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—Original Article—

A comparison of different vitrification devices and the effect of blastocoele collapse on the cryosurvival of *in vitro* produced porcine embryos

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Abstract. The aim of this study was to determine the optimum conditions for vitrifying *in vitro* produced day 7 porcine embryos using different vitrification devices and blastocoele collapse methods. Firstly embryos were collapsed by micro-pipetting, needle puncture and sucrose with and without conducting vitrification. In the next experiment, non-collapsed embryos were vitrified in an open device using either superfine open-pulled straws (SOPS) or the CryoLoop™ system, or vitrified in a closed device using either the CryoTip™ or Cryo Bio™'s high security vitrification system (HSV). The post-thaw survival of embryos vitrified in the open devices did not differ significantly (SOPS: 37.3%; CryoLoop™: 37.3%) nor did the post-thaw survival of embryos vitrified in the closed devices (CryoTip™: 38.5%; HSV: 42.5%). The re-expansion rate of embryos that were collapsed via micro-pipetting (76.0%) did not differ from those that were punctured (75.0%) or collapsed via sucrose (79.6%) when vitrification was not performed. However, embryos collapsed via sucrose solutions (24.5%) and needle puncture (16.0%) prior to vitrification were significantly less likely to survive vitrification than the control (non-collapsed) embryos (53.6%, $P < 0.05$). The findings show that both open and closed vitrification devices were equally effective for the vitrification of porcine blastocysts. Collapsing blastocysts prior to vitrification did not improve survival, which is inconsistent with the findings of studies in other species. This may be due to the extremely sensitive nature of porcine embryos, and/or the invasiveness of the collapsing procedures.

Key words: Blastocoele collapse, Porcine embryo, Vitrification, Vitrification device

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Embryo cryopreservation is a vital technology for the storage and transportation of valuable genetics for the fields of agriculture and biomedical research. While embryo cryopreservation has been incorporated successfully into advanced breeding programmes for some time now in cattle and sheep [1–3], its use in pigs is severely restricted due to the relatively poor rates of post-thaw survival achieved to date [4]. Not only would embryo cryopreservation aid in swine breeding programmes, it would advance the use of the pig as a valuable animal model for biomedical studies because of the species' immunological, morphological, physiological, and functional similarities to humans [5, 6]. Embryo cryopreservation would allow for the banking of transgenic embryonic lines for use in xenotransplantation and other such biomedical research [7].

With the importance of porcine embryos clearly evident, the successful cryopreservation of these embryos is still also far behind that of other non domestic species such as humans and mice [8, 9] with few studies describing the birth of live offspring from the transfer of vitrified-warmed porcine embryos [10–14]. There are two reasons for this; firstly, porcine embryos have endemically high concentrations of cytoplasmic lipid, which hinders the vitrification process [15, 16]. Secondly, *in vitro* produced porcine embryos are generally of poorer quality compared to *in vivo* derived porcine embryos [17, 18]. As a result, the *in vitro* produced porcine embryo is regarded as being particularly sensitive to manipulations, making them a useful model for studying the impacts of *in vitro* procedures. For example, handling conditions that do not illicit affects on mouse embryo development [19] have been shown to do so in pig embryos [20].

With the widespread adoption of embryo cryopreservation technologies, a vast array of vitrification devices has been developed, including the superfine open pulled straw (SOPS), CryoLoop™, CryoTip™ and Cryo Bio™'s High Security Vitrification System (HSV). Apart from the physical differences of the devices used to immerse embryos in liquid nitrogen (LN_2), the systems differ in that vitrification occurs in either an "open" or "closed" manner. In open systems, the embryos come into direct contact with LN_2, thus allowing rapid cooling rates to occur, whereas in closed systems, the embryos are completely sealed within a device before plunging into LN_2, which potentially reduces the cooling rate. As LN_2 has been identified as a potential source of fungal, viral and bacterial contamination, vitrification protocols are moving to closed systems [21, 22]. However, there are very few studies that have directly compared the cryosurvival of embryos vitrified using these systems.

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Along with the proliferation of different vitrification tools, various modifications to the vitrification procedures have been adopted, including the clinical practice of collapsing the blastocoele prior to vitrification. Since Vanderzwalmen *et al.* [23] demonstrated the efficacy of the procedure in human embryos, it has been widely accepted that collapsing the blastocoele prior to vitrification increases the survival of blastocysts post-thaw. Consequently, many methods were developed to initiate blastocoele collapse including micro-pipetting, needle puncture, needle aspiration, laser-pulse and exposure to concentrated sucrose solutions [24–28]. However, if equilibration time in cryopreservation media is extended, the cryoprotectants within the media are able to replace the fluid of the blastocoele, negating the need to physically collapse the blastocoelic cavity [29]. As a result, recent debates as to whether or not artificial blastocoele shrinkage is necessary have surfaced, with some arguing that the procedure could be detrimental to subsequent embryo development. Therefore, the objectives of this study were to compare the effectiveness of common "open" and "closed" vitrification tools and to determine the effect of collapsing the blastocoele cavity prior to vitrification on the cryosurvival of *in vitro* produced porcine embryos.

Materials and Methods

Chemicals

All chemicals used in this study were from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

