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Exogenous ascorbic acid enhances vitrification survival of porcine in vitro-developed blastocysts but fails to improve the in vitro embryo production outcomes

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Abstract

In this study, the effects of addition of the antioxidant ascorbic acid (AsA) were evaluated during porcine in vitro embryo production (IVP) and vitrification. In experiment 1, the effects of AsA supplementation during IVM, IVF and IVC were evaluated, using a total of 2,744 oocytes in six replicates. The IVM, IVF and embryo IVC media were supplemented or not (control) with 50 µg/mL AsA in all possible combinations. No significant effects of AsA were detected in any of the maturation, fertilization or embryo development parameters assessed. In experiment 2, we evaluated the effects of adding AsA to vitrification-warming media on the post-warming survival and quality of blastocysts. Day-6 in vitro-produced blastocysts (N=588) from six replicates were randomly divided in two groups, with vitrification and warming media either supplemented with 50 µg/mL AsA (VW+ group) or un-supplemented (VW- control). Addition of AsA increased ($P<0.05$) blastocyst survival rate after vitrification compared with that of VW- control embryos. Vitrification and warming increased ($P<0.05$) the production of oxygen species (ROS) and reduced ($P<0.05$) the glutathione levels in blastocysts. Although VW+ blastocysts displayed higher ($P<0.05$) ROS levels than those of fresh control blastocysts, the levels were lower ($P<0.05$) than those found in VW- control blastocysts. In conclusion, under the experimental conditions, supplementation of IVM/IVF/IVC media with AsA did not improve the embryo production in vitro. By contrast, the addition of AsA to chemically defined vitrification and warming media increased the survival of in vitro-produced porcine blastocysts by decreasing ROS production.

Keywords: Ascorbic acid; porcine; in vitro maturation; in vitro fertilization; vitrification.

1. Introduction

In vitro production (IVP) of porcine embryos is an important tool for agricultural, biotechnological and biomedical purposes. Although substantial progress has been achieved in embryo IVP systems [1,2,3], the overall efficiency remains unsatisfactory because of the prevailing high incidence of polyspermy during IVF [4-7], leading to low efficiency of blastocyst production and to poor quality of the resulting blastocysts [8,9]. Although many different supplements, such as follicular fluid [10], vitamins [11,12], growth factors [9] or hormones [13], have been added to IVP media in attempts to improve the IVP of porcine embryos, the general assumption is that IVC conditions remain suboptimal. One of the primary differences between in vitro and in vivo conditions is related to oxidative stress [14]. The oxygen content of IVP environments is higher than that in vivo, which results in increased production of ROS [15,16]. These high ROS levels during embryo IVP are harmful to gametes and embryos [17-19]. Thus, protecting oocytes and embryos against oxidative stress during in vitro culturing is a key step for improving embryo IVP efficiency and embryo quality. To achieve this goal, a widely used strategy is the addition of antioxidants to the media [12,20-23].

Ascorbic acid (AsA), the most important antioxidant in extracellular fluids [24], is one of the antioxidants tested in embryo IVP that shows some beneficial properties. When present during IVC, AsA increases the cleavage rate in bovine [25] and blastocyst rates in ovine [26]. In porcine, AsA shows positive effects on oocyte nuclear maturation [12] and blastocyst formation after parthenogenetic activation [27]. Addition of AsA also apparently protects embryos against oxidative stress during IVC, improving embryo developmental competence after either IVF [28] or parthenogenesis [27,29]. In some studies, the quality of blastocysts in terms of cell numbers [29] or survival after vitrification [30] also increases with supplementation with AsA during IVC. Moreover,

addition of AsA to vitrification and warming media supplemented with serum increases embryo survival rates of in vitro-produced porcine blastocysts [30]. Collectively, the above studies suggest that AsA could be an interesting molecule to use during embryo IVP and/or vitrification in porcine to avoid the excessive increase of ROS and their deleterious effects on oocytes and embryos. However, no systematic study has explored the influence of AsA on each step of the embryo IVP system and the presence of possible synergistic effects. Therefore, the aims of the present study were the following: (1) evaluate the effects of AsA supplementation to IVM, IVF and IVC media, including all possible combinations, on maturation, fertilization and embryonic developmental parameters; and (2) assess the effects of adding AsA to vitrification and warming defined media on the vitrification survival of IVP-porcine blastocysts.

