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Simple storage (CO₂-free) of porcine morulae for up to three days maintains the in vitro viability and developmental competence

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Abstract

The advancement of porcine embryo transfer (ET) technology is constrained by regulatory hurdles (liquid nitrogen transportation) or, more importantly, the technical obstacles of using vitrified embryos in combination with nonsurgical deep uterine ET technology. Maintaining embryos in culture during transport and prior ET collides with the need of CO₂ gassing and the best choice of culture medium. In this work, we describe storage conditions for short-term embryo CO₂-free storage that allowed for a majority of *in vivo*-derived porcine morulae to survive after 3 days of storage in a liquid state, and to develop to the blastocyst stage unhatched, a sanitary prerequisite for ET. The storage conditions included NCSU-23 medium supplemented with bovine serum albumin, where bicarbonate was partially replaced by HEPES to avoid the need for CO₂ gassing, and a temperature of 37 °C. These conditions were able to maintain the functionality of the stored embryos (hatching capacity after exposure to conventional culture conditions) and their developmental competence after ET (normal fetuses by day 38 of pregnancy). Use of this strategy of CO₂-free storage should allow the shipment of fresh embryos worldwide in the absence of liquid nitrogen.

Keywords: CO₂-free embryo storage; morulae; blastocyst; embryo transfer; pig.

1. Introduction

Genetic spreading by use of transfer of embryos with an intact zona pellucida (ZP) notably reduces the risk of disease transmission between the donor and recipient farms associated to commerce with live animals. Porcine embryo transfer (ET) technology has advanced dramatically over the past years, particularly through the development of a nonsurgical deep uterine (NsDU) ET technique and the refinement of procedures for embryo preservation (reviewed in [1]).

High reproductive performance of the recipients has been reported after NsDU-ET of 24 to 30 fresh (0 h to 6 h post-collection) morulae or unhatched blastocysts [2-4]. However, in commercial programs, the embryos require a longer storage period to allow for their transportation from the donor farm to the recipient farm. Because cryopreservation allows for the unlimited preservation of embryos and the shipment of embryos to locations worldwide, it should be the preferred storage method for pig embryos. Vitrification is the only procedure proven effective for the cryopreservation of porcine embryos to date. Currently, high percentages (80–95%) of untreated morulae and blastocysts survive the in vitro vitrification and warming processes (reviewed in [1,5]). Moreover, vitrified-warmed morulae and blastocysts retain their capacity to develop in vivo as demonstrated by the high farrowing rates and litter sizes achieved when they are surgically transferred into the recipients (reviewed in [1]). However, the use of vitrified-warmed embryos has several disadvantages, such as the need to comply with strict regulations for the air handling and transport of liquid nitrogen (N₂L)-dewers, the risk of devitrification when dry-shipping containers are used for air transportation or the need to have N₂L and N₂L containers on the recipient farms to warrant safe manipulation of the embryo straws during the warming process. In addition, when vitrified embryos are used in combination

with NsDU-ET, the number of transferred embryos has to be notably increased to obtain comparable results to the surgical fertility results mentioned above [6]. These drawbacks call for alternative procedures for short-term embryo liquid storage.

In vitro culture could serve as one of these procedures; however, two primary concerns must be considered. First, the use of the NsDU-ET technique implies the transfer of embryos deep into a uterine horn. Therefore, the embryos should be in a morula or blastocyst stage immediately prior to ET. Second, for sanitary reasons, the embryos must be transferred with an intact ZP, which excludes the use of hatching and hatched blastocysts. Under conventional conditions of in vitro culture [i.e., culture medium supplemented with bovine serum albumin (BSA) or fetal calf serum (FCS) at 38.5°C in humidified air with 5% CO₂], more than 20% [3] and 50% [7] of the in vivo collected morulae and blastocysts hatched at 24 h of culture, respectively, which makes this process unsuitable for practical purposes. To improve this strategy, the embryos could be collected earlier when they are at the one-, two- or four-cell stages. These embryos yield high unhatched blastocyst formation rates after 3 to 5 days of conventional culture [8,9] and are able to develop to term after surgical transfer into recipients [10,11]. However, the quality in terms of the total cell number and the in vivo developmental capacity of the resultant blastocysts was much lower than that of uncultured blastocysts [12], which suggested that the conventional conditions generally used for in vitro embryo culture were suboptimal as was subsequently proven in other studies [9,13-15]. To minimize the detrimental effects of the in vitro environment on embryonic development, we previously examined a culture period as short as 24 h using in vivo-derived morulae [3]. In that study, we evaluated different incubation temperatures and storage media and demonstrated that more than 95% of the porcine morulae progressed to the unhatched blastocyst stage after 24 h of liquid storage in a defined

medium that did not require CO₂. Furthermore, despite the embryonic developmental delay observed at the end of storage, these blastocysts yielded high fertility and prolificacy rates after NsDU-ET into recipients. Although a 24-h storage period could permit the transportation of embryos in liquid state from the origin to the recipient farm within certain distances, a longer storage period would allow for the transport of the embryos over longer distances and, therefore, a more rational use of this type of embryo.