Culture media

Oocyte *in vitro* maturation (IVM) was carried out in Medium 199 (Invitrogen, Gibco 11150-059, Grand Island, NY, USA) supplemented with 0.1 mg/ml sodium pyruvate, 76 µg/ml penicillin-G, 50 µg/ml streptomycin sulphate, 2.0 mM Glutamax (Gibco), 100 mM cysteamine, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml equine chorionic gonadotropin (eCG), 10 IU/ml human chorionic gonadotropin (hCG), 0.1% fatty acid-free bovine serum albumin (BSA; IVP grade gamma irradiated, MP Biomedicals, Auckland, New Zealand), and 10% porcine follicular fluid [30]. Additionally, the oocyte maturation medium (OMM-199) was supplemented with 1.0 mM dibutyryl cAMP (dbcAMP) for the first 22 h only. HEPES-buffered Medium 199 (Invitrogen, Gibco 12340-030) supplemented with 0.1 mg/ml sodium pyruvate, 76 µg/ml penicillin-G, 50 µg/ml streptomycin sulphate, 2.0 mM Glutamax, and 0.1% BSA was used for oocyte washing (HEPES-199). The fertilization medium consisted of Tyrode's albumin lactate pyruvate-polyvinyl alcohol (TALP-PVA) medium [31] supplemented with 2.0 mM caffeine-sodium benzoate and 3.0 mM calcium lactate [32]. Following insemination, presumptive zygotes were placed in porcine zygote medium–3 (PZM3) [17] containing 0.2 mM sodium pyruvate, 2.0 mM calcium lactate, 1.0 mM Glutamax, 5.0 mM hypotaurine, MEM amino acids (Gibco), MEM non-essential amino acids (Gibco), and 0.3% BSA. All wells of OMM-199 and TALP-PVA medium and all droplets of PZM-3 medium were covered with embryo-tested mineral oil.

Oocyte collection and in vitro maturation

Ovaries from pre-pubertal gilts were sourced from a local abattoir (Wollondilly Abattoir Pty, Picton NSW, Australia) and brought back to the laboratory within 2 h of slaughter in 0.9% NaCl (Baxter, Deerfield, IL, USA) solution supplemented with an Antibiotic-Antimycotic solution (100 IU/ml penicillin G, 0.25 µg/ml streptomycin sulphate and 0.85% amphotericin B; Gibco) at 34–38 C. Antral follicles 3–8 mm in diameter were aspirated, cumulus-oocyte complexes (COCs) were collected and washed twice in HEPES-199 before being placed in 500 µl of OMM-199 containing dibutryl cAMP in groups of 50 in Nunc™ dishes covered with mineral oil and incubated at 38.5 C in a humidified atmosphere of 6% CO_2 in air. Following 22 h of oocyte maturation, COCs were transferred to OMM-199 without dbcAMP and incubated for a further 22 h.

In vitro fertilization

Following IVM, COCs were placed in a 100 µl drop of HEPES-199 supplemented with 0.5 mg/ml hyaluronidase for 1 min, and gently aspirated in a fresh HEPES-199 droplet using a fine bore glass pipette to partially remove cumulus cells. Partially denuded oocytes were placed in TALP-PVA media in 4-well Nunc dishes (~50 oocytes per 500 µl well). A straw of frozen boar semen was thawed at 42 C in a water bath for 20 sec and the sperm solution was centrifuged at 720 *g* at 37 C for 10 min. The sperm pellet was then retrieved, and resuspended in TALP-PVA medium before being centrifuged again at 310 *g* at 37 C for 5 min. The sperm pellet was again resuspended in TALP-PVA medium and sperm concentration and motility were assessed. To each well, a total of 250 motile sperm per oocyte was added. After 30 min, oocytes and zona-bound sperm were carefully moved to a second well containing 500 µl TALP-PVA medium and incubated for a further 5 h at 38.5 C in a humidified atmosphere of 6% CO_2 in air.

In vitro culture and assessment of embryos

After 5 h, presumptive zygotes were washed in PZM-3 medium, transferred to 50 µl droplets of PZM-3 medium (10–15 zygotes/droplet), and incubated in a humidified atmosphere of 6% CO_2, 5% O_2 and 89% N_2 at 38.5 C. Day of insemination was defined as day 0. On day 4 of embryo culture, 5 µl of pre-equilibrated foetal calf serum (FCS) was added to each 50 µl culture droplet. On day 7 of culture, blastocysts were assessed as either A, B or C grade embryos dependent on the level of expansion, number of cells and compaction of cells, and amount of dark granulation present in the trophectoderm. Embryos graded A or B expanded or grade A or B hatching blastocysts were selected for use in experiments.

Blastocoele collapse

Selected blastocysts were placed in Vitrolife's RapidVit Blast™ solution 1 (Vitrolife AB, Göteborg, Sweden), and collapsed by micro-pipetting or needle puncture. Glass pipettes were pulled to give an internal diameter that was slightly smaller (about 10 µm smaller) than that of the blastocysts. The blastocysts were then pipetted up and down approximately 10 times or until blastocoele collapse was observed. For needle puncture, VitroLife's ICSI sperm injection needles (Vitrolife AB), were used. A pulled glass pipette, through which gentle suction was applied, was used to hold the blastocyst in place. Needle puncture occurred at a site away from the inner cell mass. Blastocysts that did not collapse immediately, collapsed within 5 min of being manipulated. Blastocysts collapsed using the sucrose method were exposed to 0.25 M sucrose in HEPES-199 medium for

3 min and then 0.5 M sucrose in HEPES-199 medium for a further 3 min before being placed directly into Vitrolife's RapidVit Blast™ solution 2. Exposure to the sucrose solutions resulted in partial collapse, with the blastocoele reducing in diameter by about 50% (Fig. 1). If vitrification did not take place, the embryos were exposed to 0.25 M sucrose for 3 min immediately after the 0.5 M sucrose exposure before being washed and placed in culture medium.

Vitrification and thawing of embryos

Blastocysts were vitrified and warmed using VitroLife's three step RapidVit Blast™ and three step RapidWarm Blast™ MOPS (3-(N-morpholino) propanesulfonic acid) (Vitrolife AB) buffered solutions according to the vendor instructions. Briefly, embryos selected for vitrification were placed in vitrification solution 1 (which contains no cryoprotectants) where they remained for 5–20 min. In small groups (depending on the device to be used), embryos were transferred to solution 2 (which contains ethylene glycol and propanediol as the cryoprotectants) for precisely 2 min, then placed in vitrification solution 3 (which contains ethylene glycol, propanediol and ficoll as the cryoprotectants), loaded into a device, and submerged in liquid nitrogen (LN_2) within 45 sec of being placed in vitrification solution 3. Each device was kept submerged in LN_2 until warming. Upon warming, embryos remained in solution 1 (which contains sucrose) for precisely 2 min, solution 2 (which contains sucrose) for precisely 3 min, and solution 3 (wash solution) for 5–10 min. Cryoprotectant concentrations were not detailed in the information provided with the commercial vitrification kits. All procedures were carried out in warmed media on a heated stage set to 38.5 C. Immediately after warming, embryos were washed thoroughly, transferred to 50 µl droplets of PZM-3 medium containing 20% FCS and incubated in a humidified atmosphere of 6% CO_2, 5% O_2 and 89% N_2 at 38.5 C. After 24 h of culture, re-expansion of the blastocoele in each blastocyst was assessed.

