Theriogenology 184 (2022) 110–123

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Impact of equilibration duration combined with temperature on the outcome of bovine oocyte vitrification

Tania García-Martínez [a, 1], Iris Martínez-Rodero [a, 1], Joan Roncero-Carol [a], Iván Yánez-Ortiz [a], Adam Z. Higgins [b], Teresa Mogas [a, *]

[a] Dept. of Animal Medicine and Surgery, Autonomous University of Barcelona, ES-08193, Cerdanyola del Vallès, Spain
[b] School of Chemical, Biological and Environmental Engineering, Oregon State University, Corvallis, OR, 97331-2702, USA

ARTICLE INFO

Article history:
Received 1 December 2021
Received in revised form
25 February 2022
Accepted 26 February 2022
Available online 10 March 2022

Keywords:
Membrane permeability
Osmotic behavior
Meiotic spindle
DNA fragmentation
Embryo development

ABSTRACT

The cryopreservation of mammalian oocytes and embryos has become an integral part of assisted reproduction in both humans and veterinary species. However, the methods used to cryopreserve bovine oocytes still have significant shortcomings. A wide variety of approaches has been used to try to improve and optimize methods of cryopreservation. However, these procedures employed are not always designed to specifically take account of the osmotic tolerance response of the cells according to the temperature and time of cryoprotectant (CPA) addition. When these properties are considered, optimal procedures for the addition of CPAs can be designed proactively. Based on *in silico* and *in vitro* osmotic observations, we propose shorter dehydration-based protocols at different temperatures (25°*C vs.* 38.5°*C*) towards defining an improved cryopreservation method. *In vitro* matured oocytes were exposed to equilibration solution (ES) at 25°*C* and 38.5°*C* and effects of optimized exposure times for each temperature were determined prior to vitrification/warming on oocyte spindle configuration, DNA fragmentation, and further embryo development. Upon exposure to standard ES (7.5% dimethyl sulfoxide + 7.5% ethylene glycol in TCM199 medium + 20% fetal bovine serum), original oocyte volume was recovered within 2 min 30 s at 38.5°*C* and 5 min 30 s at 25°*C*. *In vitro* matured oocytes were then exposed to the aforementioned cryoprotectants at both temperature/duration conditions and vitrified/warmed. While similar percentages of oocytes exhibiting a normally configured spindle and DNA fragmentation were observed in the fresh control group and oocytes vitrified at 38.5°*C*, significantly higher apoptosis rate and lower percentages of normal spindle configuration were observed in oocytes vitrified at 25°C when compared to control fresh oocytes. Similar cleavage rates and blastocyst yields were observed in the vitrified/38.5°*C* and fresh controls, while these rates were lower in vitrified/25°*C*. These results revealed that the limitation of the exposure time of the oocytes to the ES to the point of osmotic equilibrium volume recovery could be a more efficient approach to prepare them for vitrification. Therefore, exposure time to ES to 2 min 30 s at 38.5 °C appears to improve the quality of vitrified/warmed oocytes by protecting spindle integrity and reducing DNA fragmentation thus improving blastocyst rates and embryo quality.

© 2022 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Oocyte cryopreservation is today the most promising and cost-effective option for the storage of female germplasm. In domestic animals, cryopreservation of oocytes to create banks is a way of preserving female genetically valuable material or avoiding the loss of rare genotypes in the event of an unexpected catastrophe. The value and feasibility of genome resource banking become even more important for the preservation of ancient breeds, as over the last decades, farm animal genetic diversity has rapidly declined due

* Corresponding author.
E-mail addresses: taniagarciamartinez@gmail.com (T. García-Martínez), iris.martinez@outlook.com (I. Martínez-Rodero), joanroncerocarol@gmail.com (J. Roncero-Carol), ivan.yanez22@gmail.com (I. Yánez-Ortiz), Adam.Higgins@oregonstate.edu (A.Z. Higgins), teresa.mogas@uab.cat (T. Mogas).
[1] Both authors contributed equally.

https://doi.org/10.1016/j.theriogenology.2022.02.024
0093-691X/© 2022 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

to changing market demands and intensification of agriculture [1]. Cryopreserved oocyte banks could also help improve research endeavors by providing accessible and repeatable supplies and reducing the effects of seasonal variation in oocyte quality.

Despite some successes, there still remain shortcomings with methods used to cryopreserve bovine oocytes (reviewed by Refs. [2,3]). Many of the problems associated with the cryopreservation of mature oocytes are related to exposure to cryoprotective agents (CPAs). The ability of CPAs to avoid ice formation increases with concentration, however, so does their toxicity. CPAs can have a damaging effect on cells due to their intrinsic chemical toxicity, and they can also produce osmotic damage, which could be defined as the creation of an osmolarity that is not tolerated by the cells [4]. Both types of damage are dependent on the duration and the temperature and of the exposure, so these parameters have to be carefully controlled in vitrification protocols. It is well known that both osmotic stress and temperature fluctuations below their physiological range directly affect the developmental potential of oocytes, causing disruption in cytoskeleton structures, especially depolymerization of the meiotic spindle [5,6].

Most of the currently employed vitrification protocols for bovine oocytes are time-specific and require a given treatment duration with an equilibration solution (ES), whose composition is similar to the vitrification solution (VS) but less concentrated, and a given exposure time to VS before plunging the oocytes into liquid nitrogen; some require observation of oocyte volume response in the ES; some demand exposure to ES and VS be conducted at room temperature (RT), biological temperatures or higher than biological temperatures (reviewed by Ref. [7]). These procedures, however, rarely consider the osmotic tolerance of the bovine oocytes according to temperature and time of CPA addition and removal. When this characteristic is considered, the time needed to prepare oocytes for vitrification may be shortened while maintaining the critical cytosolic solute content necessary for successful vitrification.

Previous papers have already described in detail bovine oocyte membrane permeability parameters and their application for mathematical modeling for finding optimal cryopreservation protocols [5,8–12]. These studies reported water plasma membrane and CPA permeability characteristics of bovine oocytes in the presence of the most used CPAs such as dimethyl sulfoxide (Me_2SO), ethylene glycol (EG), propylene glycol, as well as glycerol solutions; performance at multiple temperatures (4–38°*C*) with activation energies derived; while comparing different developmental stages (*in vitro* matured, fertilized oocytes); and many osmotic volume response simulations/proposed cryopreservation protocols. From these studies, it was obvious that EG or Me_2SO were more adequate as CPAs for bovine oocytes, compared with glycerol [12]. For sugars, the use of trehalose, as a non-penetrating cryoprotectant, produced worse results compared with sucrose [13]. And the combination of Me_2SO and EG at lower concentrations with the addition of a non-penetrating CPA such as sucrose is commonly added to the standard vitrification solutions of bovine oocytes to reduce the toxic effect of a high total concentration of one CPA alone [14].

In the context of optimization of cryopreservation procedures for vitrification in the presence of combined CPAs, one goal is to determine the best exposure time to the equilibration solution at a specific temperature [15]. So, the present study aimed to optimize oocyte vitrification protocols via incorporating insights on osmotic oocyte volume responses during incubation in cryopreservation solutions. For that purpose, we first determine both predictively and experimentally the osmotic behavior of metaphase II (MII) bovine oocytes in terms of membrane permeability parameters (L_p and P_s) in response to ES CPAs at 25°*C* and 38.5°*C*, and then we assess the optimized CPA addition procedure by examining induced toxicity/osmotic stress and the whole vitrification process by analyzing oocyte spindle morphology, DNA fragmentation, and subsequent embryo development.

2. Materials and methods

2.1. Chemicals and suppliers

All chemicals and reagents used in this study were purchased from Sigma Chemical Co (St. Louis, MO, USA) except where otherwise indicated.

2.2. Oocyte collection and in vitro maturation

The methods used for the *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) of the bovine oocytes have been described elsewhere [16]. Briefly, ovaries were collected from cows at a local abattoir (Escorxador Sabadell, S.A., Sabadell, Spain) and immediately transported to the laboratory in pre-warmed (35–37°*C*) saline solution (0.9% NaCl). Cumulus-oocyte complexes (COCs) were obtained by aspirating 3–8 mm diameter follicles using an 18 g needle and washed three times in modified Dulbecco's PBS (PBS supplemented with 0.036 mg/mL sodium pyruvate, 0.05 mg/mL gentamicin, and 0.5 mg/mL bovine serum albumin, BSA). Only COCs with at least three compact layers of cumulus cells and a homogeneous cytoplasm were used. After three washes in PBS, groups of 50 COCs were transferred to 500 μL of maturation medium in four-well dishes and cultured for 24 h at 38.5°*C* in a 5% CO_2 humidified air atmosphere. The maturation medium was composed of tissue culture medium (TCM-199) supplemented with 10% (v/v) fetal bovine serum (FBS), 10 ng/mL epidermal growth factor, and 50 μg/mL gentamicin.