Overall, the objective of this study was to evaluate the *in vitro* viability and the *in vivo* developmental capacity (up to 38 days post ET) when *in vivo*-derived pig morulae were kept for up to 72 h in CO₂-free liquid state, comparing adequate different culture media and temperature ranges.

2. Materials and methods

Unless otherwise specified, reagents were obtained from Sigma-Aldrich Quimica SA (Madrid, Spain). All animal procedures complied with the European Directive 2010/63/EU for animal experiments and were *a priori* examined and approved by the Ethical Committee for Experimentation with Animals of the Murcia University (research code: 183/2015).

2.1. Animals

The embryo donors were multiparous (2 to 7 parity) Landrace × Large-White sows with similar lactation periods (21 to 24 d). After weaning, the animals were housed in individual crates in a barn with mechanical ventilation. Semen was obtained from boars (2 to 3 years of age) of proven fertility. The boars were allocated in individual pens in a climate-controlled facility (20 to 25°C).

All animals had ad libitum access to water and were fed commercial diets according to their nutrient requirements.

2.2. Estrus detection and artificial insemination

Embryos were obtained from sows with natural post-weaning estrus. The detection of estrus started the same day of weaning and was performed by skilled operators. Backpressure was applied on the females, and snout-to-snout contact of females with a mature boar was allowed. Sows were considered in estrus when they exhibited a clear standing reflex. Only sows starting estrus at day 4 or 5 after weaning were selected as embryo donors. The sows were inseminated at 0 and 24 h after the onset of estrus using the post-cervical artificial insemination procedure. Sperm doses containing 1.5×10^9 spermatozoa in 50 mL of BTS (Beltsville thawing solution; [16]) extender were produced from complete ejaculates and kept at 17°C for a maximum of 24 h.

2.3. Embryo recovery and evaluation

Donors were subjected to laparotomy in a simple operating room located next to the experimental building of the farm. Surgeries were performed on day 5 (to collect embryos at the morula stage) and 6 (to collect blastocysts) of the cycle (day 0=onset of estrus) according to the experimental design. Animals were sedated by intramuscular administration of azaperone (2 mg/kg body weight). Subsequently, sodium thiopental (7 mg/kg body weight, intravenous) was used for the induction of general anesthesia, which was maintained with isoflurane (3 to 5%).

The embryos were recovered as previously reported [17]. Briefly, once the number of corpora lutea on the ovaries was counted, the tip of each uterine horn was flushed with 30 mL of modified Tyrode's lactate (TL)-HEPES-polyvinyl alcohol (PVA) medium (TL-PVA; [3,18]).

The developmental stage and quality of the collected embryos were assessed according to the criteria of the International Embryo Transfer Society [19]. Only compacted morulae morphologically classified as excellent or good were used in the experiments.

The recovery rate was defined as the ratio of the number of embryos and oocytes and degenerate embryos recovered to the number of corpora lutea present. The fertilization rate was defined as the ratio of the number of viable embryos to the total number of embryos and oocytes and degenerate embryos collected.

2.4. Embryo storage and embryo culture

Morulae were washed ten times in TL-PVA medium and immediately stored according to the experimental design at different temperatures for up to 72 h in Eppendorf tubes containing 1.5 mL of different media. Following the storage period, the embryos were conventionally cultured in four-well plates containing NCSU-23 [20] supplemented with 0.4% BSA and 10% FCS (500 μ L per well). Cultures were performed at 38.5°C, 5% CO₂ in air and 95% humidity.

2.5. Evaluation of viability and embryonic developmental stage

The embryos were evaluated for viability and developmental progression by stereomicroscopy. Morulae that progressed during storage or during conventional culture to blastocysts with good or excellent morphology were considered viable. The in vitro survival rate was calculated as the proportion of the number of viable embryos divided by the total number of embryos evaluated.

The stage of development was scored as follows using previously outlined criteria [14]: 1, compacted morula (embryos with blastomeres totally compacted and an undistinguishable cell boundary; 2, early blastocyst (blastocysts with an initial visible blastocoele); 3, full blastocyst

(blastocysts with a differentiated blastocoele, inner cell mass and trophoblast); 4, expanded blastocyst (full blastocysts with increased outer diameter and thinned ZP); and 5, hatching/hatched blastocyst (expanded blastocyst with fractured or lost ZP). The hatching rate was defined as the proportion of the number of hatching or hatched embryos divided by the total number of embryos evaluated.

2.6. Measurement of embryonic size

Some embryos were photographed and their outer diameter (ZP included) and the width of the ZP were retrospectively measured using the open-source software ImageJ (National Institutes of Health; <http://rsbweb.nih.gov/ij/>).