CryoLoop™

Vitrification of blastocysts with the CryoLoop™ device was conducted as previously described [33]. Briefly the CryoLoop™ device is made up of a small nylon loop (20 µm in diameter), which is attached to a stainless steel rod of width 0.5 mm adhered to the lid of a cryovial. Once embryos were placed in vitrification solution 3, the loop was dipped in the same solution to create a thin film via surface tension. Between 1–3 embryos were loaded on the loop, directly plunged into the cryovial which contained LN_2 and then the lid was tightened. Upon warming, the lid of the cryovial was unscrewed, immediately removed and the loop placed in warming solution 1. The embryos were seen to drift from the loop before the device was removed from the solution.

Superfine open pulled straw

Embryos were vitrified using the SOPS as previously described [34]. Briefly, 1–10 blastocysts were placed in a droplet of vitrification solution 3 with a total volume of <1 µl. The straw was then placed directly over the droplet whereby the media and blastocysts entered the straw via capillary action. The straw was plunged into LN_2 and placed within a 0.5 ml straw which was then sealed and remained in LN_2. Upon warming, the top of the outer straw was cut, the SOPS was removed with tweezers and the tip of the SOPS containing the embryos was immediately submerged in warming solution 1. A connector on a 1 ml syringe was carefully attached to the opposite end of the SOPS and the plunger was pressed to help expel the embryos.

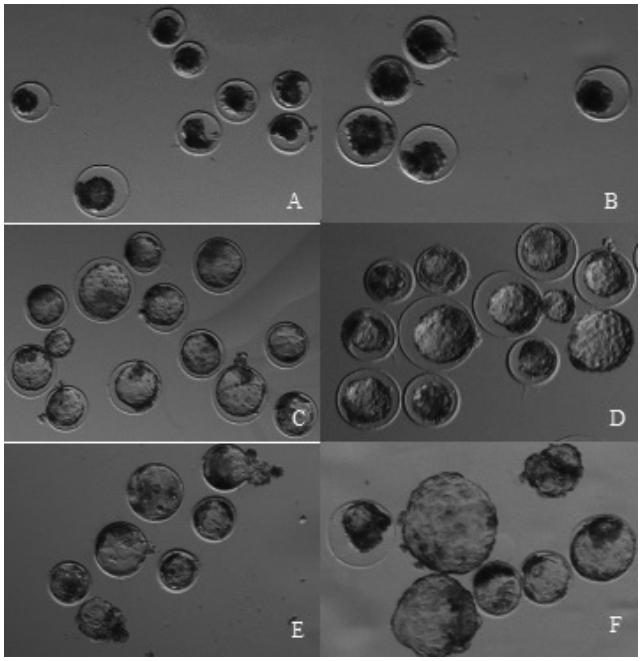

Fig. 1. Expanded blastocysts immediately after blastocoele collapse by (A) micro-pipetting, (B) needle puncture, (C) 0.25 M sucrose, (D) 0.5 M sucrose. Expanded blastocysts 24 h post collapse by (E) needle puncture and (F) sucrose solutions.

Cryo Bio System™ high security vitrification system

The use of the HSV system to vitrify blastocysts was identical to a previous description [35]. Blastocysts, 1 or 2 at a time, were placed in vitrification solution 3. Embryos were then placed on the black tip of the HSV device in a < 1 µl droplet of solution. The device was then placed within the outer sheath, which was heat sealed before being plunged immediately into liquid nitrogen. To warm, the outer sheath was cut, and the inner device containing the embryos was removed and immediately placed into warming solution 1. Embryos were seen floating off the device before it was removed from solution.

CryoTip™

Loading of blastocysts within this device was carried out to vendor instructions and as previously described [36]. A connector was used to attach a 1 ml syringe to the CryoTip™ device. The embryos were then loaded into the correct area of the device with the use of the syringe. The fine end of the device was then sealed, the connector was removed, and the large end of the device was sealed. The metal sheath was then replaced back over the end of the device containing the embryos before plunging it into LN_2. To warm, the device was removed from LN_2 and swirled in a 37 C water bath for 3 sec. The sheath was removed, both ends were cut which sharp scissors, and the end of the device containing the embryos was placed directly into the warming solution. The plunger of the syringe was pressed

Table 1. Effect of blastocoele collapse method on the post-thaw survival of blastocysts

Treatment**	Total blastocysts vitrified	Total survived post-thaw	Survival rate (%)*	Number of cells per blastocyst*
Control***	41	20	$54.7^{B} \pm 9.7$	41.0 ± 5.1
Sucrose exposure	49	13	$24.5^{A} \pm 9.9$	44.0 ± 4.1
Micro-pipetting	52	22	$44.2^{AB} \pm 7.0$	44.8 ± 14.2
Control***	32	17	$53.6^{B} \pm 2.1$	33.6 ± 7.2
Needle puncture	32	6	$16.0^{A} \pm 8.3$	33.8 ± 3.6

* Values are presented as the mean ± SEM. Values in the same column with different superscripts differ significantly (^{AB} $P < 0.05$). ** Collapsing methods compared in two different experiments. *** Non-collapsed embryos.

to expel the embryos from the device.

Post-thaw survival analysis

Survival of vitrified-warmed blastocysts was determined by assessing re-expansion of the blastocoele cavity after 24 h culture. The presence of an obvious blastocoele cavity was considered to indicate blastocyst survival. Surviving blastocysts were stained with Hoechst 33342 (4 mg/ml) in HEPES-199 to visualise the nuclei. Stained blastocysts were slide mounted and cell counts were conducted using fluorescence microscopy.