2.3. Modeling the membrane permeability of bovine MII oocytes

2.3.1. Measurement of oocyte volumetric changes following CPA exposure at 25 °C and 38.5 °C

After 22 h of IVM, oocytes were denuded of cumulus cells by gentle pipetting. Only mature oocytes showing a normal appearance and a visible first polar body were used. An oocyte was placed in a 25 μL-drop of holding medium (HM: TCM199-Hepes supplemented with 20% (v/v) FBS) covered with mineral oil, and held with a holding pipette (outer diameter, 100 μm) (MPHL-35, Life Global group, Guilford, United States) connected to a micromanipulator on an inverted microscope (Zeiss Axio Vert A1, Germany). An initial photograph was taken of the oocyte to calculate its initial volume. The oocyte was then covered with another pipette of larger inner diameter (600 μm) (G-1 Narishigue, Tokyo, Japan) connected to a different micromanipulator. Then, by sliding the dish, the oocyte was introduced in a 25 μL drop containing 1.55 M Me_2SO or 1.55 M EG Ref. [14] at 25°*C* or 38.5°*C* and left for 5 min. The volumetric response of the oocyte during the experiments was recorded every 5 s using a time-lapse video recorder (Zeiss Zen imaging software/Axiocam ERc 5s). The video was converted into image frames and a series of images taken at 5 s-intervals were used for analysis. Oocyte volume was measured through its cross-sectional area using ImageJ software. An average of 10–14 mature oocytes that remained close to spherical in shape were individually analyzed for each CPA and temperature. Osmotic responses of the oocyte were calculated by measuring oocyte volume changes when exposed to 1.55 M Me_2SO or 1.55 M EG at the different temperatures.

2.3.2. Membrane permeability parameters

The experimental data were fitted to a two-parameter (2P) transport formalism [17] to determine the permeability of bovine MII oocytes to water (L_p) and solutes (P_s). The 2P model assesses mass transfer dynamics through the cell over time by assuming there is no intramembrane interaction between water and permeable solutes. This model uses a pair of coupled differential equations to describe cell volume changes and moles of an intracellular permeating solute when the cell is exposed to a ternary solution consisting of a permeable solute (CPA), an impermeable solute (NaCl), and solvent (water).

Water flux into the cell over time is expressed as:

$$\frac{dV_w}{dt} = -L_pART(M^e - M^i) \quad (1)$$

where V_w is the cell water volume, L_p is the membrane permeability to water (hydraulic conductivity), A is the area of the plasma membrane, R is the universal gas constant, T is the absolute temperature, and M^e and M^i are the total external and internal osmolalities, respectively.

The rate of CPA transport is given by:

$$\frac{dN_s}{dt} = P_sA(M_s^e - M_s^i) \quad (2)$$

where N_s is the intracellular moles of CPA, P_s is CPA permeability, and M^i_s and M^e_s are intracellular and extracellular CPA molality, respectively.

Volumetric data for each oocyte at each concentration and temperature were fitted to the 2P model to determine L_p and P_s. The differential equations (equation (1) and (2)) were solved in Matlab software using the ode45 function, which implements an explicit Runge-Kutta formula [18,19]. To estimate permeabilities, model predictions were fitted to the data by minimizing the sum of the error squared in Matlab using the fminsearch function, which implements the Nelder-Mead simplex algorithm [20]. The various constants and parameters appearing in the equations are listed in Table 1.

2.3.3. Prediction of cell volume changes during exposure of bovine oocytes to the equilibration solution at 25 °C and 38.5 °C

To predict the cell volume response and intracellular CPA concentration when oocytes are exposed to ES (the first step of CPA addition), it is essential to define two solute equations (equations (3) and (4)), one for Me_2SO and another for EG. Accordingly, a system of three linear ordinary differential equations needs to be solved for the three variables (V_w, $N_{Me2SO,}$ and N_{EG}). These three differential equations (equation (1), (3) and (4)) were solved as described above in Matlab software using the ode45 function, which implements an explicit Runge-Kutta formula [18,19]. Temperature differs between 25°C (298 K) or 38.5°C (311K) and is indicated in the first equation of the three differential equations solved in Matlab software.

The rate of Me_2SO transport is given by:

$$\frac{dN_{Me2SO}}{dt} = P_{Me2SO}A(M^e_{Me2SO} - M^i_{Me2SO}) \quad (3)$$

And the rate of EG transport is given by:

$$\frac{dN_{EG}}{dt} = P_{EG}A(M^e_{EG} - M^i_{EG}) \quad (4)$$

In this study, the water and solute permeability used for the CPA addition predictions at 25°C or 38.5°C were previously estimated (see section 2.3.2). Water permeability was assumed to be the average value for the individual L_p values obtained when the oocytes were exposed to 1.55 M Me_2SO or 1.55 M EG at 25°C or 38.5°C (2.41 and 1.64 μm/atm × min, respectively). The values for P_{Me2SO} and P_{EG} were 0.59 and 0.78 μm/s, respectively at 25°C, and 1.38 and 1.94 μm/s, respectively at 38.5°C. The oocyte was assumed to be a perfect sphere with a radius of 56.1 μm in isotonic medium, and an osmotically inactive volume of 25% of its initial volume [8,12].

Predictions for the CPA addition process (equilibration solution) were run at 25°C or 38.5°C for bovine MII oocytes, for which the exposure time was limited to 9 min. Key parameters for oocyte survival of vitrification, such as the volumetric excursion of the oocyte and total cytoplasmic solute concentration were determined and compared between the two temperatures.

2.4. In vitro osmotic behavior following ES exposure at 25°C and 38.5°C

To determine if the model predictions were accurate, our *in silico* dehydration profiles obtained with the theoretical models were tested *in vitro* by observing the osmotic behavior of bovine MII oocytes exposed to the equilibration solution at 25°C or 38.5°C. The methodology used was the same as described in section 2.3.1 with the difference that oocytes were exposed for 9 min to the equilibration solution (ES; 7.5% (v/v) EG and 7.5% (v/v) Me_2SO in TCM-199 Hepes) at 25°C or 38.5°C. An average of 20 mature oocytes that remained close to spherical in shape were individually analyzed for each temperature.

2.5. Oocyte vitrification and warming

After 22 h of IVM, oocytes were vitrified/warmed as previously described [21]. Oocytes were partially denuded by gently pipetting in PBS. Based on the results obtained in the previous experiments, oocytes with only corona radiata cells were transferred to ES containing 7.5% (v/v) Me_2SO and 7.5% (v/v) EG for different periods of time depending on the temperature (see experimental design). Oocytes were then transferred to the vitrification solution (VS: 15% (v/v) Me_2SO, 15% (v/v) EG, and 0.5 M sucrose). After incubating for 30–40 s, up to five oocytes were loaded onto a Cryotop and almost all the solution was removed to leave only a thin layer covering the oocytes. Oocytes were immediately plunged into liquid nitrogen. The entire process from exposure to the vitrification solution to plunging in liquid nitrogen was completed within 60 s.

Warming was performed by quickly immersing the tip of the Cryotop in warming solution 1 (WS1: HM supplemented with 1 M sucrose) at 38.5°C. After 1 min, oocytes were transferred into WS2 (HM supplemented with 0.5 M sucrose) for 3 min and then to HM for 5 min. Oocytes were then transferred back into the maturation medium and allowed to mature for 2 additional hours at 38.5°C in humidified air containing 5% CO_2.

Table 1
Constant and parameters used in 2P model.

Description	Values	Symbol
Universal gas constant	8.314 m^3 Pa K^{-1} mol^{-1}	R
Absolute temperature	298 K or 311 K	T
Partial molar volume of water	18.02 x10^{12}μ$m^3$$mol^{-1}$	υ_w
Partial molar volume of CPA:		
EG[a]	55.8 × 10^{-6} m^3 mol^{-1}	υ_s
Me_2SO[a]	71.3 × 10^{-6} m^3 mol^{-1}	
Osmotically inactive volume MII oocytes):	0.25 [8,12]	V_b
Isotonic cell volume	7.40 × 10^5 μm^3 [68]	V_0

Abbreviations: CPA, cryoprotectant; EG, ethylene glycol; Me_2SO, dimethyl sulfoxide; MII, metaphase II.

[a] Partial molar volumes of cryoprotectants from Vian et al. [69].

2.6. In vitro fertilization and embryo culture

After 24 h of IVM, oocytes were *in vitro* fertilized (IVF) at 38.5°*C* in a 5% CO_2 atmosphere and cultured as previously described by Arcarons et al. [22]. Briefly, high motility and good morphology spermatozoa from Asturian bulls (ASEAVA, Llanera, Asturias, Spain) were obtained by centrifuging frozen/thawed sperm at 300×*g* for 10 min at *RT* on a discontinuous gradient consisting of 1 mL of 40% and 1 mL of 80% BoviPure (Nidacon Laboratories AB, Göteborg, Sweden) according to the manufacturer's instructions. Viable spermatozoa collected from the bottom were washed with 3 mL of BoviWash (Nidacon International, Göteborg, Sweden) and pelleted by centrifugation at 300×*g* for 5 min at *RT*. Spermatozoa were counted in a Neubauer chamber and diluted in an appropriate volume of fertilization medium to give a final concentration of 1×10^6 spermatozoa/mL. One-hundred-microliter droplets of diluted sperm were prepared under mineral oil and 20 oocytes/droplet were co-incubated at 38.5°*C*, 5% CO_2, and high humidity. The fertilization medium consisted of Tyrode's medium supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/mL fatty acid-free BSA and 1 mg/mL heparin-sodium salt.