2.7. Evaluation of total cell number

Embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 30 min at room temperature. The embryos were then washed twice with PBS supplemented with 3 mg/mL BSA and mounted in 4 μ L of Vectashield (Vector, Burlingame, CA, USA) containing 10 μ g/mL Hoechst 33342. The embryos were photographed using a fluorescence microscope with an excitation filter of 330 to 380 nm. The total cell number per blastocyst was determined by counting the number of nuclei with blue fluorescence. The cell counts were all performed by the same person using the same procedure and instruments.

2.8. Embryo transfer

Transfers were surgically performed in recipients on day 5 of the cycle. Before transfer, each recipient was treated with a long-acting amoxicillin suspension (Clamoxyl LA; Pfizer, Madrid,

Spain; 15 mg/kg, i.m.). The surgical procedure was similar to that described for embryo collection. The embryos were loaded into an ET catheter (Emtract Delphin, Gynetics, Lommel, Belgium) for transfer into the recipients. The transfer catheter was loaded with air bubbles that separated the 30- μ L drop of medium that contained the embryos from two 50- μ L drops of NCSU-BSA before and after the embryos. The embryos were deposited into the tip of a uterine horn at approximately 15 cm from the uterotubal junction. The transfer catheter was introduced into the uterus through of a small hole in the uterine wall, which was made with blunt Adson forceps. After ET, the tip of the uterine horn was reintroduced inside the abdominal cavity, the incision was sutured with continuous 1-polyglactin 910 sutures in three layers and the area was treated with chlorhexidine.

2.9. Experimental design

2.9.1 Experiment 1

This experiment was designed to evaluate the influence of storage temperature and medium on in vitro embryo survival and development of morulae stored for 48 h. Immediately after collection, groups of 7 to 10 morulae were stored for 48 h at 25°C or 37°C in TL-PVA medium (defined medium) or in NCSU-23 medium [20] supplemented with 10 mM HEPES and 0.4% of BSA (semi-defined medium; NCSU-BSA). Non-stored morulae cultured under conventional conditions (i.e., 500 μ L of NCSU-23 medium supplemented with 0.4% BSA and 10% FCS at 38.5°C in humidified air with 5% CO₂) were used as controls. Embryos from each donor were equally and randomly allocated to each of the groups. At 0 h and 48 h of storage, embryos were photographed for the subsequent determination of their outer diameter and ZP thickness. At the

end of storage, embryos were also assessed for viability and development stage, and then they were conventionally cultured for an additional 48 h to reassess viability and evaluate their hatching competence.

2.9.2. Experiment 2

Because the results of Experiment 1 showed that NCSU-BSA at 37°C was superior to the other conditions, we designed a second experiment with two aims: first, to evaluate the effectiveness of that medium (NCSU-23) supplemented with PVA instead of BSA; and second, to determine the viability of increasing the storage period until 72 h. Morulae were stored at 37°C in NCSU-BSA or NCSU-PVA for 72 h and then morphologically assessed for viability and development. Subsequently, the embryos were cultured for 24 h under conventional conditions to reassess their viability and evaluate their hatching competence following the same criteria described for Experiment 1. Morulae not subject to storage and cultured under conventional conditions were used as controls.

2.9.3. Experiment 3

This experiment was conducted to determine the *in vivo* developmental potential of the blastocysts derived from morulae stored in NCSU-BSA medium at 37°C for 72 h. At the end of storage, viable embryos that progressed to the unhatched blastocyst stage were transferred to recipients. The control group was formed by un-stored blastocysts, which were transferred to recipients within 3 h of recovery. A total of 25 blastocysts was transferred into each recipient. Twelve days after the transfer, recipients were evaluated daily for signs of estrus. Pregnancy was diagnosed by ultrasonography on Days 20 to 22 post-transfer. The uterus of each pregnant

recipient was collected at slaughter by day 38 to 41 of pregnancy, and the number of fetuses in each recipient was recorded.

2.10. Statistical analysis

The statistical analysis was performed using the IBM SPSS 19 Statistics package (IBM, Chicago, IL, USA). Binary variables were compared using Fisher's exact test. The development stage scores were analyzed using the Kruskal–Wallis test. Because this test revealed significant differences, two by two comparisons were performed using the Mann–Whitney U-test for two independent samples. Continuous variables were analyzed for normality by the Kolmogorov–Smirnov test, and groups were compared by a mixed-model ANOVA. The ANOVA model included the fixed effects of temperature and medium and their interaction (Experiment 1) or the medium (Experiment 2) and the random effect of the replicate. If the ANOVA revealed a significant effect, the means were compared using Bonferroni's test. The outer diameter and ZP thickness at 0 h and 48 h of storage within each group were compared using Student's t test for related samples, and the total cell number per blastocyst between groups was compared using Student's t test for independent samples. Differences were considered significant at $P < 0.05$. The results are expressed as percentages and $\text{mean} \pm \text{S.E.M.}$

3. Results

3.1. Experiment 1

Out of 20 sows, sixteen (80%) had morulae, three (15.0%) had blastocysts and one (5.0%) had only oocytes. The mean ovulation rate per pregnant sow was 22.1 ± 1.7 corpora lutea (range 15 to

28 corpora lutea). The recovery and fertilization rates were 94.0% and 92.4%, respectively. A total of 316 morulae were used in this experiment. The embryos recovered at the blastocyst stage were used for other research purposes.