2. Materials and methods

All experimental procedures used in this study were performed in accordance with the 2010/63/EU EEC Directive for animal experiments and were reviewed and approved by the Ethical Committee for Experimentation with Animals of the University of Murcia, Spain (research code: 1002/2012).

2.1. Reagents and culture media

Unless otherwise specified, all chemicals used in this study were purchased from Sigma–Aldrich Co. (Alcobendas, Madrid, Spain). A physiological saline solution composed of NaCl 0.9% (w/v) and 70 µg/mL kanamycin was used to transport ovaries from the slaughterhouse to the laboratory. The cumulus–oocyte complexes (COCs) were collected and washed in Tyrode’s lactate supplemented with 10-mM HEPES and 0.1% (w:v) polyvinyl alcohol (TL-PVA) [31,32]. The oocyte maturation medium was

TCM-199 (Gibco Life Technologies S.A., Barcelona, Spain) supplemented with 0.57-mM cysteine, 0.1% (w:v) PVA, 10 ng/mL EGF, 75 µg/mL penicillin G potassium, and 50 µg/mL streptomycin sulfate. The basic medium used for IVF was a modified Tris-buffered medium [33] enriched with 2.0-mM caffeine and 0.2% (w:v) BSA. A Dulbecco's PBS (Gibco, Grand Island, NY) with 4 mg/mL of BSA was used for washing spermatozoa after thawing and before re-suspension in IVF medium. The embryo culture medium was North Carolina State University 23 (NCSU23) [34] supplemented with 0.4% BSA. The basic medium for vitrification and warming was the chemically defined TL-PVA medium. The first vitrification medium (V1) was TL-PVA containing 7.5% (v:v) of ethylene glycol and 7.5% of dimethyl sulfoxide (DMSO), and the second vitrification medium (V2) was TL-PVA containing 16% (v:v) of ethylene glycol and 16% DMSO and 0.4 M sucrose. The warming medium consisted of TL-PVA supplemented with 0.13 M sucrose.

2.2. Cumulus-oocyte complexes (COCs) collection and in vitro maturation

Ovaries were collected from pre-pubertal gilts at a local slaughterhouse and transported to the laboratory at the University of Murcia at 35 °C within 1 h of collection. Then, medium-sized follicles (3–6 mm in diameter) were sectioned with a sterile scalpel blade into TL-PVA to collect COCs. Oocytes surrounded by two or more layers of compact cumulus cells and with evenly granulated cytoplasm were selected and washed in maturation medium. Groups of 75-80 COCs were transferred into a well of a four-well multidish (Nunc, Roskilde, Denmark) containing 500 µL of maturation medium supplemented with 10 IU/mL eCG (Folligon; Intervet International B.V., Boxmeer, The Netherlands) and 10 IU/mL hCG (Veterin corion; Divasa Farmavic S.A., Barcelona, Spain) for 22 h. The oocytes were then incubated for another 20 to 22 h in maturation

medium without hormones. Maturation was performed under mineral oil at 38.5 °C in 5% CO₂ in air and 95% to 97% relative humidity.

2.3. In vitro fertilization

After oocyte maturation, cumulus cells were removed with 0.1% hyaluronidase in maturation medium by vortexing for 2 min at 1,660 rounds/min. The denuded oocytes were washed three times in IVM medium and three times in IVF medium. Then, groups of 30 oocytes were placed into 50 µL drops in IVF medium in a 35 mm × 10 mm Petri dish (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) under mineral oil and were maintained in an incubator (38.5 °C, 5% CO₂ in air and 95% to 97% relative humidity) until addition of spermatozoa. Semen from a mature boar cryopreserved in 0.5 mL straws as described by Roca et al. [35] was thawed (two straws per replicate) in a circulating water bath at 37 °C for 20 s. Immediately after thawing, 100 µL of semen was washed three times by centrifugation at ×1,900 g for 3 min in 10 mL of Dulbecco's PBS. The resulting pellet was suspended in IVF medium. After appropriate extension, 50 µL of this sperm suspension was added to the medium with the oocytes such that the final sperm concentration was 3×10^5 spermatozoa/mL; thus, each oocyte was exposed to 1,000 spermatozoa. Oocytes were co-incubated with spermatozoa for 5 h under mineral oil at 38.5 °C in an atmosphere of 5% CO₂ in air and 95% to 97% relative humidity.