At 18–20 h post-insemination, oocyte survival was evaluated morphologically. The criteria used to classify oocytes as surviving or degenerated have been described elsewhere [23]. Briefly, oocytes with intact oolemma, intact zona pellucida, and homogenous and dark cytoplasm were considered as surviving oocytes. Only surviving oocytes were transferred to 25-μL drops of the culture medium (1 embryo/μL) covered by 3.5 mL of mineral oil. The culture medium was synthetic oviduct fluid (Caisson Labs, Smithfield, UT, USA) supplemented with 0.96 μg/mL BSA, 88.6 μg/mL sodium pyruvate, 2% (v/v) non-essential amino acids, 1% (v/v) essential amino acids, 0.5% gentamicin and 2% (v/v) FBS. Presumptive zygotes were incubated at 38.5°*C* in a humidified 5% CO_2 and 5% O_2 atmosphere for 8 days. Embryo development was recorded at 48 h post-insemination (cleavage) and Days 7 and 8 (blastocysts) post-insemination (pi). Day 8 blastocysts were fixed and immunostained to assess total cell number (TCN), inner cell mass (ICM) number, trophectoderm (TE) cell number, and apoptosis rate (AR).

2.7. Spindle configuration

After 24 h of IVM, oocytes were completely denuded of cumulus cells by gentle pipetting before immunostaining for tubulin and chromatin detection as described previously by García-Martínez et al. [21]. Briefly, oocytes were fixed in 2% (w/v) paraformaldehyde–phosphate buffer saline (PFA-PBS) for 30 min at 38.5°*C*. Oocytes were then permeabilized in Triton X-100 (2.5% (v/v) in PBS) for 20 min and blocked in 3% BSA (w/v) in PBS for 30 min at 38.5°*C*. The fixed oocytes were incubated with mouse *anti*-α-tubulin monoclonal antibody (TU-01, Invitrogen, CA, USA; 1:250 dilution) overnight at 4°*C*, followed by incubation with the anti-mouse IgG antibody Alexa Fluor™ 488 (Molecular Probes, Paisley, UK; 1:5000) at 38.5°*C* for 1 h. Oocytes were washed three times in pre-warmed PBS supplemented with 0.005% (v/v) of Triton X-100 at 38.5°*C* for 20 min after each incubation. Groups of 20 oocytes were mounted on poly L-lysine-treated coverslips fitted with a self-adhesive reinforcement ring in a 3-μL drop of Vectashield containing 125 ng/mL 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) (Vysis Inc., Downers Grove, USA) and flattened with a coverslip. Preparations were sealed with clear nail varnish and stored at 4°*C* protected from light until observation within the following 2 days. An epifluorescence microscope (Axioscop 40FL; Carl Zeiss, Göttingen, Germany) was used to examine tubulin (Alexa Fluor™ 488; excitation 488 nm; emission 525 nm) and chromatin (DAPI; excitation 405 nm; emission 460 nm). The criteria used to classify chromosome and microtubule distributions have been described elsewhere [24]. In brief, the meiotic spindle was defined as normal when the classic symmetrical barrel shape was observed, with chromosomes aligned regularly in a compact group along the equatorial plane. In contrast, abnormal spindles were recorded when there was microtubule decondensation or partial or total disorganization, or as absent when there was a complete lack of microtubules. Chromosome organization was considered abnormal when chromosomes were dispersed or had an aberrant, less condensed appearance or lacking when chromosomes were missing. Detailed images of these normal and abnormal patterns are shown in Fig. 1.

2.8. TUNEL detection of fragmented oocyte DNA

Oocyte DNA fragmentation was detected by terminal deoxynucleotidyl transferase (TdT) mediated dUTP-digoxigenin nick end-labeling (TUNEL) using a kit (*in situ* Cell Death Detection Kit, Fluorescein) according to the manufacturer's instructions (as described in Vendrell-Flotats et al. [25] with some modifications). Briefly, after 24 h of maturation, oocytes were completely denuded of cumulus cells by gentle pipetting and fixed in 2% (w/v) paraformaldehyde in PBS for 30 min at 38.5°*C*. After three washes in PBS with 0.3% polyvinylpyrrolidone (PVP), fixed oocytes were permeabilized with 0.5% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 1 h at *RT*. The oocytes were then washed again in PBS-PVP solution and incubated in the TUNEL reaction cocktail at 38.5°*C* for 1 h in the dark. Positive and negative control samples were included in each assay. Oocytes exposed to DNase I (50 mL of RQ1 RNase-free Dnase (50 U/mL)) for 1 h at *RT* served as positive controls, and oocytes incubated in the absence of the terminal TdT enzyme served as negative controls. After washing in PBS-PVP, controls and samples were mounted on poly L-lysine-treated coverslips fitted with a self-adhesive reinforcement ring in a 3-μL drop of Vectashield containing 125 ng/mL of DAPI (Vectorlabs, Burlingame, CA, USA) and flattened with a coverslip. Preparations were sealed with clear nail varnish and stored at 4°*C* in the dark until their observation within the following 2 days. An epifluorescence microscope (Axioscop 40FL; Carl Zeiss, Göttingen, Germany) was used to detect TUNEL-positive nuclei (fluorescein isothiocyanate-conjugated TUNEL label; excitation 488 nm; emission 517 nm) while nuclei were localized using the DAPI filter (DAPI; excitation 405 nm; emission 460 nm). Nuclei were scored as having either intact (TUNEL [−]; blue stain) or fragmented (TUNEL [+]; green stain) DNA. The percentage of TUNEL-positive oocytes was calculated as the ratio between TUNEL[+] oocytes and the total number of oocytes analyzed in each group.

2.9. Differential staining of blastocysts and DNA fragmentation

Day 8 bovine blastocysts were immunostained for differential cell counts and apoptosis analysis as previously described by Martinez-Rodero et al. [26] with some modifications. Unless otherwise stated, all steps were conducted at 38.5°*C*. After fixation in 2% (v/v) paraformaldehyde diluted in PBS for 15 min, embryos were washed three times in PBS. For permeabilization, embryos were incubated with 0.01% Triton X-100 in PBS supplemented with 5% normal donkey serum (PBS-NDS) for 1 h at *RT*. Then, the embryos were washed in PBS (× 3) and incubated at 4°*C* overnight with mouse anti-SOX2 primary antibody (1:100; MA1-014, Invitrogen, CA, USA) and rabbit *anti*-caspase 3 primary antibody (1:250, CST9664S, Cell Signaling Technologies, CA, USA) in a humidified chamber. Afterwards, once washed in 0.005% Triton X-100 in PBS-NDS for 20 min, the embryos were incubated with the secondary