The embryonic viability at 48 h of storage under different temperatures and media conditions is shown in Figure 1. At the end of the storage period, the TL-PVA medium at 25°C was harmful ($P<0.05$) for embryonic viability (73.9%) compared with the control (93.2%) or the other experimental groups (range: 90.9% to 98.3%). Following conventional culture conditions, the NCSU-BSA medium group at 37°C was the only group with embryonic viability rates similar (96.7%) to that of the control group (93.2%).

Embryonic development at 48 h of storage was delayed ($P<0.01$) in all experimental groups compared with the controls, with the delay was more pronounced at 25°C than at 37°C (Figure 2A) regardless of the storage medium. The developmental delay continued at 24 h and 48 h of conventional culture in all experimental groups except for embryos stored at 37°C in NCSU-BSA, which achieved similar development as the control embryos during conventional culture (Figure 2B-D).

Consistent with these data, a comparable pattern was observed for the outer diameters and the thicknesses of the ZP of the embryos at the end of storage period (Table 1). Significant interactions were not observed between temperature and medium. In addition, differences in the diameter and ZP thickness were not observed between 0 h and 48 h of storage for embryos stored at 25°C. In contrast, embryos stored at 37°C, like the control embryos, had a larger diameter ($P<0.001$) and a smaller ZP thickness ($P<0.001$) at 48 h than at 0 h of storage.

Although most embryos stored at 37°C reached the blastocyst stage, none of them, unlike controls, hatched at the end of storage (Figure 2E-G). The embryos stored at 37°C in NCSU-BSA

had the same hatching ability as the control embryos at the end of the conventional culture (~85%), and these values were much higher ($P<0.01$) than those observed in the other experimental groups (<25%) (Figure 2G). Despite similarities observed by the end of the conventional culture between embryos stored at 37°C in NCSU-BSA and controls, differences were observed ($P<0.001$) in the total cell numbers between blastocysts that hatched in each group (96.6 ± 3.1 and 149.6 ± 12.9 cells, respectively).

3.2. Experiment 2

A total of 14 donor sows was used in this experiment. Embryos at the morula and blastocyst stages were collected from 11 (78.6%) and 3 (21.4%) sows, respectively.

The mean number of corpora lutea in the donor sows was 18.5 ± 1.4 (range 12 to 29 corpora lutea). The ratio of collected embryos to the total number of corpora lutea counted was 84.9%, and the fertilization rate was 95.6%. Only embryos at the morula stage ($n=167$) were used in this experiment. The embryos collected at the blastocyst stage were used for other studies.

In vitro survival of the morulae stored for up to 72 h is shown in Figure 3. The viability of embryos stored for 48 h in NCSU-BSA was not different from that observed in the control group (98.3% and 98.0%, respectively), but it was higher ($P<0.02$) than that of the embryos stored in NCSU-PVA medium (84.5%). This pattern continued at 72 h of storage, with the differences in viability between the NCSU-PVA group (53.4%) and the other two groups (98.3% and 90.0% for the NCSU-BSA and control groups, respectively) becoming more pronounced ($P<0.001$). In the NCSU-PVA group, the percentage of viable embryos decreased after 24 h of

conventional culture (43.1%), whereas all viable embryos from the NCSU-BSA group remained viable after 24 h of conventional culture (Figure 3), which was similar to the control.

A developmental delay was observed in the stored embryos compared with the controls ($P < 0.02$), and it was more evident for the embryos stored in NCSU-PVA medium (Figure 4A). This developmental delay continued in the embryos stored in NCSU-PVA medium after 24 h of conventional culture ($P < 0.003$) but disappeared in the embryos from the NCSU-BSA group (Figure 4B-D). Despite the developmental delay, some embryos (6.7%) stored in NCSU-BSA medium hatched at the end of storage (Figure 4E-G). Although the hatching ability after conventional culture was similar for the embryos from NCSU-BSA and control groups (86.0% and 76.4%, respectively), a significant reduction in the total cell number per hatched blastocyst was observed (85.5 ± 4.8 and 143.6 ± 6.6 cells, respectively). In contrast, none of the embryos stored in the NCSU-PVA medium hatched at the end of storage or after 24 h of conventional culture.

Representative images showing the development of morulae stored in NCSU-BSA medium at 37 °C for 72 h are depicted in Figure 5.