2.4. In vitro culture and assessment of embryonic development

After gamete co-incubation, presumptive zygotes were washed by three rounds of mechanical pipetting in IVC medium to remove spermatozoa that were not bound to the zona pellucida. Presumptive zygotes were then transferred (30 zygotes per well) into a

four-well multidish containing 500 μ L of glucose-free embryo culture medium (NCSU23-BSA) supplemented with 0.3-mM pyruvate and 4.5-mM lactate for 2 days and then changed to fresh embryo culture medium containing 5.5-mM glucose for an additional 5 day period. Embryo culture was performed under paraffin oil in an incubator at 38.5 °C, 5% CO₂ in air, and 95% to 97% relative humidity.

2.5. Assessment of maturation, fertilization and embryo development

To evaluate the maturation and fertilization, representative aliquots of oocytes and presumptive zygotes were fixed at 44 h of IVM and at 18 h after IVF, respectively, in a solution of acetic acid:ethanol (1:3) for 72 h at room temperature. Fixed oocytes and presumptive zygotes were stained with 1% (w:v) lacmoid in 45% acetic acid and examined under a phase-contrast microscope at magnification $\times 400$. Oocytes with chromatin enclosed in a nuclear membrane or those with condensed chromatin but without extruded polar body were classified as immature oocytes. Oocytes were considered mature when their chromosomes were organized at metaphase and they showed an extruded first polar body (MII).

Presumptive zygotes were considered penetrated when they contained one or more swollen sperm heads and/or male pronuclei and two polar bodies. Sperm penetration rate was the ratio of the number of penetrated oocytes relative to the total number of mature oocytes inseminated. Monospermic rate was calculated as the ratio of oocytes with one female pronucleus, one male pronucleus and two polar bodies to the total number of matured oocytes penetrated. The efficiency of fertilization was the ratio of the number of monospermic oocytes relative to the total number of matured oocytes inseminated.

The cleavage (ratio of the number of embryos cleaved to two to four cells of the total number of oocytes inseminated) and blastocyst formation rates (ratio of the number of blastocysts of the total number of cleaved embryos) were morphologically evaluated using a stereomicroscope at Day 2 and Day 7 post-insemination (Day 0), respectively. The total efficiency was described as the percentage of the total number of inseminated oocytes that reached the blastocyst stage. Blastocysts were fixed in 4% (v:v) paraformaldehyde in PBS for 30 min at room temperature (24 °C) to assess total cell numbers. After fixation, embryos were washed with PBS containing 3 mg/mL BSA (PBS-BSA), placed on a slide in a drop of 4 µL of VECTASHIELD (Vector Labs, Burlingame, CA, USA) containing 10 µg/mL Hoechst 33342, and covered with a coverslip. Stained embryos were examined with fluorescence microscopy using a 330 to 380-nm excitation filter. The total number of nuclei that showed blue fluorescence was counted.

2.6. Vitrification and warming; assessment of survival and hatching rates

Vitrification was performed according to the method described previously [36,37]. All media used for vitrification and warming were held at 38.5 °C. Briefly, groups of five to six embryos were washed twice in TL-PVA and consecutively equilibrated in V1 for 3 min and in V2 for 1 min for vitrification. During the final equilibration, embryos were placed in a 1 µL drop and loaded in the narrow end of a super open pulled straw (SOPS; Minitüb, Tiefenbach, Germany) by capillary action. Subsequently, straws containing the embryos were horizontally plunged into liquid nitrogen (LN₂). After storage in LN₂ for one week, the straws were removed and warmed by the one-step warming method [38,39]. Briefly, the straws were vertically submerged in one well of a four-well multidish containing 800 µL of warming medium and equilibrated for 5 min. Then,

1 vitrified-warmed blastocysts were washed in TL-PVA and cultured in vitro to assess the
2 embryo viability. Vitrified-warmed blastocysts that reformed their blastocoelic cavities
3 after warming and displayed a normal or thinning zona pellucida with an excellent or
4 good appearance during the culture were considered viable. The survival rate was
5 defined as the ratio of viable embryos to the total number of vitrified-warmed cultured
6 blastocysts. Additionally, after warming, the hatching rate (ratio of hatching or hatched
7 embryos at the end of the culture to the total number of cultured embryos) was
8 evaluated.

