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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.

Keywords: Morulae; Blastocyst; Double vitrification; Dry shipper; Porcine.

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 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

1 treatment [5]. Moreover, recent studies clearly showed similar survival rates after 24 h
2 in culture between vitrified and fresh embryos [6].

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

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

20 The shipment of embryos in liquid nitrogen appears optimal for long-distance
21 transportation of vitrified pig embryos [11]. However, a major practical concern is the
22 decision by the International Air Transport Association (IATA) to forbid the transport
23 of tanks with liquid nitrogen. Since then, the only option for the air transport of
24 cryopreserved embryos is to use special containers, the so-called dry shippers (DSs).
25 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

1 anesthesia was induced with sodium thiopental (B. Braun VetCare SA, Barcelona,
2 Spain; 7 mg/kg body weight, i.v.) and maintained with isoflurane (IsoFlo®, Madrid,
3 Spain). The reproductive tract was exposed and the corpora lutea in each ovary were
4 counted. Embryos were collected as described previously [18] by flushing the tip of
5 each uterine horn with 30 mL of Tyrode's lactate (TL)-HEPES-polyvinyl alcohol
6 (PVA) [18,19].

7 8 **2.5. Embryo assessment**

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

17 18 **2.6. Vitrification and warming**

19 The embryos were washed three times with TL-HEPES-PVA medium before
20 vitrification and maintained at 39 °C. Vitrification was performed within 4 h after
21 embryo recovery. Groups of three to six embryos were vitrified by the method
22 described by Cuello et al. [21]. Briefly, the embryos were washed twice in TL-HEPES-
23 PVA medium and sequentially equilibrated in the first vitrification medium (TL-
24 HEPES-PVA+7.5% dimethyl sulfoxide+7.5% ethylene glycol) for 3 min and in the
25 second vitrification medium (TL-HEPES-PVA+16% dimethyl sulfoxide+16% ethylene

glycol+0.4 M sucrose) for 1 min. Finally, the embryos were placed in a 1.0 to 1.5 μ L drop and loaded into the narrow end of a superfine open pulled straw (SOPS; Minitüb, Tiefenbach, Germany) by capillary action. Immediately, the straw containing the embryos was plunged horizontally into liquid nitrogen.

After storage, embryos were warmed in a four-well multi-dish plate (Nunc A/C, Roskilde, Denmark). Briefly, the straw containing the embryos were removed from the liquid nitrogen and immediately (less than one second) vertically submerged in the well containing 1 mL of TL-HEPES-PVA at 39 °C supplemented with 0.13 M sucrose, for 5 min. Finally, embryos were placed in another well containing 800 μ L of TL-HEPES-PVA.

2.7. In vitro embryo culture and evaluation of in vitro development post-warming

Post-warmed embryos were cultured in a four-well multi-dish plate containing 500 μ L of embryo culture medium (NCSU-23) [22] supplemented with 0.4% bovine serum albumin (BSA) and 10% fetal calf serum at 39 °C in humidified air with 5% CO₂. The embryos were evaluated morphologically with a stereomicroscope to determine their viability and developmental stage. Compacted or cavitating morulae that had progressed to the blastocyst stage during in vitro culture and UBLs that reformed their blastocoelic cavities post-warming with an excellent or good appearance were considered viable. Survival rate was determined as the ratio of viable embryos to the total number of cultured embryos. Hatching rate was defined as the ratio of hatching and hatched embryos at the end of the culture to the total number of cultured embryos.