Experimental design

Effect of blastocoele collapse: The effects of the different blastocoele collapse methods on blastocoele re-expansion were first examined without vitrification being performed. Blastocysts were collapsed by micro-pipetting (n = 38), needle puncture (n = 38) or sucrose exposure (n = 43) as described above. Three replicates were performed.

The effect of blastocoele collapse on post-thaw survival was then examined. In 6 replicates, blastocysts were either subjected to collapse via micro-pipetting (n = 52), collapse via the sucrose method (n = 49), or not collapsed (n = 41) (control), prior to vitrification. In another 3 replicates, blastocysts were either subjected to collapse via needle puncture (n = 32), or not collapsed (n = 32) (control), prior to vitrification. In order to allocate adequate numbers of grade A and B blastocysts to each group, it was not possible to perform all three blastocoele collapse methods in the same replicates. For these experiments blastocysts were vitrified using the SOPS device.

Effect of vitrification device: Two commonly used open vitrification devices were compared. Forty-two blastocysts were vitrified using the CryoLoop™ and 45 blastocysts were vitrified using SOPS. A total of 3 replicates were performed.

Two closed vitrification devices were then compared. Twenty-two blastocysts were vitrified using the CryoTip™ and 29 blastocysts were vitrified using the HSV system. A total of 4 replicates were performed.

Statistical analysis

Analysis was performed by arcsine transformation of the percentage data followed by a two way ANOVA. Statistical analysis was performed using GenStat 16th Edition (Numerical Algorithms Group, Oxford, UK). Percentage data were arcsine transformed and subjected to two-way ANOVA. When a significant difference was detected, the Fisher's protected post hoc test was performed for pair-wise comparisons. A P value less than 0.05 was considered to indicate a statistically significant difference.

Results

Effect of blastocoele collapse

The blastocoele re-expansion rates did not differ between the groups (Fig. 1). The rates of blastocoele re-expansion following needle puncture, micro-pipetting and sucrose exposure were 75.0%, 76.0% and 79.6%, respectively.

The effect of blastocoele collapse prior to vitrification on post-thaw survival is shown in Table 1. In the first trial, the post-thaw survival rates of embryos collapsed by micro-pipetting did not differ significantly from that of the control embryos, although the post-thaw survival rate following sucrose exposure was significantly lower than that of the control embryos (24.5% *vs.* 54.7%, respectively). The total numbers of cells in surviving blastocysts did not differ significantly between the micro-pipetted, sucrose exposed and control embryos (44.8 ± 14.2, 44 ± 4.1 and 41 ± 5.1, respectively). In the second trial, the post-thaw survival rate of embryos that were collapsed via needle-puncture was significantly lower than that of control embryos (16.0% *vs*. 53.6%, respectively). The total numbers of cells in surviving embryos did not differ between the groups (33.8 ± 3.6 and 33.6 ± 7.2, respectively).

Effect of vitrification device

The effect of vitrification device on the post-thaw survival of blastocysts is shown in Table 2. The post-thaw survival rates of blastocysts vitrified in the CryoLoop™ and SOPS open devices were the same (37.3% and 37.3%, respectively). The post-thaw survival rates of blastocysts vitrified in the CryoTip™ and HSV system closed devices also did not differ significantly (38.5% and 42.5%, respectively). The total numbers of cells in the surviving blastocysts did not differ significantly between the groups (Fig. 2).

Discussion

The results presented here demonstrate that the commonly used open vitrification devices, the CryoLoop™ and the Superfine Open Pulled Straw, and closed vitrification devices, the CryoTip™ and the HSV system, are equally effective for cryopreserving *in vitro* produced porcine embryos. The results also show that contrary to previous reports in other species, collapsing the blastocoelic cavity prior to vitrification did not improve the cryosurvival of *in vitro* produced porcine embryos.

Table 2. The effect of vitrification device on the post-thaw survival of blastocysts

Device**	Total blastocysts vitrified	Total survived post-thaw	Survival rate (%)*	Number of cells per blastocyst*
CryoLoop™ (open)	42	14	37.3 ± 6.5	39.2 ± 7.2
SOPS (open)	45	16	37.3 ± 3.0	40.9 ± 4.7
CryoTip™ (closed)	22	9	38.5 ± 8.5	53.6 ± 9.5
HSV system (closed)	29	15	42.5 ± 8.5	48.4 ± 4.9

* Values are presented as the mean ± SEM. ** Open and closed devices were compared in separate experiments.

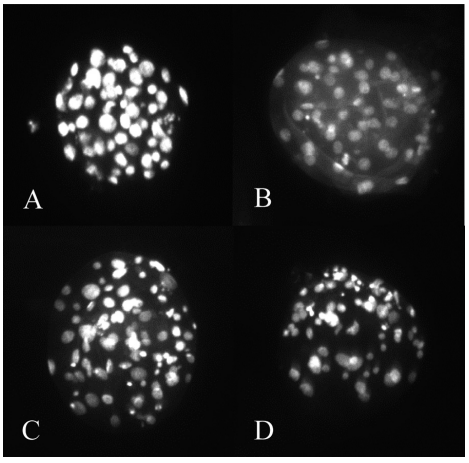

Fig. 2. Blastocysts 24 h post thaw, Hoechst stained and viewed by fluorescence microscopy. Embryos vitrified with (A) CryoLoop™ (B) SOPS (C) CryoTip™ and (D) CryoBio's™ HSV.