3.3. Experiment 3

For the transfers, we used 86 morulae collected from five donors at day 5, which were stored for 72 days in NCSU-BSA, and 75 blastocysts recovered from five donors at day 6, which were transferred just after collection (control group). Blastocysts derived from stored morulae and control blastocysts were transferred to a total of six recipients (three per group). One pregnant recipient from the NCSU-BSA group returned to estrus on day 28. The remaining recipients were pregnant and maintained the pregnancy until slaughter. The mean number of fetuses

collected in the recipients in the NCSU-BSA group was 11.0 ± 4.0 , of which 10.0 ± 3.0 fetuses were diagnosed as alive. The recipients transferred with control embryos had 15.3 ± 0.9 fetuses, of which 12.7 ± 1.3 were alive. These results indicate that in pregnant recipients, 40.0% of the stored embryos were able to develop until day 38 of pregnancy and form live fetuses, whereas the percentage was 50.8% in the control embryos.

4. Discussion

The legal regulatory challenges associated with the transportation of N₂L and, more importantly, the remaining technical obstacles to the use of cryopreserved embryos in combination with NsDU-ET [6] have encouraged the development of alternative methods for short-term embryo storage in a liquid state [3,21]. In the present study, we chose a particular stage of embryo development and two culture media for the storage of porcine embryos in a liquid state. We selected embryos at the morula stage for several reasons: first, to avoid the adverse effects of long culture periods of earlier stage embryos on the quality of the resulting blastocysts; second, because under conventional culture conditions, most morulae should have an autonomy of at least 24 h without progression to the hatching blastocyst stage; third, because high in vitro embryo viability (>95%) of porcine morulae stored for 24 h at 37°C in a chemically defined medium was obtained in a previous study. Moreover, despite the storage conditions tested produced a developmental delay, the resulting blastocysts kept their capacity to develop to term following NsDU-ET at similar levels to that of the control blastocysts [3]; and fourth, because although fresh morulae together with NsDU-ET have provided optimal reproductive results, the use of blastocysts is more advisable for ET programs because these embryos tolerate a wider

donor-recipient asynchrony [22]. Therefore, blastocysts derived from stored morulae ought to have a great potential for commercial ET.

From a practical perspective and to bypass additional liquid-state embryo shipment requirements, it is more convenient to use storage media capable of maintaining proper pH levels without the need for CO₂ gas; therefore, we used two media supplemented with HEPES: TL-PVA medium, which is a chemically defined medium frequently used for porcine embryo recovery and handling [4,22,23], and an adaptation of the extensively used porcine embryo culture NCSU-23 medium [20] supplemented with BSA, in which bicarbonate was partially replaced by HEPES (NCSU-BSA). In addition, we decided to prolong the storage period from the 24 h used in our previous study [3] to 72 h because a longer period should allow for the global distribution of embryos and their efficient utilization in a liquid state.

In Experiment 1, the embryo survival rate at the end of 48 h of storage was not different between the controls and the experimental groups with the exception of the TL-PVA-25°C group, which showed the lowest survival. However, when viable embryos following storage were conventionally cultured for 24 h to 48 h to reassess their viability, only the embryos from the NCSU-BSA group stored at 37°C maintained the same survival rate as the controls. These results demonstrated that a temperature of 25°C during 48 h of storage was harmful for embryonic viability regardless of the storage medium used. In our previous study [3] and in a study by Pomar et al. [21], high embryonic survival rates were achieved after 24 h of storage at 25°C using media supplemented with BSA. However, in both cases, the embryos were not further cultured to reassess viability; therefore, the results do not indicate the effects of storage on embryo functionality. In the present study, we also obtained high survival rates for embryos stored at 25°C in a BSA medium (~95%). However, almost 20% of these embryos degenerated

between 24 and 48 h of conventional culture. This finding suggests that some embryos with apparently normal morphology were functionally damaged during storage.

Storage medium played an important role when the preservation temperature was 37°C, with the NCSU-BSA medium showing much more effective results than the TL-PVA medium. We previously demonstrated that TL-PVA provides a chemically defined medium capable of maintaining high in vitro and in vivo viability of porcine morulae stored at 37°C for 24 h. However, it becomes clear from our present results that the TL-PVA medium at 37°C was not efficient to support a 48 h storage period. Although these conditions supported embryo viability up to 48 h of storage (>90% survival rate), a considerable proportion of embryos from the TL-PVA medium group that were classified as viable at the end of storage period was not able to further progress and degenerated under conventional culture conditions. This finding suggests that embryo injuries occurred during preservation in TL-PVA medium at 37°C, which is similar to the results for storage at 25°C. In contrast, our present results also indicate that it is feasible to achieve high survival rates after 48 h of storage at 37 °C in a medium containing BSA with successive embryo development similar to that of the control embryos. To avoid any risk of viral contamination, it would be preferable to use of media without serum or serum components [24,25]. Nevertheless, BSA clearly possesses fewer hazards than serum for disease transmission via ET [25] and is infinitely less dangerous than the exchange of live animals between farms. As proof, BSA is currently incorporated in many commercial media used in bovine ET, a booming worldwide technology.