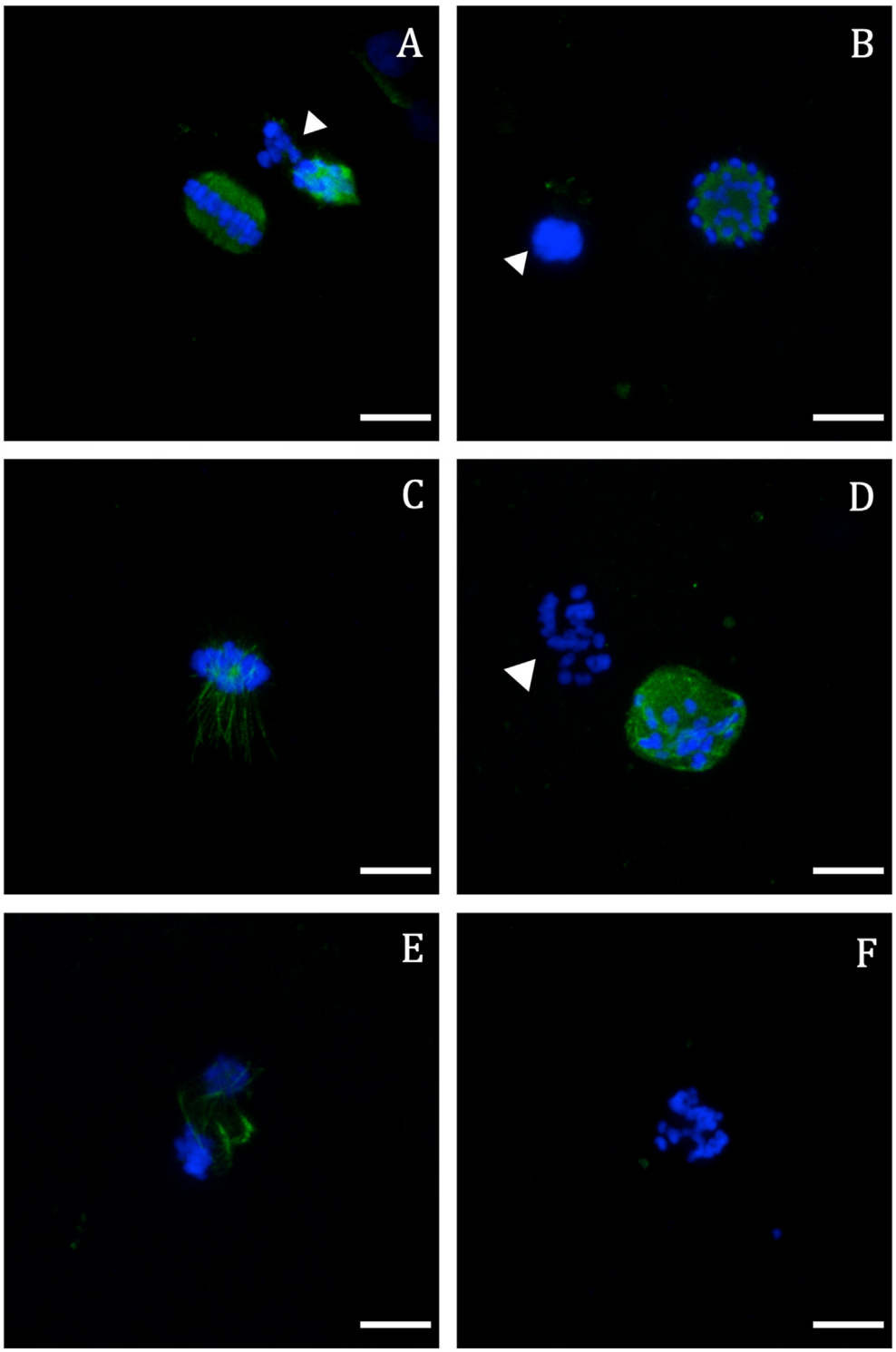

Fig. 1. Representative confocal laser-scanning photomicrographs of spindle microtubule and chromosome configurations in IVM bovine oocytes after CPA exposure or vitrification. (A) Normal barrel-shaped MII spindle with microtubules forming a clear meiotic spindle with compact chromosomes arranged at the equator of the structure. (B) Abnormal spindle morphology showing partly disorganized chromosomes. (C) Abnormal spindle structures with completely disorganized microtubules and partly disorganized chromosomes. (D) Abnormal spindle structure associated with completely disorganized microtubules and chromosomes. (E) Abnormal spindle structure associated with a disrupted microtubule arrangement and chromosomes appearing condensed. (**F**) Chromosomes with an aberrant, less condensed appearance. Note the absence of microtubules. Scale bar, 10 μm. Green, tubulin (Alexa Fluor™ 488); blue, chromosomes (DAPI). The white arrowheads indicate polar bodies. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

antibody goat anti-mouse IgG Alexa Fluor™ 568 (1:500; A-11004, ThermoFisher, Waltham, MA, USA) and a goat anti-rabbit IgG Alexa Fluor™ 488 antibody (1:500, A-11001, ThermoFisher, Waltham, MA, USA) for 1 h in a humidified chamber. Embryos were then washed thoroughly in 0.005% Triton X-100 in PBS-NDS for 5 min, mounted on poly-L-lysine treated coverslips fitted with a self-

adhesive reinforcement ring in a 3-μL drop of Vectashield containing 125 ng/ml DAPI (Vectorlabs, Burlingame, CA, USA), and flattened with a slide. The preparation was sealed with clear nail varnish and stored at 4°*C* protected from light until observation within the following 2 days. Confocal images of 0.5 μm-serial sections were captured with a confocal laser-scanning microscope (Leica TCS SP5, Leica Microsystems CMS GmbH, Mannheim, Germany) to examine the ICM nucleus (SOX2-Alexa Fluor™ 568; excitation 561 nm; emission 603 nm), cell nucleus (DAPI; excitation 405 nm; emission 460 nm) and DNA fragmentation (caspase 3-Alexa Fluor™ 488; excitation 488 nm; emission 525 nm). TCN, ICM cell number, and apoptotic cells were analyzed using Imaris 9.2 software (Oxford Instruments, UK). Individual nuclei were counted and assessed as intact (caspase-3(−); blue/red stain) or fragmented (caspase-3(+), green stain) DNA, TE cells (SOX2(−OX2(, green stain) DNA, TE cells (SOXs, UK) (Fig. 2). The AR was calculated as the ratio of caspase-3(+) cells/total number of cells.

2.10. Experimental design

The experimental design followed in this study is described in Fig. 3. After predicting the osmotic response of bovine Metaphase-II oocytes with a two-parameter permeability model, and reproducing the *in silico* predictions by in vivo recording the osmometric behavior of bovine oocytes, we limited the duration of the exposure to ES to 5 min 30 at 25 °C s or to 2 min 30 s at 38.5 °C. In a first trial of experiments, the effect of exposure of IVM bovine oocytes to ES to 5 min 30 at 25 °C s or to 2 min 30 s at 38.5 °C followed by vitrification/warming was assessed on spindle and chromosome configurations and DNA fragmentation. To this aim, bovine oocytes *in vitro* matured for 22 h were randomly assigned to two groups: 1) oocytes exposed to ES for 5 min 30 s followed by exposure to VS, WS2, and HM media at 25°*C* as previously described; and 2) oocytes exposed to ES for 2 min and 30 s followed by exposure to VS, WS2, and HM media at 38.5°*C* as previously described. In both experimental groups, exposure to WS1 was performed at 38.5°*C*. A sample of the oocytes in each group was vitrified/warmed using the Cryotop method and allowed to recover for 2 additional h (VIT25 and VIT35.8, respectively) while the other half were only exposed to the vitrification and warming solutions without plunging them in liquid nitrogen to assess CPA toxicity (CPA25 and CPA38.5, respectively). Fresh, non-vitrified *in vitro* matured oocytes served as the Control group. A sample of oocytes from each of the five treatment groups (Control, CPA25, CPA38.5, VIT25, and VIT38.5) was collected at 24 h of IVM to assess spindle and chromosome configurations (6 replicates) and DNA fragmentation (4 replicates).

In a second trial of experiments, we tested the effect of exposure of bovine oocytes *in vitro* matured for 22h to ES to 5 min 30 at 25 °C s or to 2 min 30 s at 38.5 °C prior to vitrification/warming on further embryo development. For that purpose, oocytes in the groups Control, VIT25 and VIT38.5 groups were inseminated and *in vitro* cultured for 8 days. Cleavage rates were determined at 48 hpi and blastocysts rates at 168 hpi (day 7; D7) and 192 hpi (day 8; D8) and are provided as the percentage of zygotes cultured. Early and non-expanded blastocysts (mid blastocysts) were graded as blastocysts, while expanded, hatching, and hatched blastocysts were termed advanced blastocysts [27]. Day 8 blastocysts were fixed and immunostained to assess TCN, ICM cell number, TE cell number, and AR (3 replicates).

2.11. Statistical analysis

All statistical tests were performed using R software (V 4.0.3, R Core Team, Vienna, Austria). The Kolmogorov-Smirnov test and the