It is generally accepted that collapsing the blastocoele cavity prior to vitrification improves the post-thaw survival of blastocyst stage embryos. In humans, pregnancy rates achieved following transfer of embryos vitrified at the blastocyst stages were negatively correlated with the extent of blastocoele expansion [37]. Subsequently, the post-thaw survival rates of human blastocysts, as well as the pregnancy rates achieved, have been increased significantly by collapsing the blastocoele prior to vitrification via the needle puncture [23], micro-pipetting [38], sucrose exposure [28], and laser pulse methods [26]. The reduced post-thaw survival of non-collapsed blastocysts is thought to be due to damage caused by intra-blastocoelic ice formation during vitrification [23]. Improvements in post-thaw survival following artificial blastocoele shrinkage has also been described in equine [39, 40], murine [29] and bovine [41, 42] embryos. Barfield and Seidel [41] showed that the cryosurvival rate of bovine embryos nearly doubled when blastocysts were collapsed via needle aspiration prior to vitrification. The results of these previous studies clearly indicate that the presence of a blastocoelic cavity hinders the vitrification process, and as a result, artificial blastocoele collapse is commonly implemented in clinical practice worldwide.

Despite the findings, recent debate has focused on whether it is necessary to intentionally damage the embryo to induce blastocoele collapse. While the embryo may be able to repair this damage after warming, perhaps this damage could be avoided as the blastocoelic volume reduces in the high osmolarity equilibration solutions used regardless [28]. Sufficient time in equilibration solution would allow the blastocoelic contents to be replaced by the permeating cryoprotectants, thereby preventing ice crystal formation [29]. However, extended exposure to cryoprotectants can exert toxic effects on the embryo, thus a balance between the beneficial and detrimental effects of the cryoprotectants must be considered [43].

This study compared three simple methods of blastocoele collapse, which can be practised in animal research laboratories without the use of expensive equipment; needle puncture, micro-pipetting and sucrose exposure proved to be equally effective in terms of the blastocoele re-expansion rates when vitrification was not performed. However, when vitrification was performed after blastocyst collapse, needle puncture and sucrose exposure significantly decreased the post-thaw survival rates, whilst micro-pipetting did not significantly affect the post-thaw survival rate, compared to that of control embryos. To our knowledge, there is only one previous report on the effect of blastocoele collapse prior to vitrification on the cryosurvival of porcine embryos. Using needle aspiration to induce blastocoele collapse, and the CryoTop™ method of vitrification, Lina *et al*. [44] reported an increase in the post-thaw survival of porcine parthenogenetic embryos, compared with the un-manipulated control parthenotes. The reason for the reduced post-thaw survival rates in the present study in unclear, but may be attributed to the blastocoele collapse methods, differences in the *in vitro* origin of the embryos, and/or the vitrification/warming conditions used. Porcine embryos, especially those produced *in vitro*, are notoriously fragile and are particularly sensitive to manipulations [45]. This is supported by our finding that 20 to 25% of blastocysts failed to re-expand after blastocoele collapse when vitrification was not even performed. Therefore, we believe that the damage caused to manually induce the blastocoele collapse outweighed the benefit that it provided in terms of a reduction in damaging ice crystal formation.

Many studies have demonstrated that the success of embryo vitrification is dependent on rapid cooling and warming rates [46, 47]. In order for this to occur the vitrification solution containing the embryos must be of minimal volume and contain cryoprotectants in high concentrations [48]. Numerous tools have been developed that allow rapid cooling rates to be achieved by using small volumes of solution (<1 µl), many of which bring the solution containing the embryos into direct contact with liquid nitrogen and are thus referred to as "open" systems [49, 50]. In recent years there has been a shift towards the use of "closed" devices [51], which prevent the solution containing the embryos from coming into direct contact with the LN_2. This reduces the possibility of contamination from LN_2-borne pathogens [52], but creates an insulating layer between the embryo and LN_2, which may reduce the cooling rate achievable and thus reduce the efficacy of vitrification [53]. There is a paucity of information

in the literature about the relative effectiveness of the various open and closed devices, and no direct comparisons have been reported using porcine embryos. Using bovine blastocysts, Herrera-Puerta *et al*. [54] achieved higher re-expansion rates with the CryoLoop™ device than with the Open Pulled Straw (OPS), but the difference was not statistically significant. Similarly, the CryoLoop™ was more efficient than the OPS when vitrifying murine blastocysts [55]. It should be noted that the diameter of the Super-fine Open Pulled Straw (SOPS) is considerably less than that of the OPS, which may account for the lack of differences observed in the present study. When open and closed tools were compared, the post-thaw survival of human blastocysts using either the open Cryoleaf™ device or the closed HSV device did not differ [51]. Similarly, there was no significant difference in post-thaw survival when human blastocysts were vitrified using either the CryoTop™ or the CryoTip™ [21]. AbdelHafez *et al*. [36] also found no significant difference in post-thaw survival rates when 8 cell and blastocyst stage murine embryos were vitrified in either the CryoTip™ or the HSV system. Here we report that the CryoLoop™, SOPS, CryoTip™ and HSV system were equally effective for vitrifying *in vitro* produced day 7 porcine embryos.

This study was performed to establish a reliable and practical protocol for the vitrification of day 7 porcine embryos in our laboratory. Having shown that the various tools were equally effective, the practicality and cost of each device was considered. The decision of which tool to select for subsequent experiments was based purely on practicality and cost. Several embryos that were vitrified in the CryoTip™ device were lost upon sealing the device or upon warming. This is a technical issue and would be resolved with higher levels of expertise and training. Other studies have also described low recovery rates when using this device [36]. Furthermore both closed systems and the CryoLoop™ device only allowed two to three expanded blastocysts to be vitrified at any one time. In contrast, the SOPS allowed up to 10 embryos to be successfully vitrified in less than 1µl of vitrification solution. Moreover, the SOPS is relatively inexpensive, and, if necessary, can be used as a closed device [56]. For this reason we recommend using SOPS to cryopreserve porcine embryos.

The post-thaw survival rates of *in vitro* produced blastocysts reported here are comparable to those of other reports in the pig [4, 57–60]. In contrast to these results, Maehara *et al*. [13] reported an extremely high post-thaw survival rate of IVP porcine embryos following vitrification at the morula stage using a novel hollow fibre device. The unique properties of the hollow fibre device, which appears to avoid the surface tension effects seen when loading embryos in a volume less than 0.1 µl, may be the key to improving post-thaw success in cryosensitive porcine embryos.