10 **2.7. Differential staining**

11 The number of inner cell mass (ICM) and trophectoderm (TE) cells of the vitrified-
12 warmed blastocysts was determined using an indirect immunofluorescence protocol
13 [39]. Blastocysts were fixed with paraformaldehyde as described before for total cell
14 evaluation. Fixed embryos were permeabilized (1.5% Triton X-100 and 0.15% Tween
15 20 in PBS) at 4 °C overnight. Permeabilized blastocysts were incubated at room
16 temperature first in a 2 N HCL solution for 20 min and then in 100 mM Tris-HCl (pH
17 8.5) for 10 min. After denaturation and washing (3 times for 2 min in PBS-BSA),
18 blastocysts were incubated for 6 h in blocking solution (PBS containing 1% BSA, 10%
19 Normal Donkey Serum and 0.005% Tween 20) at 4 °C. After washing (3 times for 2
20 min in PBS-BSA), blastocysts were incubated with the primary CDX-2 antibody (1:200
21 in the commercial dilution solution; BioGenex, San Ramon, CA, USA) for 1.5 days.
22 Then, blastocysts were washed (3 times for 2 min in PBS-BSA) and incubated with
23 donkey anti-mouse IgG-Alexa Fluor® 568 conjugate (1:1000 in blocking solution;
24 Invitrogen, Rockford, USA). Finally, blastocysts were washed twice for 15 min in PBS-
25 BSA, placed on a slide in 4 µL of Vectashield (Vector Labs, Burlingame, CA, USA)

containing 10 µg/mL Hoechst 33342, and covered with a coverslip. Stained blastocysts were examined with a fluorescence microscope using an excitation wavelength of 330-380-nm to count the total number of nuclei stained with Hoechst and a 536 nm excitation filter to count trophectoderm cells that were stained with Alexa Fluor 568 showing red fluorescence.

2.8. Measurement of intracellular GSH and ROS levels

Intracellular GSH and ROS levels of embryos were determined by staining with CellTracker Blue (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin; CMF2HC; Invitrogen, ThermoFisher scientific, Massachusetts, USA) and H2DCFDA (2', 7'-dichlorodihydrofluorescein diacetate; Invitrogen), respectively. Blastocysts were washed in TL-PVA and incubated in the dark for 30 min in TL-PVA medium containing 10 µM CellTracker Blue and 10 µM H2DCFDA at 38.5 °C. Stained embryos were washed three times in TL-PVA. Then, groups of 5 embryos were transferred into 10-µL droplets of TL-PVA medium, and the fluorescence was immediately observed with fluorescence microscopy with UV filters (370 nm for GSH and 460 nm for ROS). Fluorescence emissions were recorded as TIFF files using a digital camera connected to the fluorescent microscope. The fluorescence intensities of each blastocyst were analyzed by ImageJ software (Version 1.51h; National Institutes of Health, Bethesda, MD, USA).

2.9. Experimental design

2.9.1. Experiment 1

1 In the first experiment, the effects of AsA supplementation in IVM, IVF and IVC media
2 on maturation, fertilization and embryo development were evaluated. For the
3 evaluation, oocyte maturation, fertilization, and embryo culture were performed in the
4 presence or absence of 50 µg/mL AsA in all possible combinations, which involved a
5 total of 8 experimental groups. A total of 2,744 oocytes were used in six replicates. A
6 random subset of oocytes (N=149) and presumed zygotes (N=1,142) from each group
7 was fixed and stained at 44 h of IVM and at 18 h after IVF to evaluate the maturation
8 and fertilization parameters, respectively. The remaining presumptive zygotes
9 (N=1,602) were cultured to assess in vitro embryo development. Day 7 blastocysts were
10 fixed and stained to assess their total cell number.