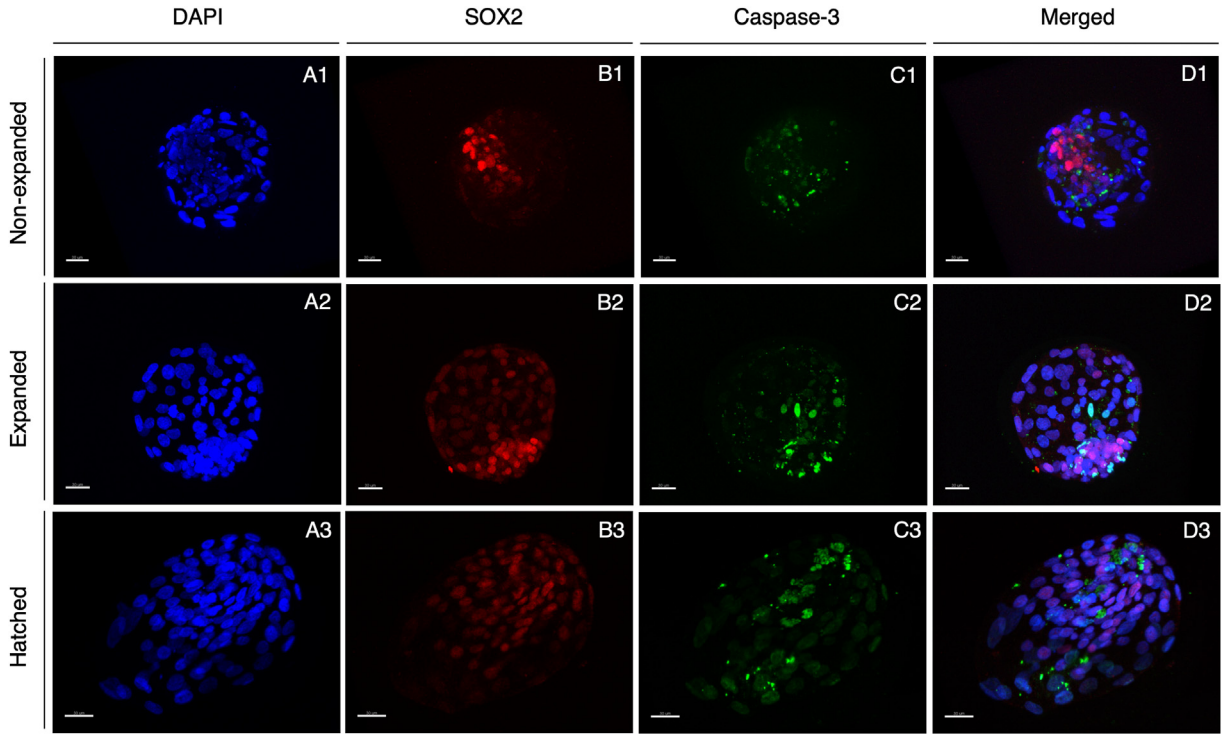

Fig. 2. Representative images of D8 non-expanded, expanded, and hatched blastocysts derived from oocytes vitrified/warmed in the 25°*C* and 38.5°*C* equilibration protocols. Fluorescence of anti-SOX2 (red) was examined using the Alexa Fluor™ 568 filter to detect ICM cells (B1–B3). Fluorescence of anti-active-caspase-3 antibody (green) was examined by the Alexa Fluor™ 488 filter to detect caspase activity (C1–C3), while DAPI staining (blue) was examined by the DAPI filter for total cell counts (A1-A3). An overlay is provided in (D1-D3). (A1,B1,C1,D1) Non-expanded blastocyst; (A2,B2,C2,D2) Expanded blastocyst; (A3,B3,C3,D3) Hatched blastocyst. Scale bar, 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

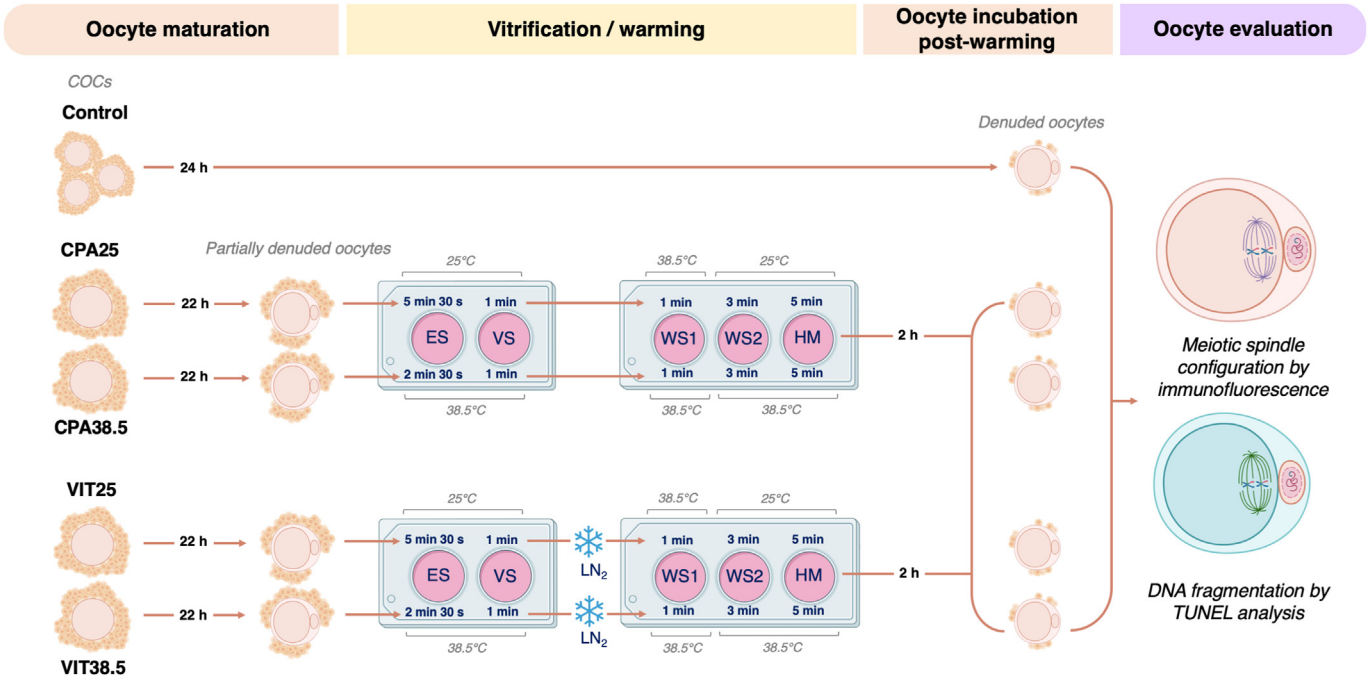

Fig. 3. Experimental design. IVM oocytes were randomly assigned to five groups: 1) Control, fresh, non-vitrified oocytes; 2)VIT25: oocytes exposed to ES for 5 min 30 s at 25 °C and vitrified/warmed; 3) CPA25: non-vitrified oocytes exposed to ES for 5 min 30 s at 25 °C; 4)VIT38.5: oocytes exposed to ES for 2 min 30 s at 38.5 °C and vitrified/warmed; and 5) CPA38.5: non-vitrified oocytes exposed to ES for 2 min 30 s at 38.5 °C. At 24 h of IVM, oocytes from each of the five treatment groups were denuded and immunostained to assess spindle morphology and DNA fragmentation.

Levene test were first used to check the normality of the data and homogeneity of variance, respectively. When required, data were linearly transformed into the arcsin $\sqrt{x}$ function prior to running statistical tests. However, if after applying this function, the data continued to show a non-normal distribution, they were analyzed using a non-parametric test. For the *in vitro* osmotic behavior experiment, an ANOVA of repeated measures was used, considering the given oocyte examined as a random factor. A parametric test (one-way ANOVA) was used to examine differences between groups in spindle configuration, DNA fragmentation, and embryo development. Non-normally distributed data were analyzed with a non-parametric test (Kruskal-Wallis). For the permeability estimates and the TCN, ICM, and TE cell number, and AR experiments, a two-way ANOVA was used. Secondly, we conducted a pairwise comparison using the Bonferroni test for parametric analyzes and the Wilcoxon test for non-parametric analyzes. Results are expressed as means ± standard error of the mean (SEM). Significance was set at $P \leq 0.05$.

3. Results

3.1. Modeling membrane permeability of bovine MII oocytes

3.1.1. Membrane permeability parameters

Our calculated L_p and P_s for MII bovine oocytes in the presence of Me_2SO or EG at different temperatures are given in Table 2. Overall, L_p values varied significantly between the temperatures ($P < 0.05$) for each CPA. In general, values of L_p for MII oocytes exposed to 1.55 M Me_2SO or 1.55 M EG at 25°*C* were much smaller than for MII oocytes in the presence of Me_2SO or EG at 38.5°*C*. Moreover, we also found that temperature had a significant effect on the permeability of solutes in both CPAs ($P < 0.05$). In general, solute permeability increased by a factor of about two as the temperature increased from 25°*C* to 38.5°*C* (Table 2). Also, the Lp for MII oocytes exposed to Me_2SO was higher than for oocytes exposed to EG at 25 °C ($P < 0.05$) whereas at 38.5°*C* no differences in water permeability were detected between the CPAs. When solute permeability was compared between CPAs at each temperature, only at 38.5°*C* was P_s for EG higher than for Me_2SO ($P < 0.05$).

3.1.2. In silico versus in vitro results

For our comparison of the effects of temperature on the cell osmotic response, mature oocytes were exposed to ES containing 7.5% Me_2SO + 7.5% EG at the two temperatures (25°*C* and 38.5°*C*). As these CPAs are permeable, there is simultaneous water transport (because of the osmotic gradient) and CPA influx (because of concentration gradients) and this produces cell volume changes.

The permeability parameters that we determined using single CPA solutions containing either Me_2SO or EG allowed us to make predictions for exposure to the ES mixture, which contains both CPAs. Predicted relative volumes and total intracellular solute concentrations in oocytes subjected to ES at 25°*C* or 38.5°*C*, as a function of time, are shown in Fig. 4. Our model predictions show

Table 2
Water permeability (L_p) and solute permeability (P_s) of bovine MII oocytes in the presence of 1.55 M Me_2SO or 1.55 M EG at 25°*C* or 38.5°*C*.