A notable difference between the present study and other porcine embryo vitrification studies is our use of commercially available vitrification and warming solutions. We have found there to be no significant difference in the post-thaw survival rates of IVP porcine embryos vitrified in either commonly used in-house media [61] or the Vitrolife™ media (unpublished data). Hence, the VitroLife™ media were used in this study for convenience and because we reasoned that the independently tested, quality assured solutions would provide more consistent results than solutions prepared in-house. To our knowledge this is the first report of the use of commercially available human embryo vitrification solutions to vitrify porcine embryos.

In conclusion, the results show that collapsing the blastocoele of porcine embryos prior to vitrification did not enhance cryosurvival; on the contrary, the needle puncture and sucrose exposure methods had a negative effect on post-thaw survival. Additionally, comparisons of common vitrification devices revealed that the SOPS, CryoLoop™, CryoTip™ and HSV systems were equally effective for cryopreserving *in vitro* produced Day 7 porcine embryos. The findings also provide evidence that closed devices are as effective as open devices, alleviating the concern that thermal insulation of samples impairs the effectiveness of closed systems. We recommend the use of the SOPS device as it is relatively inexpensive, easy to handle, enables a greater number of blastocysts to be vitrified at once, and can be used as a closed device if necessary.

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References

1. **Martinez AG, Valcarcel A, Furnus CC, de Matos DG, Iorio G, de las Heras MA.** Cryopreservation of in vitro-produced ovine embryos. *Small Rumin Res* 2006; **63**: 288–296. [CrossRef]
2. **Martinez EA, Vazquez JM, Roca J, Cuello C, Gil MA, Parrilla I, Vazquez JL.** An update on reproductive technologies with potential short-term application in pig production. *Reprod Domest Anim* 2005; **40**: 300–309. [Medline] [CrossRef]
3. **Mapletoft RJ, Hasler JF.** Assisted reproductive technologies in cattle: a review. *Rev Sci Tech* 2005; **24**: 393–403. [Medline]
4. **Cuello C, Gomis J, Almiñana C, Maside C, Sanchez-Osorio J, Gil MA, Sánchez A, Parrilla I, Vazquez JM, Roca J, Martinez EA.** Effect of MEM vitamins and forskolin on embryo development and vitrification tolerance of in vitro-produced pig embryos. *Anim Reprod Sci* 2013; **136**: 296–302. [Medline] [CrossRef]
5. **Nagashima H, Hiruma K, Saito H, Tomii R, Ueno S, Nakayama N, Matsunari H, Kurome M.** Production of live piglets following cryopreservation of embryos derived from in vitro-matured oocytes. *Biol Reprod* 2007; **76**: 900–905. [Medline] [CrossRef]
6. **Prather RS, Hawley RJ, Carter DB, Lai L, Greenstein JL.** Transgenic swine for biomedicine and agriculture. *Theriogenology* 2003; **59**: 115–123. [Medline] [CrossRef]
7. **Men H, Zhao C, Si W, Murphy CN, Spate L, Liu Y, Walters EM, Samuel MS, Prather RS, Critser JK.** Birth of piglets from in vitro-produced, zona-intact porcine embryos vitrified in a closed system. *Theriogenology* 2011; **76**: 280–289. [Medline] [CrossRef]
8. **Shaw JM, Nakagata N.** Cryopreservation of transgenic mouse lines. *Methods Mol Biol* 2002; **180**: 207–228. [Medline]
9. **Liebermann J, Tucker MJ.** Comparison of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. *Fertil Steril* 2006; **86**: 20–26. [Medline] [CrossRef]
10. **Berthelot F, Martinat-Botté F, Perreau C, Terqui M.** Birth of piglets after OPS vitrification and transfer of compacted morula stage embryos with intact zona pellucida. *Reprod Nutr Dev* 2001; **41**: 267–272. [Medline] [CrossRef]
11. **Beebe LFS, Cameron RDA, Blackshaw AW, Keates HL.** Changes to porcine blastocyst vitrification methods and improved litter size after transfer. *Theriogenology* 2005; **64**: 879–890. [Medline] [CrossRef]
12. **Misumi K, Suzuki M, Sato S, Saito N.** Successful production of piglets derived from vitrified morulae and early blastocysts using a microdroplet method. *Theriogenology* 2003; **60**: 253–260. [Medline] [CrossRef]
13. **Maehara M, Matsunari H, Honda K, Nakano K, Takeuchi Y, Kanai T, Matsuda T, Matsumura Y, Hagiwara Y, Sasayama N, Shirasu A, Takahashi M, Watanabe M, Umeyama K, Hanazono Y, Nagashima H.** Hollow fiber vitrification provides a novel method for cryopreserving in vitro maturation/fertilization-derived porcine embryos. *Biol Reprod* 2012; **87**: 133. [Medline] [CrossRef]
14. **Misumi K, Hirayama Y, Egawa S, Yamashita S, Hoshi H, Imai K.** Successful production of piglets derived from expanded blastocysts vitrified using a micro volume air cooling method without direct exposure to liquid nitrogen. *J Reprod Dev* 2013; **59**: 520–524.

[Medline] [CrossRef]