2.8. Assessment of cellular apoptosis and total cell number in blastocysts

Apoptotic cells were stained by terminal deoxynucleotidyl transferase and fluorescein dUDP nick-end labelling (TUNEL) as described in Brison and Schultz [23], and Byrne et al. [24], using an *APO-BrdU^{TM}* TUNEL Assay Kit (A23210; Invitrogen, Oregon, USA). Briefly, viable blastocysts were fixed in 4% paraformaldehyde and washed in phosphate buffer solution (PBS)-BSA (3 mg/mL). Then, embryos were permeabilized overnight at 4 °C using 1.5% Triton X-100 and 0.15% Tween 20 in PBS. Afterwards, blastocysts were washed in PBS-BSA for 10 min. Fixed and permeabilized embryos from the same donors were used as positive control. After permeabilization, positive control embryos were incubated in DNase I (50 U/mL) in PBS-BSA for 20 min at 39 °C in a petri-dish covered with mineral oil. Then, both control and experimental embryos were placed in PBS-BSA with 0.5% Tween 20 for 10 min and incubated in TUNEL reaction droplets (10 µL) for 1 h at 38.5 °C in the dark. Finally, all the embryos were washed in PBS-BSA three times for 5 min each time at room temperature and mounted on a slide in droplets with Hoechst (33342)-Vectashield (10 µg/mL). Samples were examined under a fluorescence microscope (Eclipse E800, Nikon, Japan) using excitation filters of 465–495 nm and 330–380 nm for green and blue fluorescence, respectively. The total number of nuclei that displayed blue fluorescence was counted. Cells were considered TUNEL positive when their nuclei exhibited green fluorescence. Blastocysts were considered undamaged when they did not show TUNEL positive nuclei or areas. The percentage of apoptotic cells was calculated as TUNEL positive nuclei out of the total number of nuclei stained with Hoechst (apoptotic index).

2.9. Dry shipper features and management

The DS used was an ST Repro 6.4 model (Reproductive Technologies, Navasota, Texas) that holds 6.4 L of liquid nitrogen total capacity, 4.2 L of liquid nitrogen

absorbed, and has the capacity to hold 1,600 0.25 mL-straws. Twenty-four hours before the beginning of the experiment, the Dewar was gradually pre-chilled, adding small amounts of liquid nitrogen into the canister chamber to avoid excessive bubbling, until it was apparently full. To reach full absorption (a weight of 10.6 kg), the canister chamber was refilled 2 h later. The Dewar was maintained overnight at 4 °C. Finally, the canister chamber was refilled once again. Two hours later, the remaining liquid nitrogen was removed, and the straws containing the embryos were transferred from the liquid nitrogen tank to the DS. The temperature was checked within the canister to confirm that it was approximately -150 °C.

2.10. Statistical analysis

Statistical analyses were performed using IBM SPSS 19 Statistics (SPSS, Chicago, IL, USA). The data expressed in percentages were analyzed by Fisher's exact test. Continuous variables were evaluated using the Kolmogorov–Smirnov test to assess the assumption of normality, and groups were compared using unpaired Student's t-tests corrected for inequality of variances (Levene's test). The threshold for significance was set at $P < 0.05$. Results are expressed as percentages and least square means $\pm$ standard deviation (SD).

3. Results

A total of 26 donor sows were used in this study. The mean ovulation rate in these sows was 21.1 ± 3.9 . The ratio of collected embryos to the number of corpora lutea was 93.9%. The total number of oocytes and embryos collected was 501, of which 3.2% were unfertilized oocytes and degenerated embryos, 45.7% were CCMs, 45.7% were UBLs and 5.4% were hatched blastocysts.

3.1. Experiment 1

This experiment was performed to evaluate the effects of the re-vitrification of warmed embryos on the in vitro embryo survival. For that, collected embryos (N=255) were assigned to one of two groups: a group that underwent a single vitrification and warming (vitrification control group) and a fresh embryo control group. Each group was thereafter divided into two sub-groups according to their embryonic developmental stage (CCM or UBL). Embryos from the fresh control group were not vitrified, and embryo viability was evaluated at 24 h of in vitro culturing. Viability and hatching rates of embryos from the single vitrification group were evaluated at 24 h post-warming. Subsequently, unhatched viable blastocysts were re-vitrified and re-warmed (N=136) and the viability of those embryos was assessed at 24 h of in vitro culturing. Finally, we evaluated hatching rates of re-vitrified and fresh control embryos at 48 h of in vitro culturing (see Fig. 1, for schematic representation of the experimental design).