CPA	Temperature	n° oocytes	Membrane permeability parameters	
			L_p (μm/atm × min)	P_s (um/s)
Me_2SO	25°*C*	13	$1.91 \pm 0.13^{a,1}$	$0.59 \pm 0.05^{a,1}$
	38.5°*C*	10	$2.47 \pm 0.18^{b,1}$	$1.38 \pm 0.11^{b,1}$
EG	25°*C*	14	$1.38 \pm 0.12^{a,2}$	$0.78 \pm 0.09^{a,1}$
	38.5°*C*	10	$2.34 \pm 0.16^{b,1}$	$1.94 \pm 0.24^{b,2}$

Unless indicated otherwise, data are given as the mean ± SEM. [a,b] Different superscript letters indicate significant differences between temperatures for the same CPA ($P < 0.05$). [1,2] Different superscript numbers indicate significant differences in water or solute permeability between Me_2SO and EG CPA for the same temperature ($P < 0.05$). Me_2SO, dimethyl sulfoxide; EG, ethylene glycol.

that oocytes exposed to ES shrink to a minimum of 53% of their isotonic volume after 8 s of exposure, whereas at 38.5°C the oocytes are predicted to shrink to 60% within 5 s. Moreover, simulations predict that oocytes at 38.5°C swell back to their isotonic volume faster than at 25°C, recovering their original volume after 2.33 min or 5 min, respectively. Total intracellular solute molarity is achieved faster at 38.5°C than at 25°C. However, in both cases, the solute concentration gets close to the equilibrium value within the first minute of exposure to ES (Fig. 4).

As in the simulations, the *in vitro* oocyte osmotic response of bovine MII oocytes exposed to ES depicted in Fig. 4 indicates when oocytes attained their minimum volume (Vmin ~61% at 25°C after 15 s and 65% at 38.5°C after 10 s) and when they reach their new final equilibrium volume (5 min 30 s at 25°C and 2 min 30 s at 38.5°C). As expected, we observed the reduced volume of oocytes in all groups attributable to water outflow. However, after this initial shrinkage, the cells swell as CPA permeates the cell membrane driven by the concentration gradient (Fig. 4). These relative volume changes are dependent on temperature and were in good agreement with the model predictions.

3.2. Spindle configurations observed in IVM bovine oocytes vitrified/warmed in the 25°C or 38.5°C equilibration protocols

To assess whether reducing the preparation time of oocytes for vitrification at a certain temperature would modify oocyte morphofunctionality, oocytes were exposed to the ES for 5 min 30 s at 25°C and for 2 min 30 s at 38.5°C and then analyzed for their spindle configurations compared to those of non-treated controls. In this set of experiments, we used 987 oocytes. Results are summarized in Table 3.

Percentages of bovine oocytes reaching the MII stage did not differ between treatments, except for oocytes exposed to ES at 25°C for 5 min 30 s which showed a significantly lower percentage of nuclear maturation than fresh, non-vitrified oocytes. Neither were differences observed in the percentages of oocytes exhibiting a normally configured spindle between the control group and oocytes exposed or vitrified at 38.5°C. However, oocytes exposed or vitrified at 25°C showed a significantly lower proportion of oocytes with normal spindle and chromosome configurations compared to the control group, due primarily to a higher proportion of oocytes with decondensed or disorganized microtubules or chromosomes in the treated groups.

No significant differences among the treatment groups were observed in proportions of oocytes with a disorganized, decondensed, or absent spindle configuration, except in the VIT25 group, in which a significantly higher proportion of oocytes featured disorganized spindle and chromosome configurations compared to the control group.

3.3. Percentages of TUNEL-positive oocytes observed in IVM bovine oocytes vitrified/warmed in the 25°C or 38.5°C equilibration protocols

CPA exposure or vitrification/warming of bovine oocytes after exposure to the equilibration solution for 5 min 30 s at 25°C significantly increased the percentage of oocytes with fragmented DNA compared to the fresh control group. Although without significance, percentages of TUNEL-positive oocytes in the 38.5°C vitrified group were similar to those in the fresh non-vitrified treatments (Fig. 5A). Representative images of DNA fragmentation detected by TUNEL analysis are pictured in Fig. 5B.

3.4. Embryo development in IVM bovine oocytes vitrified/warmed in the 25°C or 38.5°C equilibration protocols

Table 4 compares the effects on early *in vitro* embryo development of exposing bovine oocytes *in vitro* matured for 22 h to CPA solutions at 25°C or 38.5°C prior to vitrification/warming. Vitrification after exposure of oocytes to CPA solutions at 25°C resulted in significantly lower cleavage rates and D7 and D8 blastocyst yields when compared to the control non-vitrified oocyte group.

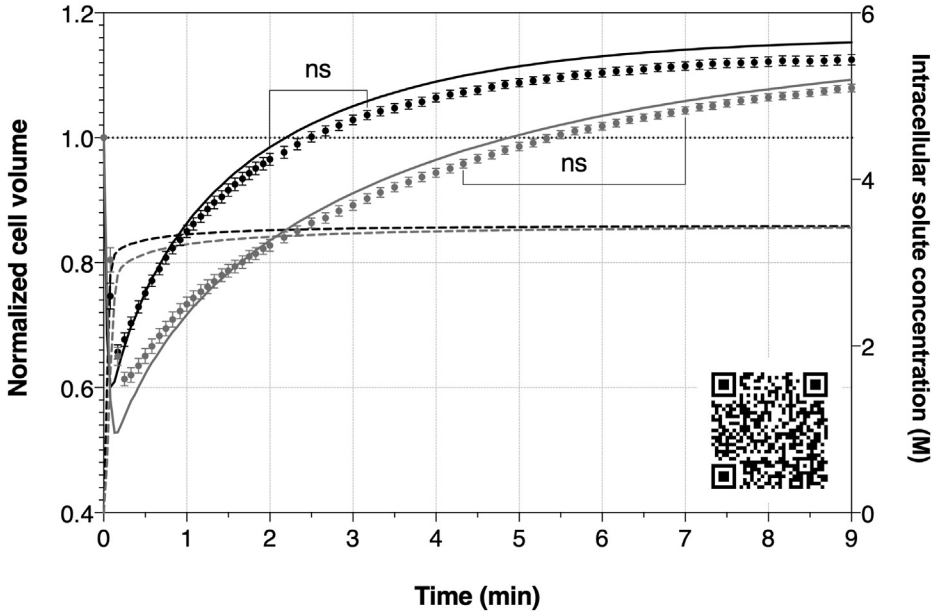

Fig. 4. Comparison between model predictions and *in vitro* results obtained in bovine MII oocytes exposed for 9 min to ES at 25°C or 38.5°C. Simulation of the osmotic response (solid line) of bovine MII oocytes when transferred to ES and predicted intracellular CPA concentration (dotted line) at 25°C (gray) and 38.5°C (black). The figure also shows experimental volume changes produced in bovine MII oocytes after exposure to ES at 25°C (gray circles) and 38.5°C (black circles). Data are the means of relative volumes ± SEM. Ns: means of relative volumes that do not differ statistically from the volume in isotonic solution. QR code links to a representative time-lapse video for MII bovine oocytes exposed to ES at 25°C or 38.5°C.

Table 3
Spindle morphology and chromosome alignments observed in IVM bovine oocytes vitrified/warmed in the 25°C or 38.5°C equilibration protocols.

Treatment	N° oocytes	MII (%)	Normal spindle configuration (%)	Microtubule distribution (%)			Chromosome distribution (%)		
				Dispersed	Decondensed	Absent	Dispersed	Decondensed	Absent
Control	227	181 (79.19 ± 1.59)[a]	120 (66.69 ± 2.28)[a]	43 (22.19 ± 2.00)[a]	15 (9.53 ± 3.87)	3 (1.59 ± 1.30)	43 (22.19 ± 2.00)[a]	18 (11.12 ± 3.52)	0 (0.00 ± 0.00)
CPA25	183	109 (62.16 ± 4.26)[b]	46 (42.00 ± 2.63)[b]	36 (33.21 ± 5.46)[ab]	19 (16.85 ± 6.27)	8 (7.94 ± 3.75)	38 (34.49 ± 5.31)[ab]	25 (23.51 ± 4.68)	0 (0.00 ± 0.00)
CPA38.5	211	151 (70.30 ± 2.64)[ab]	90 (61.16 ± 5.40)[a]	41 (27.54 ± 4.18)[a]	14 (8.05 ± 2.90)	6 (3.26 ± 1.76)	41 (27.54 ± 4.18)[a]	20 (11.30 ± 2.98)	0 (0.00 ± 0.00)
VIT25	164	110 (66.54 ± 3.53)[ab]	43 (38.38 ± 1.52)[b]	50 (45.76 ± 2.61)[b]	13 (12.79 ± 2.78)	4 (3.07 ± 1.46)	50 (45.76 ± 2.61)[b]	17 (15.86 ± 3.10)	0 (0.00 ± 0.00)
VIT38.5	198	143 (71.42 ± 5.22)[ab]	72 (53.49 ± 4.00)[ab]	46 (32.26 ± 1.62)[ab]	16 (10.19 ± 1.49)	9 (4.07 ± 2.61)	46 (32.26 ± 1.62)[ab]	25 (14.25 ± 3.91)	0 (0.00 ± 0.00)

Unless indicated otherwise, data are given as the mean ± SEM. [a,b] Within columns, values with different superscript letters differ significantly ($P < 0.05$). Rates of oocytes with the given morphology were calculated from the total number of oocytes reaching the MII stage. Control, fresh, non-vitrified oocytes; CPA25: non-vitrified oocytes exposed to the equilibration solution at 25°C for 5 min 30 s; CPA38.5: non-vitrified oocytes exposed to the equilibration solution at 38.5°C for 2 min 30 s; VIT25: oocytes exposed to the equilibration solution at 25°C for 5 min 30 s and vitrified/warmed; VIT38.5: oocytes exposed to the equilibration solution at 38.5°C for 2 min 30 s and vitrified/warmed.