15. **Nagashima H, Kashiwazaki N, Ashman RJ, Grupen CG, Nottle MB.** Cryopreservation of porcine embryos. *Nature* 1995; **374**: 416. [Medline] [CrossRef]
16. **Nakano K, Matsunari H, Nakayama N, Ogawa B, Kurome M, Takahashi M, Matsumoto M, Murakami H, Kaji Y, Nagashima H.** Cloned porcine embryos can maintain developmental ability after cryopreservation at the morula stage. *J Reprod Dev* 2011; **57**: 312–316. [Medline] [CrossRef]
17. **Yoshioka K, Suzuki C, Tanaka A, Anas IMK, Iwamura S.** Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biol Reprod* 2002; **66**: 112–119. [Medline] [CrossRef]
18. **Grupen CG.** The evolution of porcine embryo in vitro production. *Theriogenology* 2014; **81**: 24–37. [Medline] [CrossRef]
19. **Swain JE, Pool TB.** New pH-buffering system for media utilized during gamete and embryo manipulations for assisted reproduction. *Reprod Biomed Online* 2009; **18**: 799–810. [Medline] [CrossRef]
20. **Bartolac LK, Grupen CG.** Effect of pH buffer composition on the maintenance of porcine embryo viability during extended out-of-incubator culture. *In*: Proceedings of the Annual Meeting of the Society of Reproductive Biology; 2012; Gold Coast, Australia. Abstract 43:84.
21. **Kuwayama M, Ieda S, Zhang J, Kato O.** The CryoTip method: Aseptic vitrification of oocytes and embryos. *Fertil Steril* 2005; **84**(Suppl 1): S187. (abstract 98). [CrossRef]
22. **Criado E, Moalli F, Polentarutti N, Albani E, Morreale G, Menduni F, Levi-Setti PE.** Experimental contamination assessment of a novel closed ultravitrification device. *Fertil Steril* 2011; **95**: 1777–1779. [Medline] [CrossRef]
23. **Vanderzwalmen P, Bertin G, Debauche C, Standaert V, van Roosendaal E, Vandervorst M, Bollen N, Zech H, Mukaida T, Takahashi K, Schoysman R.** Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. *Hum Reprod* 2002; **17**: 744–751. [Medline] [CrossRef]
24. **Palini S, Galluzzi L, De Stefani S, Bianchi M, Wells D, Magnani M, Bulletti C.** Genomic DNA in human blastocoele fluid. *Reprod Biomed Online* 2013; **26**: 603–610. [Medline] [CrossRef]
25. **Kader AA, Choi A, Orief Y, Agarwal A.** Factors affecting the outcome of human blastocyst vitrification. *Reprod Biol Endocrinol* 2009; **7**: 99. [Medline] [CrossRef]
26. **Mukaida T, Oka C, Goto T, Takahashi K.** Artificial shrinkage of blastocoeles using either a micro-needle or a laser pulse prior to the cooling steps of vitrification improves survival rate and pregnancy outcome of vitrified human blastocysts. *Hum Reprod* 2006; **21**: 3246–3252. [Medline] [CrossRef]
27. **Hiraoka K, Hiraoka K, Kinutani M, Kinutani K.** Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. *Hum Reprod* 2004; **19**: 2884–2888. [Medline] [CrossRef]
28. **Iwayama H, Hochi S, Yamashita M.** In vitro and in vivo viability of human blastocysts collapsed by laser pulse or osmotic shock prior to vitrification. *J Assist Reprod Genet* 2011; **28**: 355–361. [Medline] [CrossRef]
29. **Li L, Zhang X, Zhao L, Xia X, Wang W.** Comparison of DNA apoptosis in mouse and human blastocysts after vitrification and slow freezing. *Mol Reprod Dev* 2012; **79**: 229–236. [Medline] [CrossRef]
30. **Grupen CG, Armstrong DT.** Relationship between cumulus cell apoptosis, progesterone production and porcine oocyte developmental competence: temporal effects of follicular fluid during IVM. *Reprod Fertil Dev* 2010; **22**: 1100–1109. [Medline] [CrossRef]
31. **Bavister BD.** A consistently successful procedure for in vitro fertilization of golden hamster eggs. *Gamete Res* 1989; **23**: 139–158. [Medline] [CrossRef]
32. **Grupen CG, Fung M, Armstrong DT.** Effects of milrinone and butyrolactone-I on porcine oocyte meiotic progression and developmental competence. *Reprod Fertil Dev* 2006; **18**: 309–317. [Medline] [CrossRef]
33. **Lane M, Schoolcraft WB, Gardner DK.** Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril* 1999; **72**: 1073–1078. [Medline] [CrossRef]
34. **Cuello C, Alminana C, Gomis J, Gil MA, Maside C, Gomez S, Sanchez-Osorio J, Roca J, Vazquez JM, Martinez EA.** Effect of MEM vitamins and forskolin on in vitro embryo production and SOPS-vitrification ability of in vitro derived porcine blastocysts. *Reprod Fertil Dev* 2010; **22**: 324. [CrossRef]
35. **Van Landuyt L, Stoop D, Verheyen G, Verpoest W, Camus M, Van de Velde H, Devroey P, Van den Abbeel E.** Outcome of closed blastocyst vitrification in relation to blastocyst quality: evaluation of 759 warming cycles in a single-embryo transfer policy. *Hum Reprod* 2011; **26**: 527–534. [Medline] [CrossRef]
36. **AbdelHafez F, Xu J, Goldberg J, Desai N.** Vitrification in open and closed carriers at different cell stages: assessment of embryo survival, development, DNA integrity and stability during vapor phase storage for transport. *BMC Biotechnol* 2011; **11**: 29. [Medline] [CrossRef]
37. **Vanderzwalmen P, Zech H, Lejeune B, Prapas Y, Prapas N, Nijs M, Vandamme B, Segal-Bertin G, Debauche C, Van Roosendaal E, Chatziparasidou A, Stecher A, Puissant F, Wissman D, Schoysman R.** Pregnancy and implantation rates after transfers on day 4 and 5 of fresh and vitrified embryos. *Hum Reprod* 1999; **14**(Suppl 3): 333–334. (Abstract 123).
38. **Schiewe MC, Miller F, Hubert GD, Buyalos RP.** Pre-freeze manual collapsing of the blastocele cavity increases cryosurvival of human blastocysts. *Fertil Steril* 2005; **84**(Suppl 1): S182. (abstract 84). [CrossRef]