In vitro survival rates of vitrified and re-vitrified embryos at 24 h post-warming are presented in Table 1. A high proportion (>85%) of embryos survived the first vitrification and warming and achieved comparable survival rates to those of their fresh counterparts. At 24 h post-warming the 6.4% and 20.8% of blastocysts derived from vitrified CCMs and UBLs, respectively, were hatched (Fig. 2). These embryos were considered as viable but were not used for subsequent vitrification. A subsequent second vitrification and warming was harmful to the embryos since their survival rates were significantly decreased ($P<0.01$), regardless of embryonic stage, when compared with those of the control groups. At 48 h of culturing, hatching rates were similar in re-vitrified blastocysts derived from vitrified CCMs and fresh control groups (50.8% and 55.3%, respectively). Significant 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; Fig. 3).

3.2. Experiment 2

This experiment evaluated the efficacy of transport of embryos in a DS dewar in maintaining the viability of vitrified embryos for a 3-day storage period. After vitrification, SOPS straws containing the embryos were stored in a liquid nitrogen tank for one month. Then, the straws were transferred from the liquid nitrogen tank to the DS (DS group; N=102) or to another liquid nitrogen tank (control group; N=101) for an additional three days. In both groups, the embryos were divided according to their developmental stage (CCM or UBL). After warming, embryos from the DS and the control groups were cultured for 24 h to assess embryo survival and hatching rates. Finally, an aliquot of viable embryos from each group was subjected to a TUNEL assay to evaluate nuclei with DNA degradation and the apoptotic index. (see Fig. 4, for schematic representation of the experimental design).

Figure 5 shows the survival rates of CM-CAVs and UBLs stored for three days in the DS. Embryos from the DS group presented similar survival rates to those of the liquid nitrogen control group (range: 88-100%), regardless of the embryonic stage tested. The storage of CCMs and UBLs in DS did not affect embryo development towards the end of culture compared to the control group (Fig. 6A). The pattern of the frequency distribution of embryonic development was comparable between the groups (Fig. 6B). Although there were no differences in the total cell number of the resulting blastocysts between the DS and liquid nitrogen groups, the apoptotic index was affected, being slightly higher ($P<0.05$) in those embryos stored in the DS, regardless of the developmental stage considered (Fig. 7).

4. Discussion

This is probably the first study detailing the embryonic survival and development of porcine CCMs and UBLs subjected to double vitrification. The present study also shows, for the first time, the effectiveness of DS Dewars for the storage of vitrified porcine CCMs and UBLs for a three-day period, which is of major practical importance for the commercial air transportation of embryos.

Previous investigations have demonstrated that the success of vitrification depends on the embryonic developmental stage and that embryos at the morula and blastocyst stages are more resistant to cooling than earlier stages and are therefore more suitable for vitrification [2,11,25]. In the present study, the post-warming survival rates of CCMs and UBLs were comparable to those achieved in previous studies [26-28]. Taken together, the results from experiments 1 and 2 indicated that approximately 90% and 95% of CCMs and UBLs, respectively, survived vitrification. These results are consistent with the general assumption that blastocysts usually have higher survival rates than morulae [6,28]. However, in the second experiment, we did not observe differences in survival rates between the two developmental stages. This observation is not surprising because similar results had previously been reported [21]. The use of morulae in different stages of development (uncompacted, partially compacted, fully compacted or with initial cavitation) might be responsible for that apparent discrepancy. In sum, our results confirm those of earlier studies [27], namely, that the SOPS vitrification procedure is able to maintain the post-warming viability of vitrified morulae and blastocysts without any previous treatment. Moreover, an adequate in vivo developmental capacity of these embryos following embryo transfer has also been reported [29,30].

1 Re-vitrification has been successfully accomplished in murine [7,31], and human
2 embryos [9]. These studies demonstrated no differences in the post-warming survival
3 rates of mouse, rat and human embryos between the first and second vitrification or in
4 human pregnancy rates between re-vitrified and control embryos. To the best of our
5 knowledge, this is the first study where re-vitrification of porcine embryos has been
6 attempted. Our results indicate that although high survival rates were achieved after the
7 first vitrification process, a subsequent second vitrification was detrimental to embryo
8 viability since the post-warming survival rates in embryos from the re-vitrified group
9 were lower than those of the single vitrification and fresh control groups. These results
10 contrast with the abovementioned studies in murine and human embryos. The reasons
11 for this discrepancy are not clear but are probably linked to species-specific differences
12 in the sensitivity of embryos to low temperatures. It is known that porcine embryos,
13 which contain high levels of intracellular lipids, are particularly sensitive to thermal
14 effects [32,33].