Our present results also indicate a significant embryonic developmental delay at 48 h of storage in all experimental groups compared with the controls, with the delay more severe at 25°C than at 37°C regardless the storage medium. These results are consistent with those

reported for porcine morulae stored for 24 h under similar conditions [3] and for stored embryos in other species [26,27]. The differences in embryo development were in accordance with the size of the embryos, which confirms the results of previous studies [3,21].

Interestingly, the viable embryos from all experimental groups were able to further progress after storage when they were incubated under conventional culture conditions. However, only those embryos stored at 37°C in NCSU-BSA medium achieved a similar degree of development as the control embryos at the end of conventional culture, although having 1.5-fold lower total cell number. This finding indicates an important mitotic reduction that could be related to dysregulations of the genome or embryonic metabolism during storage, which has been previously reported for embryos of other species under hypothermic conditions [27,28]. Nevertheless, the fact that the embryos stored at 37°C in NCSU-BSA medium had a similar hatching rate at the end of the conventional culture as the control embryos predicts their correct functionality because the hatching rate has been shown to represent a sensitive biological indicator of blastocyst quality and developmental potential [29].

The present study showed that approximately 30% and 80% of the control morulae hatched at 48 h and 72 h of conventional culture, respectively. In contrast, none of the stored embryos hatched at 48 h of storage, regardless of the temperature and medium used. This finding is highly relevant from a sanitary standpoint, since the ZP must remain intact until the embryos are transferred to recipients to preserve appropriate sanitary conditions [30].

In our second experiment, we evaluated the reliability of NCSU-23 medium supplemented with PVS instead of BSA in an attempt to develop a storage method lacking animal components. In addition, we extended the storage period to 72 h to provide sufficient time for the efficient international exchange of liquid-kept embryos. The results from this

experiment clearly indicated that the survival rates of embryos stored in NCSU-PVA medium were reduced at 48 h and 72 h of storage compared with those obtained in the NCSU-BSA and control groups. Moreover, approximately 40% of embryos stored in the absence of BSA survived after 24 h of conventional culture, which means that the final viability in that group was decreased more than 2 fold in relation to that of the other groups. In contrast, the viability of embryos stored in the presence of BSA was similar to that of the control embryos at each evaluated time-point. In addition to viability, two other fundamental differences between both media were noted. First, the developmental delay observed in embryos stored for 72 h in NCSU-BSA medium compared with the controls vanished after 24 h of conventional culture, whereas this was not observed in the embryos maintained in NCSU-PVA medium. Second, the hatching capacity of embryos in the presence of BSA was similar to that of the controls but was totally inhibited in the absence of BSA, even after conventional culture. Taken together, these results indicate a protective effect of BSA on the embryos under the storage conditions used in our study. This observation is not surprising considering the numerous benefits BSA provides to the in vitro embryo culture (reviewed in [31]), including its antioxidant, nutritive and pH buffer roles during embryonic development. Additionally, other studies have also reported the benefits of BSA prolonging viability of sperm stored above the freezing point [32] and protecting sperm DNA [33] and membrane integrity [34]. However, we must note that a proportion of the stored morulae, although small, hatched at 72 h of storage in the NCSU-BSA medium, and these embryos should therefore be discharged for sanitary reasons in commercial ET programs.

Our results firmly suggest that despite keeping embryos in NCSU-BSA medium at 37 °C delayed their development after 72 h of storage, a proportion of the stored embryos were competent for further develop in vivo, at least, until day 38 of pregnancy. This fact was not

surprising because we previously reported that in vivo embryo development was not affected after the transfer of developmentally delayed pig embryos stored for 24 h [3]. Similar findings have also been reported in mouse embryos [27]. However, because of the limited number of recipients used in our study, we did not compare the in vivo developmental potential of the stored embryos to that of the un-stored control blastocysts, and we can state only that a proportion of the stored embryos under the conditions used in our study were developmentally competent.

4.1. Conclusions

In conclusion, morulae stored at 37°C in a semi-defined medium containing BSA maintained their in vitro viability and developmental competence for up to 72 h. Furthermore, none of the blastocysts derived from morulae stored under these conditions hatched at 48 h of storage, and only a small proportion of them hatched at 72 h. Additionally, these embryos were able to develop into normal fetuses by day 38 of pregnancy. Although these findings open new possibilities for porcine embryo preservation in liquid state, further research is needed to determine the capacity of these embryos to undergo full-term development following NsDU-ET.