39. **Choi YH, Velez IC, Riera FL, Roldán JE, Hartman DL, Bliss SB, Blanchard TL, Hayden SS, Hinrichs K.** Successful cryopreservation of expanded equine blastocysts. *Theriogenology* 2011; **76**: 143–152. [Medline] [CrossRef]
40. **Choi YH, Velez IC, Macias-Garcia B, Hinrichs K.** Application of an open device to vitrify equine in vitro-produced embryos. *Reprod Fertil Dev* 2012; **25**(Suppl 1): 178. (abstract 61). [CrossRef]
41. **Barfield JP, Seidel GE.** Blastocoele collapse improves post-thaw survival of slow frozen and vitrified in vitro-produced bovine embryos. *Reprod Fertil Dev* 2013; **26**(Suppl 1): 181. (abstract 136). [CrossRef]
42. **Min SH, Kim JW, Lee YH, Park SY, Jeong PS, Yeon JY, Park H, Chang KT, Koo DB.** Forced collapse of the blastocoel cavity improves developmental potential in cryopreserved bovine blastocysts by slow-rate freezing and vitrification. *Reprod Domest Anim* 2014; **49**: 684–692. [Medline] [CrossRef]
43. **Liang Y, Ning F-Y, Du W-J, Wang C-S, Piao S-H, An T-Z.** The type and extent of injuries in vitrified mouse oocytes. *Cryobiology* 2012; **64**: 97–102. [Medline] [CrossRef]
44. **Lin L, Dua Y, Kragh PM, Lia J, Bolund L, Yang H, Zhang X, Kuwayama M, Vajta G.** Induced blastocoel collapse improves survival rates of porcine blastocysts after vitrification. *Reprod Fertil Dev* 2008; **20**(Suppl 1): 121. (abstract 81). [CrossRef]
45. **Vajta G, Callesen H.** Establishment of an efficient somatic cell nuclear transfer system for production of transgenic pigs. *Theriogenology* 2012; **77**: 1263–1274. [Medline] [CrossRef]
46. **Mazur P, Seki S.** Survival of mouse oocytes after being cooled in a vitrification solution to -196°C at 95° to 70,000°C/min and warmed at 610° to 118,000°C/min: A new paradigm for cryopreservation by vitrification. *Cryobiology* 2011; **62**: 1–7. [Medline] [CrossRef]
47. **Leibo SP, Pool TB.** The principal variables of cryopreservation: solutions, temperatures, and rate changes. *Fertil Steril* 2011; **96**: 269–276. [Medline] [CrossRef]
48. **Jeziorkowski M, Antosik P, Jaskowski JM.** Development of cryoconservation methods in pig embryos. *Med Weter* 2006; **62**: 757–761.
49. **Quan GB, Li WJ, Lan ZG, Wu SS, Shao QY, Hong QH.** The effects of meiotic stage on viability and developmental capability of goat oocytes vitrified by the Cryoloop method. *Small Rumin Res* 2014; **116**: 32–36. [CrossRef]
50. **Gomis J, Cuello C, Sanchez-Osorio J, Gil MA, Parrilla I, Angel MA, Vazquez JM, Roca J, Martinez EA.** Forskolin improves the cryosurvival of in vivo-derived porcine embryos at very early stages using two vitrification methods. *Cryobiology* 2013; **66**: 144–150. [Medline] [CrossRef]
51. **Chen Y, Zheng X, Yan J, Qiao J, Liu P.** Neonatal outcomes after the transfer of vitrified blastocysts: closed versus open vitrification system. *Reprod Biol Endocrinol* 2013; **11**: 107. [Medline] [CrossRef]
52. **Dorfmann A, Carroll K, Sisson M, Geltinger M, Yap S, Iwaszko M.** Collapsing of the blastocoel by laser prior to vitrification with the CryoTip improves embryo survival and pregnancy rates after embryo transfer. *Hum Reprod* 2010; **25**(Suppl 1): 181. (abstract 168).
53. **Vanderzwalmen P, Ectors F, Grobet L, Prapas Y, Panagiotidis Y, Vanderzwalmen S, Stecher A, Frias P, Liebermann J, Zech NH.** Aseptic vitrification of blastocysts from infertile patients, egg donors and after IVM. *Reprod Biomed Online* 2009; **19**: 700–707. [Medline] [CrossRef]
54. **Herrera-Puerta E, Chavarria N, Urrego R, Rodriguez-Osorio N.** Comparison between cryoloop and open pulled straw vitrification methods for bos indicus blastocysts. *Reprod Fertil Dev* 2014; **26**(Suppl 1): 137–138. (abstract 47). [CrossRef]
55. **Ghasem S, Negar K.** Effect of vitrification on number of inner cell mass in mouse blastocysts in conventional straw, closed pulled straw, open pulled straw and cryoloop carriers. *J Pak Med Assoc* 2013; **63**: 486–489. [Medline]
56. **Isachenko V, Montag M, Isachenko E, Zaeva V, Krivokharchenko I, Shafei R, van der Ven H.** Aseptic technology of vitrification of human pronuclear oocytes using open-pulled straws. *Hum Reprod* 2005; **20**: 492–496. [Medline] [CrossRef]
57. **Morató R, Mogas T.** New device for the vitrification and in-straw warming of in vitro produced bovine embryos. *Cryobiology* 2014; **68**: 288–293. [Medline] [CrossRef]
58. **Castillo-Martin M, Yeste M, Morato R, Mogas T, Bonet S.** The effect of L-ascorbic acid during culture, cryopreservation, or both on porcine embryos produced in vitro. *Reprod Fertil Dev* 2012; **25**(Suppl 1): 177. (abstract 60). [CrossRef]
59. **Men H, Agca Y, Riley LK, Critser JK.** Improved survival of vitrified porcine embryos after partial delipation through chemically stimulated lipolysis and inhibition of apoptosis. *Theriogenology* 2006; **66**: 2008–2016. [Medline] [CrossRef]
60. **Esaki R, Ueda H, Kurome M, Hirakawa K, Tomii R, Yoshioka H, Ushijima H, Kuwayama M, Nagashima H.** Cryopreservation of porcine embryos derived from in vitro-matured oocytes. *Biol Reprod* 2004; **71**: 432–437. [Medline] [CrossRef]
61. **Beebe LFS, Bouwman EG, McIlfatrick SM, Nottle MB.** Piglets produced from in vivo blastocysts vitrified using the Cryologic Vitrification Method (solid surface vitrification) and a sealed storage container. *Theriogenology* 2011; **75**: 1453–1458. [Medline] [CrossRef]