15 Surprisingly, re-vitrified blastocysts derived from vitrified CCMs had higher post-
16 warming hatching rates at the end of the culture period than re-vitrified blastocysts
17 derived from vitrified UBLs. The effects of a lengthy period (aging) during the first and
18 second post-warming cultures of vitrified and re-vitrified blastocysts could be one of the
19 main explanations of these results since, as suggested by Hardy et al. [34], culture time
20 markedly increases embryonic cell death.

21 Despite the detrimental effects of re-vitrification, approximately 60% of the embryos
22 survived the second vitrification. Although the data suggest the possibility for
23 cryopreservation of unexpected, extra warmed embryos for future re-warming and
24 transfer, further studies are required to evaluate the real potential of transferring re-
25 vitrified porcine embryos.

1 In the second experiment, we did not find differences in embryo viability at 24 h post-
2 warming between vitrified embryos stored for three days in a DS or in a liquid nitrogen
3 tank. These results are in accordance with those of AbdelHafez et al. [16], who did not
4 find a detrimental effect of storage when mouse embryos (8-cell and blastocyst stages)
5 were stored in a DS for 4 days. Moreover, the results demonstrated that storage in a DS
6 neither affected the survival rate nor produced a delay in the subsequent embryo
7 development. Hatching rates were similar in the DS and control groups, supporting that
8 assumption. Although the evaluation of embryo hatching rates after in vitro culture of
9 the vitrified-warmed embryos has been proposed as a valid assessment of the efficiency
10 of vitrification and as a suitable parameter for the estimation of the quality of those
11 blastocysts in absence of ET [35], an additional assessment of cell death was conducted
12 in our study. The TUNEL assay of broken DNA has been used in numerous cell types to
13 detect the final stages of cell apoptosis [36]. Previous studies have demonstrated that the
14 apoptotic index of in vivo-derived fresh porcine blastocysts is approximately 1%
15 [35,37]. This suggests that apoptosis occurs in a small number of blastomeres as a
16 natural process to remove unnecessary or damaged individual cells. In the present study,
17 the apoptotic index in the control embryos (embryos stored in the liquid nitrogen tank)
18 was comparable to that reported in the literature for fresh embryos [34,36]. However,
19 the apoptotic index in embryos stored in the DS was 1.5 times greater. These results
20 contrast with those reported by AbdelHafez et al. [16], in mouse embryos, who did not
21 find an increase in the apoptotic index of embryos stored in a DS for 4 days. Differences
22 among species in the sensitivity of embryos to temperature fluctuations [32,33] could
23 explain the discrepancy. Although the apoptotic index values were below 3% and likely
24 have little biological significance, further studies are necessary to verify whether these
25 values have some consequences for in vivo embryo development.

4.1. Conclusions

In conclusion, although re-vitrification was detrimental to a significant proportion of embryos, our results indicate that more than 60% of porcine blastocysts derived from vitrified and warmed CCMs and UBLs survive re-vitrification and re-warming. Therefore, embryos that were either fortuitously warmed or those warmed embryos that were not transferred into recipients due to special circumstances may be re-vitrified and used in future ETs. This study also shows the efficacy of transport of embryos in the DS for short storage (three days) of vitrified CCMs and UBLs. The use of DS Dewar should allow the safe worldwide air transport of porcine embryos. However, further studies are needed to evaluate the in vivo results after ET.

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Role of the funding source

Funding sources did not have any involvement in the study design, in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the article for publication.