11 12 2.9.2. Experiment 2

13 In the second experiment, the effect of adding AsA to vitrification-warming media on
14 the post-warming survival and quality of in vitro-produced porcine blastocysts was
15 evaluated. The IVP of blastocysts was performed without AsA. This experiment was
16 performed in a total of six replicates. Day-6 in IVP full-expanded blastocysts (N=588),
17 morphologically classified as excellent or good, were randomly divided into one of two
18 groups in which vitrification and warming media were supplemented with 50 µg/mL
19 AsA (VW+ group) or not supplemented (VW- control). This AsA concentration was
20 selected based on previous experiments (Kere et al., 2013). After warming, VW+
21 (N=281) and VW- control (N=307) blastocysts were cultured in vitro for 24 h to assess
22 the embryo survival and hatching rates. A random subset (N=21) of vitrified-warmed
23 blastocysts classified as viable from each group were subjected to differential staining to
24 assess the total number of cells, the number of cells in the inner cell mass (ICM) and the
25 number of cells in the trophectoderm (TE). Finally, the intracellular GSH and ROS

levels from VW+, VW- control and some fresh in vitro-produced blastocysts (Fresh control) were measured.

2.10. Statistical analyses

Statistical analysis was performed using the IBM SPSS 19 statistical software package (SPSS, Chicago, IL, USA). Continuous variables are expressed as the mean $\pm$ SD. Binary variables (maturation, penetration, monospermy, cleavage, blastocyst formation, efficiency, survival and hatching rates) were obtained by calculating the percentage in every well of each experimental group and in each of the replicates. These data are expressed as the mean $\pm$ standard deviation (SD). The Kolmogorov-Smirnov test was used to test for normally distributed data. Means of more than two groups were compared using a mixed-model analysis of variance (ANOVA), followed by the Bonferroni post hoc test. Pairwise comparisons of means were performed using Student's t-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Experiment 1.

Effects of AsA supplementation on IVM, fertilization and embryo development.

The addition of AsA had no effect on the percentage of MII oocytes at 44 h of maturation between treatment (81.4%) and control (83.5%). Supplementation of IVM, IVF and IVC media also had no effect on any fertilization parameter (Table 1). Rate of sperm penetration was close to 70% with monospermy approximately 60% in all groups. The overall IVF efficiency of the IVP system ranged from $37.6 \pm 8.5\%$ to $47.3 \pm 13.9\%$ with no differences between treatment and control groups.

The embryonic development parameters are presented in Table 2. Addition of AsA did not affect the development to the 2-4 cell stage or blastocyst formation at the end of the culture period. The total efficiency of blastocyst production was always approximately 30%. The quality of the in vitro-produced blastocysts in terms of total cell number (range from 44.1 ± 20.4 to 53.0 ± 26.2 cells) did not vary with AsA supplementation.

3.2. Experiment 2

Effects of AsA supplementation to vitrification and warming media on blastocyst survival, hatching rate and embryo quality.

The addition of AsA during vitrification and warming increased ($P < 0.05$) blastocyst survival rate compared with that of VW- control embryos (Table 3). However, the blastocysts survival and hatched rates 24 h after warming were not affected by AsA addition. Total cell numbers and the distribution of cells between the TP and the ICM (Table 4) were also comparable between the two vitrification groups.

The vitrification and warming procedures increased ($P < 0.05$) intracellular ROS and decreased ($P < 0.05$) GSH levels, compared with those of the controls (Fig. 1). Addition of AsA to the vitrification-warming media decreased ($P < 0.05$) ROS production but did not affect GSH. Those embryos vitrified and warmed without AsA (VW- control) displayed the highest ($P < 0.05$) intracellular ROS values, whereas those treated with AsA had intermediate ROS levels.

4. Discussion

In the present study, the effects of AsA treatment were assessed during porcine IVM, IVF and/or IVC on variables of maturation, fertilization and embryonic development. Despite previous reports of some positive effects of exogenous antioxidants within IVP,

1 the present study showed that the supplementation of media with AsA did not improve
2 the overall efficiency of our IVP system. However, AsA had a clear beneficial effect
3 during vitrification and warming, increasing the vitrification survival of in vitro-
4 produced blastocysts.