Although not significantly different from those obtained after exposure at 25°C, cleavage rate and percentages of day 7 and day 8 blastocysts resulting from vitrified oocytes previously exposed to CPAs at 38.5°C were similar to those obtained from fresh control oocytes. Further, the impact of the temperature of exposure to CPAs during vitrification/warming on kinetics blastocyst formation was examined. Vitrification/warming using the 38.5°C protocol tended to increase ($P \leq 0.1$) the proportion of advanced blastocysts (expanded, hatching, and hatched) despite no significant differences detected among the experimental groups (see Table 5).

3.5. Differential staining of blastocysts and DNA fragmentation

In Table 5 we provide total cell numbers (TCN), numbers of ICM cells, numbers of TE cells and apoptotic rates (AR) recorded in Day 8 blastocysts derived from oocytes vitrified/warmed in the 25°C and 38.5°C equilibration protocols. Photographs obtained after differential cell staining of blastocysts are displayed in Fig. 2. While no differences were observed in TCN and numbers of TE cells in blastocysts derived from vitrified/warmed oocytes in the 38.5°C protocol, advanced blastocysts were produced with similar TCN and TE and ICM cell numbers than those derived from control oocytes. Although not significantly different from advanced blastocysts derived from vitrified/warmed oocytes subjected to the 38.5°C protocol, those vitrified/warmed in the 25°C equilibration protocols gave rise to advanced blastocysts with significantly lower ICM cell counts and higher TCN and TE cell counts than blastocysts derived from the non-vitrified group. Further, a significant increase was observed in TCN, ICM cell numbers, and TE cell numbers as the blastocyst stage progressed from blastocyst to advanced blastocyst, regardless of the treatment group. Although blastocysts derived from vitrified (38.5°C) oocytes showed significantly higher apoptosis rates than those derived from fresh non-vitrified oocytes, blastocysts derived from oocytes vitrified in the 25°C protocol showed the highest AR compared to the other treatment groups, regardless of blastocyst stage.

4. Discussion

Procedures designed to prepare bovine oocytes for vitrification strive to attain both appropriate intracellular glass-forming conditions and prevent an impaired capacity for fertilization and development into viable embryos. Because of the possible effects of numerous factors during the equilibration stage such as the CPAs employed and their concentrations, as well as the temperature and time of exposure to these CPAs, success with bovine oocyte survival and development has been limited and variable among studies (reviewed by Ref. [3]). The temperature and exposure time of CPAs play a crucial role in the whole vitrification process. At higher temperatures, oocyte shrinkage is reduced but the potential toxicity of CPAs increases. Besides, each CPA will have an optimal exposure time and temperature range resulting in minimal cell damage.

In this work, based on *in vitro* observations, we examined the used of shorter, dehydration-based protocols at different temperatures (25°C *vs.* 38.5°C) as a first step toward developing an optimal cryopreservation method for MII bovine oocytes. Results indicated that when vitrification was carried out at 38.5°C, reducing the ES exposure time of MII bovine oocytes to 2 min 30 s gave rise to similar results on spindle morphology, DNA fragmentation and embryo development compared to the used of fresh control oocytes. When oocytes were exposed to ES for longer time (9 or 10 min) at 38.5°C, apoptosis rates were significantly lower [25,28]. Hence, the toxicity of CPAs can be reduced by modifying equilibration times and temperatures [29]. Similarly, using the same methodology a longer ES exposure time (9 or 15 min) to ES at 25°C results in lower [30] or similar [25,31] cleavage and blastocyst rates to those observed in this study in our VIT25 group yet reduced blastocyst yields have been reported when IVM bovine oocytes are exposed to ES at 38.5°C–39°C for 9 or more minutes prior to vitrification [21,22,32,33]. Thus, the effects of temperature on cytotoxicity are clearly dependent on exposure time [34]. Accordingly, significantly decreased rates of embryo development have been described for oocytes overexposed to CPAs at 38.5°C [21,22,32,33] while prolonged exposure (9 or 15 min) at 25°C also impaired embryo development [25,31] although showing a much less dramatic effect than at 38.5°C.

Rational design approaches combine mathematical models and cell biophysical parameters to predict optimized CPA addition and removal procedures. As the damage induced by extending cell volumes beyond osmotic tolerance limits is relatively well understood, the most common approach has been to use membrane transport equations and osmotic tolerance limits have been mostly used to predict multi-step procedures able to prevent osmotic damage [35].

I*n silico* and *in vitro* analysis of the osmotic behavior of MII oocytes has several limitations. Several assumptions need to be made when calculating the relative volume of an oocyte from a 2D image of the shrinking-swelling process, so results may not be accurate [36,37]. Also as osmotic behavior is usually determined separately for low concentrations of each CPA [38–40] under different hyperosmotic CPA conditions, this behavior is likely to vary [41]. For example, at CPA concentrations needed for oocyte vitrification, CPAs could affect cytoplasmic phospholipid bilayer permeability