Author contributions

A Nohalez, H Rodriguez-Martinez, EA Martinez and C Cuello, conceived, designed and directed the study. A Nohalez, CA Martinez, I Parrilla, MA Gil, EA Martinez and C Cuello performed the experiments. A Nohalez, EA Martinez and C Cuello analyzed and interpreted the data. A Nohalez, EA Martinez and C Cuello wrote the manuscript. J Roca and H Rodriguez-Martinez critically revised the manuscript. All authors approved the manuscript for publication.

Declaration of interest

None of the authors have any conflicts of interest to declare.

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Figure legends

Fig. 1: Schematic representation of the experimental design used in Experiment 1.

Fig. 2. Representative bright field images showing compacted-cavitating morulae and unhatched blastocysts freshly collected (A and C, respectively) and at 24 h of in vitro culture after vitrification-warming (B and D, respectively). Scale bar is 100 μ m.

Fig. 3. Post-warming hatching rates at 48 h of culturing of re-vitrified (RVW) blastocysts derived from vitrified compacted or cavitating morulae (CCMs) and unhatched blastocysts (UBLs). Control embryos were fresh (not vitrified) CCMs and UBLs cultured in vitro for 48 h. The number of embryos evaluated is in parentheses. Different letters indicate significant differences ($P < 0.01$).

Fig. 4: Schematic representation of the experimental design used in Experiment 2.

Fig. 5. Post-warming survival rates. Survival rates at 24 h post-warming of compacted or cavitating morulae (CCMs) and unhatched blastocysts (UBLs) that were vitrified and warmed and then stored for three days in a dry shipper or in a liquid nitrogen tank (control). Numbers of embryos used in parentheses.

Fig. 6. Post-warming embryonic development. A) Summary of the developmental stages at 24 h post-warming of compacted or cavitating morulae (CCMs) and unhatched blastocysts (UBLs) that were vitrified and warmed and then stored for three days in a dry shipper (N= 48 and 46, respectively) or in a liquid nitrogen tank (control; N= 56 and

42, respectively). The developmental stage was scored according to the following classes: 1, early blastocyst; 2, full blastocyst; 3 expanded blastocyst; 4, peri-hatching blastocyst; 5, hatched blastocyst. Values are expressed as the mean $\pm$ SD. B) Frequency distribution of the different embryonic developmental stages achieved at 24 h post-warming by compacted or cavitating morulae (CCMs) and unhatched blastocysts (UBLs) that were vitrified and warmed and then stored for three days in a dry shipper (DS) or in a liquid nitrogen tank (control).

Fig. 7. Post-warming total cell number and apoptotic index. A) Representative fluorescence images of TUNEL staining of a vitrified blastocyst stored in a dry shipper for three days. The Hoechst fluorophore stains all nuclei and displays blue fluorescence. TUNEL stains nuclei with DNA fragmentation and displays green fluorescence. Bar: 50 μ m. B) Total cell number and apoptotic index at 24 h post-warming in the resulting blastocysts from vitrified and warmed compacted or cavitating morulae (CCMs) and unhatched blastocysts (UBLs) stored for three days in a dry shipper or in an LN tank (control). The number of embryos analyzed is in parentheses. Values are expressed as the mean $\pm$ SD. Different letters indicate significant differences ($P < 0.05$).

Table 1. Survival rates of vitrified and warmed (VW) and subsequently re-vitrified and warmed (RVW) embryos at 24 h post-warming.

Group	Initial embryo developmental stage			
	CCMs		UBLs	
	N	Survival rates (%)	N	Survival rates (%)
VW	78	84.6 ^a	101	95.0 ^a
RVW*	61	63.9 ^b	75	60.0 ^b
Control [#]	38	92.1 ^a	38	94.7 ^a

*Blastocysts derived from vitrified and warmed compacted and cavitating morulae (CCMs; N=61) and from vitrified and warmed unhatched blastocysts (UBLs; N=75) at 24 h post-warming were re-vitrified. [#] Control embryos were fresh CCMs and UBLs cultured for 24 h.

^{a,b} Different subscripts in the same column represent significant differences (P<0.01).

A

B

C

D

(A) **(B)**

A

Hoechst 33342

TUNEL

Merge

B