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Author contributions

CA Martinez, C Cuello, H Rodriguez-Martinez, EA Martinez and MA Gil conceived, designed and directed the study. CA Martinez, A Nohalez, I Parrilla, X Lucas, J Sanchez-Osorio, C Cuello, EA Martinez and MA Gil performed the experiments. CA Martinez, C Cuello, H Rodriguez-Martinez, EA Martinez and MA Gil analyzed and interpreted the data. CA Martinez wrote the manuscript. J Roca, C Cuello, H Rodriguez-Martinez, EA Martinez and MA Gil revised the manuscript. All authors approved the manuscript for publication. EA Martinez, MA Gil, J Roca and J Sanchez-Osorio obtained the funding to carry out the study.

Declaration of interest

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

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Figure legends

Fig. 1. Survival rates of in vivo-produced porcine morulae after storage for 48 h at different temperatures and media.

Morulae were stored in TL-PVA and NCSU-BSA (both media supplemented with 10 mM HEPES) at 25°C and 37°C for 48 h (dark grey bars). After storage, the viable stored embryos from each group were cultured for an additional 24-48 h under conventional culture conditions (NCSU-23 culture medium supplemented with 0.4% BSA and 10% FCS, at 38.5°C in humidified air with 5% CO₂) to reassess their in vitro survival (light grey bars). Control morulae were cultured under conventional conditions (NCSU-23 culture medium supplemented with 0.4% BSA and 10% FCS, at 38.5°C in humidified air with 5% CO₂) for 48 h (black bar). Viable control morulae were then conventionally cultured in fresh culture medium for an additional 24-48 h (white bar). n=total number of embryos analyzed. Bars with different small letters (a, b) are significantly different (P<0.05). Bars with different capital letters (A, B) are significantly different (P<0.05).

Fig. 2. Embryonic developmental stages of in vivo-produced porcine morulae stored for 48 h in a liquid state.

(A) Summary of the developmental stages of morulae at the end of the storage period. Morulae were stored for 48 h in closed tubes containing TL-PVA or NCSU-BSA media at 25°C or 37°C.

*After storage, the embryos from each group were cultured under conventional conditions (NCSU-23 medium supplemented with 0.4% BSA and 10% FCS at 38.5°C in humidified air with 5% CO₂) for up to 48 h to re-evaluate the embryonic development. #Controls included morulae cultured under conventional conditions for 48 h and then maintained in fresh conventional culture medium for an additional 24 h to 48 h. The developmental stage was scored according to

the following classes: 1, morula; 2, early blastocyst; 3, full blastocyst; 4, expanded blastocyst; and 5, hatching or hatched blastocyst. ^{a,b,c} Different letters in the same column indicate significant differences ($P<0.01$). Values are expressed as the mean $\pm$ SEM.

(B-D) Bar graphs showing the proportion of the different embryonic developmental stages reached in each group at 48 h of storage (B) and after 24 h (C) and 48 h (D) of conventional culture. Bl: blastocyst.

(E-G) Bar graphs showing the hatching-hatched blastocyst rates reached in each group from morulae stored for 48 h (E) and after 24 h (F) and 48 h (G) of conventional culture. ^{a,b} Different letters represent significant differences ($P<0.001$).

Fig. 3. Survival rates of in vivo produced porcine morulae after storage for 48 h and 72 h at 37°C in different storage media.

Morulae were stored in NCSU-BSA and NCSU-PVA (both media supplemented with 10 mM HEPES) at 37°C for 48 h (hatch-marked bars) and 72 h (striped bars). After storage, the viable stored embryos from each group were cultured for an additional 24 h under conventional culture conditions (NCSU-23 culture medium supplemented with 0.4% BSA and 10% FCS at 38.5°C in humidified air with 5% CO₂) to reassess in vitro survival (pointed bars). Control morulae were cultured under conventional conditions (NCSU-23 culture medium supplemented with 0.4% BSA and 10% FCS at 38.5°C in humidified air with 5% CO₂) for 48 h (black bar) and 72 h (grey bar). Viable control morulae were then conventionally cultured in fresh culture medium for an additional 24 h (white bar). n=total number of embryos analyzed.

Bars with asterisks indicate significant differences compared with the NCSU-BSA and control groups (* $P<0.02$; ** $P<0.001$).

Fig. 4. Embryonic developmental stages of in vivo-produced porcine morulae stored for up to 72 h in a liquid state.

(A) Summary of the developmental stages of morulae at 48 h of storage and the end of the storage period (72 h). Morulae were stored for 72 h in closed tubes containing NCSU-BSA or NCSU-PVA media at 37°C. *After storage, the embryos from each group were cultured under conventional conditions (NCSU-23 medium supplemented with 0.4% BSA and 10% FCS at 38.5°C in humidified air with 5% CO₂) for 24 h to re-evaluate the embryonic development. #Controls included morulae cultured under conventional conditions for 72 h and then maintained in fresh conventional culture medium for an additional 24 h. The developmental stage was scored according to the following classes: 1, morula; 2, early blastocyst; 3, full blastocyst; 4, expanded blastocyst; and 5, hatching or hatched blastocyst. ^{a,b,c} Different letters in the same column indicate significant differences ($P < 0.003$). ^{d,e,f} Different letters in the same column indicate significant differences ($P < 0.02$). Values are expressed as the mean $\pm$ SEM.