5 In the current study, AsA supplementation to oocyte IVM medium did not significantly
6 affect the maturation or fertilization rates or the subsequent embryo development after
7 IVF. These results are consistent with previous investigations in ovine [26], bovine [39]
8 and porcine [11], which did not demonstrate any effect of AsA supplementation during
9 IVM on the developmental competence of porcine oocytes and embryos. Some studies
10 report positive effects of AsA during maturation using parthenogenetically activated
11 [27] or denuded oocytes [12]. Kere et al. [27] observed an increase of the cleavage rate,
12 blastocyst formation and blastocyst total cell number after parthenogenetic activation of
13 porcine oocytes matured with AsA. The effect of AsA on parthenogenetically activated
14 oocytes could be due to the higher sensitivity of these oocytes to oxidative
15 environments than those oocytes subjected to IVF [40]. Consistent with this hypothesis,
16 Tao et al. [12] demonstrated improved nuclear maturation of porcine denuded oocytes
17 when AsA was added to the IVM medium. In this case, AsA during IVM was also
18 beneficial under an increased oxidative stress condition due to the absence of cumulus
19 cells surrounding the oocytes during IVM [41]. Together with our present results, these
20 previous observations suggest that AsA has a beneficial effect during maturation when
21 the generation of ROS is extremely high or protective mechanisms against oxidative
22 stress are lacking.

23 We believe this is the first study to evaluate the effects of the addition of AsA during
24 IVF of porcine oocytes. Previous studies on the effects of antioxidants during the IVF
25 period have reported contradictory results. With regard to IVF, it is important to note

1 that physiological levels of ROS are required to induce hyperactivation and capacitation
2 of spermatozoa [42,20], both pre-requisites for fertilization. Although some antioxidants
3 added to sperm before or during IVF have improved subsequent developmental capacity
4 of bovine and ovine embryos [43], excess antioxidants impair fertilization, normal
5 pronuclear formation and embryo development in bovine [44]. In our study, the
6 supplementation of IVF media with AsA did not have any effect on the fertilization
7 parameters or on the developmental competence of fertilized oocytes.

8 With respect to the embryo culture, the results followed the same pattern as those for
9 IVM and IVF. Thus, the addition of AsA to the IVC medium did not alter the IVC
10 parameters and did not affect the number of cells in the blastocysts. These observations
11 are similar to those reported by Castillo-Martín et al. [30]; however, they are in contrast
12 to Hossein et al. [28] who obtained increased blastocyst formation when the ICV
13 medium was enriched with AsA. To understand these discrepancies, we should consider
14 the overall efficiency of each IPV system. Thus, our total blastocyst production
15 efficiency (30-35%) and that reported by Castillo-Martín et al. [30] (20%) are
16 considered adequate for a porcine IVP system, and in these circumstances, AsA did not
17 exert any effect. By contrast, under less efficient conditions in which the blastocyst
18 formation rate was close to 9% [28], the addition of AsA was beneficial. A positive
19 effect of AsA was also observed with embryos derived from parthenogenesis [27,29],
20 which may be more sensitive to oxidative stress than IVF-derived embryos because of
21 their biological differences [45]. It is possible that AsA only exerts positive effects
22 under highly oxidant conditions in which the detrimental effects of ROS are
23 exacerbated. Following this logic, the present study showed a positive effect of AsA
24 during vitrification and warming using chemically defined media, and these results are
25 consistent with those obtained by Castillo-Martín et al. [30] using a vitrification and

warming system based on supplementation with fetal serum and AsA. During vitrification and warming, embryos are subjected to important disturbances in the redox status due to an increase of ROS and/or a decrease in the GSH levels [30,46,47]. In this study, vitrification and warming increased, as expected, the oxidative stress altering intracellular GSH and ROS levels compared with those of fresh blastocysts. However, addition of AsA mitigated this high oxidative stress by reducing intracellular ROS production of blastocysts and increasing the embryo survival rate after warming. Supplementation of AsA to cryopreservation media of mouse and bovine embryos had also beneficial effects by reducing peroxidation and increasing cryotolerance [48,49]. These results indicate that the addition of AsA or other antioxidants during vitrification and warming could be an efficient strategy to reduce the oxidative stress related to this technology and therefore to improve embryo survival.

In conclusion, under our experimental conditions and within a highly efficient porcine in vitro embryo production system, the supplementation of IVM/IVF/IVC media with AsA at a concentration of 50 µg/mL failed to further increase the IVP-outcomes. By contrast, the addition of AsA to chemically defined vitrification and warming media increased the vitrification survival by decreasing the ROS production. Thus, AsA supplementation is recommended for vitrification and particularly for unstable or low performing IVP systems.

