of particular interest among these factors are embryo re-vitrification and air embryo shipment in the vapor phase of liquid nitrogen; these practical questions are of utmost importance since they are probably the weakest links of the ET chain.

In some circumstances during ET, such as when the number of warmed embryos exceeds the number of embryos necessary to be transferred to the recipients or when some recipients cannot receive embryos due to health problems or difficulties during the insertion of the non-surgical ET catheter, a number of unexpected supernumerary embryos could have been warmed. Although these extra warmed embryos could be used to increase the number of transferred embryos per recipient, the ideal would be that they could be re-vitrified and stored again for future ET. In this sense, the possibility of re-vitrification should provide more flexibility for transfer to the respective recipients. In addition, re-vitrification could be used as an embryo stress resistance and quality test as suggested by Isachenko et al. [7]. Recent studies have used re-vitrification procedures with considerable success for human [8,9], ovine [10] and murine [7] embryos. However, the effects of re-vitrification on post-warming embryo survival rates in pigs have not yet been elucidated.

The shipment of embryos in liquid nitrogen appears optimal for long-distance transportation of vitrified pig embryos [11]. However, a major practical concern is the decision by the International Air Transport Association (IATA) to forbid the transport of tanks with liquid nitrogen. Since then, the only option for the air transport of cryopreserved embryos is to use special containers, the so-called dry shippers (DSs). These dewars have an insert containing a hydrophobic absorbent covered with a hydrophobic membrane. The absorbent material repels humidity but absorbs liquid nitrogen, allowing maintenance of a vapor phase of liquid nitrogen at −150 °C [12]. Although this temperature appears to be adequate for the safe shipment of biological specimens that are conventionally frozen, it could be critical for vitrified porcine embryos, which are particularly sensitive to temperature variations [11]. Vitrified porcine embryos are traditionally stored at −196 °C in very small volumes of medium and, therefore, an oscillation in the storage temperature can induce medium devitrification, ice crystal growth and the death of the embryos. The DS Dewars have been used for cryopreserved human semen [13,14] and oocytes [15] and for vitrified mouse embryos [16]. However, to the best of our knowledge, there are no published studies on the effectiveness of dry shippers for the transport of vitrified porcine embryos.

The aims of this study were to (1) evaluate the in vitro survival of in vivo-derived porcine embryos at the compacted or cavitating morula (CCM) and unhatched blastocyst (UBL) stages after re-vitrification and (2) to assess the effectiveness of the DSs in maintaining the viability and quality of vitrified in vivo-derived porcine CCMs and UBLs for a 3-day storage period.

2. Methods

2.1. Chemicals

All chemicals used in these experiments were purchased from Sigma-Aldrich Quimica SA (Madrid, Spain) unless otherwise noted.

2.2. Animals

The embryos were obtained under field conditions at two commercial farms located in southeastern Spain (Agropor SA, and Porcisan SA, Murcia, Spain). Crossbred Landrace x Large-White sows (parity 2 to 6) with normal weight and sanitary status were used in the experiments. Sows were individually allocated into crates in a mechanically ventilated confinement facility. Animals were fed a commercial ration according to their nutritional requirements. All sows had water available *ad libitum*. Boars were housed in individual pens in a commercial artificial insemination center (AIM Iberica, Murcia, Spain).

All experimental procedures were performed in accordance with the 2010/63/EU EEC directive for animal experimentation and were reviewed and approved in advance by the Ethical Committee for Experimentation with Animals of the University of Murcia, Spain (research code: 183/2015).

2.3. Detection of estrus and artificial insemination

The embryo donors were examined (beginning the day after weaning) for estrus twice a day by exposing sows to a mature boar and applying back pressure. Sows in estrus were post-cervically inseminated at 4 and 24 h after the onset of estrus. Insemination doses (1.5×10^9 spermatozoa in 45 mL) were prepared in the AI center from ejaculates diluted in BTS extender (Beltsville Thawing solution) [17]. Sperm doses were conserved for a maximum of 24 h at 18 °C.

2.4. Embryo recovery

Surgeries were performed by mid-ventral laparotomy on day 6 of the estrous cycle, considering day 0 to be the onset of estrus. Donors were previously sedated with azaperone (Stresnil®, Landegger Strasse, Austria; 2 mg/kg body weight, i.m.). General anesthesia was induced with sodium thiopental (B. Braun VetCare SA, Barcelona, Spain; 7 mg/kg body weight, i.v.) and maintained with isoflurane (IsoFlo®, Madrid, Spain). The reproductive tract was exposed and the corpora lutea in each ovary were counted. Embryos were collected as described previously [18] by flushing the tip of each uterine horn with 30 mL of Tyrode's lactate (TL)-HEPES-polyvinyl alcohol (PVA) [18,19].

2.5. Embryo assessment

The recovered embryos were evaluated under a stereomicroscope for developmental stage and quality. One-cell eggs or poorly developed embryos were considered unfertilized oocytes or degenerated embryos, respectively. Embryos with an appropriate morphology according to the criteria determined by the International Embryo Transfer Society [20] were classified as viable. Vitrification was only performed on viable CCMs embryos with blastomeres totally compacted and an undistinguishable cell boundary or with initial cavitation and UBLs (embryos with a differentiated blastocoele, inner cell mass and trophpoblast).