(B-D) Bar graphs showing the proportion of the different embryonic developmental stages reached in each group at 48 h (B) and 72 h (C) of storage and after 24 h of conventional culture (D). Bl: blastocyst.

(E-G) Bar graphs showing the hatching-hatched blastocyst rates reached in each group from morulae stored for 48 h (E) and 72 h (F) and after 24 h of conventional culture (G). ^{a,b} Different letters represent significant differences ($P < 0.001$).

Fig. 5. Preservation of in vivo-derived morulae in NCSU-BSA medium at 37°C for up to 72 hours.

(A) Representative bright field images showing freshly collected pig morulae (a) and blastocysts at different developmental stages following the conventional culture of morulae for 48 h (b), 72 h (c) and 96 h (d).

(B) Representative bright field images showing freshly collected pig morulae (a') and blastocysts at different developmental stages following the storage of morulae in NCSU-BSA medium at 37°C for 48 h (b') and 72 h (c'). At the end of storage, the resulting blastocysts were conventionally cultured for 24 h to reassess their viability and hatching competence (d').

Fig. 6. Fetuses collected after embryo transfer. (A) Representative images of fetuses collected at day 41 of pregnancy from a recipient transferred with 25 un-cultured blastocysts (control). (B) Representative images of fetuses collected at day 38 of pregnancy from a recipient transferred with 25 blastocysts derived from morulae stored for 72 h in NCSU-BSA medium at 37°C. Scale bar is 1 cm.

Tables

Table 1. Outer diameters and zona pellucida (ZP) thicknesses of in vivo-derived morulae stored for 48 h at different temperatures and in different media.

Temperature (°C)	Medium	Time of storage					
		0 h			48 h		
		No.	Outer diameter (μm)	ZP thickness (μm)	No.	Outer diameter (μm)	ZP thickness (μm)
38.5	Control	44	151.2 $\pm$ 0.7	14.5 $\pm$ 0.3	31	238.0 $\pm$ 7.2 ^{aA}	4.8 $\pm$ 0.4 ^{aA}
25	TL-PVA	53	152.8 $\pm$ 0.5	14.0 $\pm$ 0.2	39	154.7 $\pm$ 0.9 ^b	14.0 $\pm$ 0.3 ^b
	NCSU-BSA	60	153.2 $\pm$ 0.6	14.8 $\pm$ 0.3	56	154.9 $\pm$ 0.9 ^b	14.3 $\pm$ 0.2 ^b
37	TL-PVA	55	152.5 $\pm$ 0.6	13.5 $\pm$ 0.2	50	178.4 $\pm$ 2.0 ^{cA}	8.4 $\pm$ 0.3 ^{cA}
	NCSU-BSA	60	151.9 $\pm$ 0.5	14.3 $\pm$ 0.3	59	196.1 $\pm$ 2.4 ^{cA}	7.8 $\pm$ 0.3 ^{cA}

Controls included morulae cultured under conventional conditions (NCSU-23 medium supplemented with 0.4% BSA and 10% FCS at 38.5°C in humidified air with 5% CO₂) for 48 h.

^{a,b,c} Different superscripts in the same column represent significant differences ($P < 0.002$). ^A

Capital letter superscript indicates significant differences ($P < 0.001$) compared with the same variable at time 0 h. Data are shown as the mean $\pm$ S.E.M.

A

Temperature (°C)	Medium	Embryonic developmental stage		
		Storage	Conventional culture*	
		48 h	24 h	48 h
38.5	Control [#]	4.2±0.1 ^a	4.7±0.1 ^a	4.8±0.1 ^a
25	TL-PVA	1.4±0.1 ^b	3.3±0.1 ^b	3.7±0.1 ^b
	NCSU-BSA	1.4±0.1 ^b	3.2±0.1 ^b	4.2±0.1 ^c
37	TL-PVA	3.4±0.1 ^c	3.8±0.1 ^c	4.2±0.1 ^c
	NCSU-BSA	3.5±0.1 ^c	4.6±0.1 ^a	4.8±0.1 ^a

B

C

D

E

F

G

A

Medium	Embryonic developmental stage		
	Storage		Conventional culture*
	48 h	72h	24 h
Control [#]	4.1±0.1 ^a	4.6±0.1 ^d	4.9±0.1 ^a
NCSU-BSA	3.5±0.1 ^b	4.1±0.1 ^e	4.8±0.1 ^a
NCSU-PVA	3.1±0.1 ^c	3.6±0.1 ^f	4.0±0.0 ^b

B

E

C

F

D

G

A **B**

— 100 μm