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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. Intracellular reactive oxygen species (ROS) and glutathione (GSH) levels of Day 6 porcine blastocysts vitrified-warmed in media without ascorbic acid (VW- control; white bars) or in media supplemented with 50 µg/mL ascorbic acid (VW+ group; grey bars). Non-vitrified Day 6 in vitro-produced blastocysts were also assessed (fresh control; black bars). Different letters indicate significant differences among groups ($P<0.01$). Data are expressed as the mean $\pm$ SD (six replicates).

Table 1

Fertilization parameters of porcine oocytes cultured in media with (+50 µg/mL) or without (-) ascorbic acid (AsA).

AsA supplementation			Oocytes (N)	Oocytes (%)		Efficiency (%)
IVM	IVF	IVC		Penetrated	Monospermic	
+	+	+	149	69.1 ± 14.5	71.2 ± 10.1	47.3 ± 13.9
+	+	-	123	63.5 ± 13.9	73.7 ± 14.1	43.2 ± 5.2
+	-	+	143	72.4 ± 13.5	60.9 ± 18.1	39.7 ± 13.3
+	-	-	130	73.0 ± 11.2	59.1 ± 17.0	40.0 ± 8.9
-	+	+	161	63.3 ± 10.9	68.0 ± 12.3	37.6 ± 8.5
-	+	-	148	78.3 ± 14.7	63.1 ± 10.7	45.1 ± 8.1
-	-	+	141	72.0 ± 5.4	65.4 ± 15.8	42.0 ± 10.2
-	-	-	147	72.4 ± 15.3	64.7 ± 12.2	46.3 ± 5.7

Penetrated: Number of oocytes penetrated/total inseminated oocytes.

Monospermic: Number of oocytes with only one male pronucleus/total oocytes penetrated.

Efficiency: Number of monospermic oocytes/total oocytes inseminated.

Data are expressed as the mean ± SD (six replicates).

Table 2

Embryonic development achieved after oocyte maturation, fertilization and embryo culture with (+, 50 µg/mL) or without (-) ascorbic acid (AsA).

AsA supplementation			Oocytes (N)	Embryonic development		
IVM	IVF	IVC		Cleavage (%)	Blastocyst (%)	Total efficiency (%)
+	+	+	194	47.4 ± 5.0	73.8 ± 19.1	35.4 ± 9.8
+	+	-	200	45.4 ± 7.5	65.7 ± 22.0	29.5 ± 10.4
+	-	+	196	52.3 ± 10.9	67.1 ± 16.3	36.2 ± 14.7
+	-	-	188	49.1 ± 12.9	75.7 ± 17.6	37.5 ± 13.6
-	+	+	202	47.9 ± 14.5	66.0 ± 16.1	32.1 ± 13.5
-	+	-	200	45.9 ± 9.9	61.4 ± 12.7	27.7 ± 10.3
-	-	+	199	51.6 ± 8.9	71.5 ± 18.3	37.2 ± 12.2
-	-	-	223	47.4 ± 5.0	73.8 ± 19.1	35.4 ± 9.8

Cleavage: Number of 2,4-cell embryos/total inseminated oocytes cultured.

Blastocyst: Number of blastocysts/total cleaved embryos.

Total Efficiency: Number of blastocysts/total inseminated oocytes cultured.

Data are expressed as the mean ± SD (six replicates).

Table 3

Survival and hatching rates and quality (number and distribution of cells) after 24 h of culturing of Day 6 in vitro-produced porcine blastocysts vitrified-warmed in media supplemented with 50 µg/mL ascorbic acid (AsA; VW+) or without supplementation (VW- control).

Group	N	Survival rate (%)	Hatching rate (%)	Cells in blastocysts		
				Total cell number	TP ¹	ICM ²
VW+	307	51.1 ± 20.9 ^a	10.7 ± 12.0	58.7 ± 21.1	44.2 ± 18.2	14.5 ± 6.5
VW-control	281	34.8 ± 21.4 ^b	6.0 ± 8.1	62.6 ± 14.4	46.3 ± 12.1	16.7 ± 7.5

^{a,b} Different superscripts in the same column indicate a significant difference (P<0.05).

¹TP: trophectoderm.

²ICM: inner cell mass.

Data are expressed as the mean ± SD (six replicates).