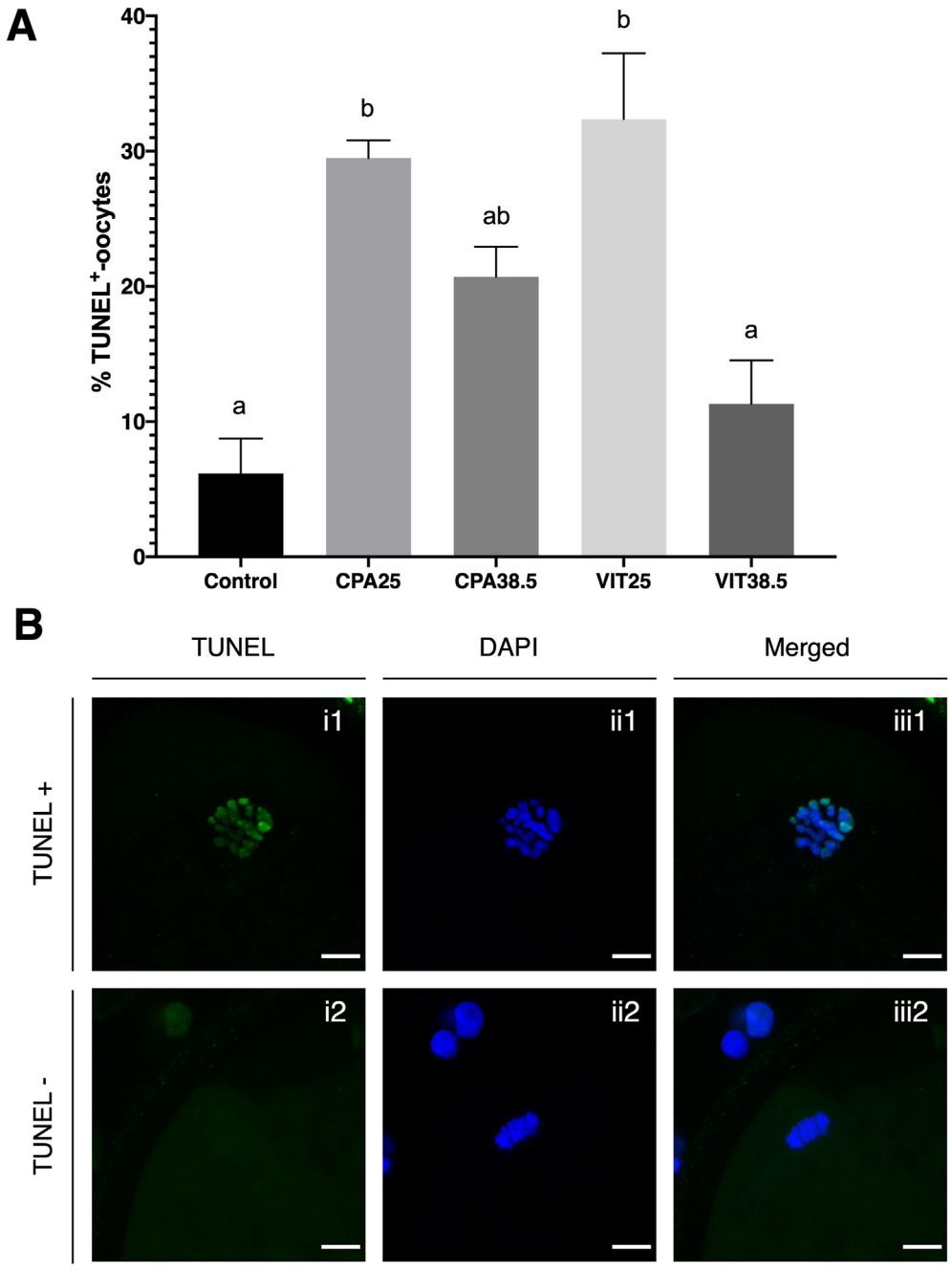

Fig. 5. TUNEL detection of fragmented oocyte DNA. (A) Effects of exposing IVM bovine oocytes to the equilibration solution for 5 min 30 s at 25°*C* or 2 min 30 s at 38.5°*C* before vitrification/warming on percentages of TUNEL-positive oocytes. Data are presented as the mean ± SEM. [a,b] Values with different letters differ significantly ($P < 0.05$). Treatment groups: Control: fresh, non-vitrified oocytes; CPA25: non-vitrified oocytes exposed to the equilibration solution at 25°*C* for 5 min 30 s; CPA38.5: non-vitrified oocytes exposed to the equilibration solution at 38.5°*C* for 2 min 30 s; VIT25: oocytes exposed to the equilibration solution at 25°*C* for 5 min 30 s and vitrified/warmed; VIT38.5: oocytes exposed to the equilibration solution at 38.5°*C* for 2 min 30 s and vitrified/warmed. Control: n = 106; CPA25: n = 92; CPA38.5: n = 110; VIT25: n = 164 and VIT38.5: n = 175 oocytes. (B) Representative images of IVM bovine oocytes stained with TUNEL ((i1,i2); green) and DAPI ((ii1,ii2); blue). (i1,ii1): TUNEL-positive oocyte; (i2,ii2): TUNEL-negative oocyte. An overlay is given in (iii1,iii2). Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

[42]. Further, the biophysical parameters of individual oocytes also seem to be highly variable [43]. Thus, permeability models and *in vitro* observations may not accurately reflect the physiological situation.

Despite these limitations, our *in silico* and *in vitro* results were fairly consistent. The time required for the oocytes to reach the equilibrium cell volume upon exposure to standard ES increased as the temperature decreased, such that 2 min 30 s was needed at 38.5°*C* and 5 min 30 s at 25°*C*. This is important because during this interval intracellular water efflux is close to completion and the permeation of low molecular weight CPAs leads to similar intracellular and extracellular solute concentrations. Thus, lengthening exposure to CPA solutions does not improve cytosolic glass formation. This means that rather than the usual 9–15 min of CPA exposure time used for bovine oocytes, a better approach could be oocyte exposure to non-vitrifying hypertonic solutions just until the equilibrium cell volume is reached to prepare them for vitrification. In the present study, we modified the equilibration protocol

Table 4
Developmental competence of embryos derived from IVM bovine oocytes vitrified/warmed after the 25°C or 38.5°C equilibration protocol.

	n	Cleavage rate	D7 blastocysts	D8 blastocysts	n_{D8}	D8 blastocysts	
						Blastocysts	Advanced
Control	170	141 (83.06 ± 2.07)[a]	49 (28.17 ± 3.34)[a]	60 (33.78 ± 5.07)[a]	60	31 (17.44 ± 1.89)[a]	29 (16.33 ± 3.90)
VIT25	158	86 (55.18 ± 5.09)[b]	19 (12.35 ± 2.54)[b]	20 (13.23 ± 1.81)[b]	20	11(7.31 ± 1.40)[b]	9 (5.92 ± 2.19)
VIT38.5	96	62 (64.92 ± 6.54)[ab]	18 (18.83 ± 2.23)[ab]	22 (23.94 ± 4.88)[ab]	22	10 (10.38 ± 2.71)[ab]	12 (13.56 ± 2.42)

Unless indicated otherwise, data are given as the mean ± SEM. [a,b] Within columns, values with different superscript letters differ significantly ($P < 0.05$). Cleavage rates (48 hpi) and D7 and D8 blastocyst yields were based on the number of oocytes that survived at Day 1 post-insemination. n_{D8}: total number of blastocysts observed at day 8 post-insemination. Oocyte survival was assessed on the basis of integrity of the oocyte membrane and zona pellucida, along with discoloration of the cytoplasm. Blastocysts: early and non-expanded blastocysts (intermediate-stage blastocysts); Advanced: expanded, hatching, and hatched blastocysts. Control: embryos derived from fresh, non-vitrified oocytes; VIT25: embryos derived from oocytes exposed to the equilibration solution at 25°C for 5 min 30 s and vitrified/warmed; VIT38.5: embryos derived from oocytes exposed to the equilibration solution at 38.5°C for 2 min 30 s and vitrified/warmed.

of a new vitrification/warming method designed to represent the current standard practice to prepare bovine oocytes [14] according to the *in vitro* osmotic behavior of oocytes at different temperatures. These modifications to the protocol were validated by examining oocyte spindle status, oocyte DNA fragmentation and further embryo development after vitrification/warming of MII bovine oocytes.

The main hurdle in developing successful protocols for the cryopreservation of mammalian oocytes is preserving the integrity of the meiotic spindle when oocytes are cooled below physiological temperature. Temperature fluctuations directly affect the cytoskeletal and microtubular systems of mature bovine [6] and human [44] oocytes. While the effects of cooling on the spindle seem reversible in the mouse oocyte, with normal spindle formation occurring after step-wise re-warming [45], bovine and human oocytes are less resilient because of the irreversibility of temperature-induced spindle disruption [6,44,46]. In our study, exposure of oocytes to CPAs at 25°C for 5 min 30 s yielded the lowest percentage of oocytes reaching the MII stage. Aman and Parks [6] reported that the spindles of MII bovine oocytes started to depolymerize when cooled to 25°C for 1 min or more, and the extent of depolymerization (or disassembly) was also directly related to CPA exposure duration [6]. We also observed that CPA exposure or vitrification after equilibration at 25°C for 5 min 30 s also resulted in reduced proportions of oocytes displaying normal spindle configurations. Other authors have described higher proportions of spindle alterations in MII bovine oocytes vitrified after similar or longer exposures times to the equilibration solution at 25°C [47,48] when compared to their fresh counterparts. However, no significant increase was noted here in chromosomal abnormalities after CPA exposure at 38.5°C for 2 min 30 s before vitrification, suggesting that the bovine oocytes were able to recover from cooling or CPA-induced damage to the oocytes' spindle microtubules. In prior work, we observed that bovine oocytes vitrified after 10 min of exposure to ES at 38.5°C led to similar rates of maturation and normal spindle configurations to those detected here at 38.5°C [21,22]. This may indicate that IVM bovine oocytes are more sensitive to the temperature of exposure to ES and VS than to the detrimental effects of CPA toxicity.

In this study, vitrification after CPA exposure at 38.5°C for 2 min 30 s did not significantly affect the occurrence of DNA fragmentation in oocytes when compared to fresh controls. Contrarily, exposure or vitrification of oocytes at 25°C significantly increased the percentage of DNA fragmentation after warming. Some authors have questioned whether CPA toxicity is related to the osmotic stress of adding and removing CPAs. Although osmotic stress can indeed cause cytotoxicity, experimental evidence suggests that CPAs are more chemically toxic [49]. While the mechanisms of CPA toxicity remain to be clearly established [50], hydrophobic interactions between CPAs and proteins, and a longer lifetime of CPA–water hydrogen bonds [51] have been proposed. These agents have also been accused of varying intracellular pH [52], increasing intracellular calcium contents [53], and causing formaldehyde formation in cryopreservation solutions [54] (or reviewed by Ref. [50]).

The results of our embryo development experiments designed to assess oocyte competence after vitrification/warming indicate comparable cleavage and blastocyst rates of vitrified bovine oocytes after exposure to CPA solutions at 38.5°C to those of control fresh oocytes. Moreover, although not significant, shorter CPA exposures at 38.5°C gave rise to almost double the percentage of blastocysts obtained after longer CPA exposure at 25°C. Our results clearly indicate the importance of temperature and time of CPA addition prior to vitrification. Cytotoxicity has been shown to increase with increased temperatures for relatively long exposure times [34,55]. This has led to the general assumption that CPA exposure should be carried out at low temperatures (25°C). However, the temperature of addition significantly affects the time needed to achieve full equilibration. At a higher temperature, equilibration occurs much more quickly, allowing for shorter incubation times and reducing cell exposure to CPAs during their addition and removal. Due to faster mass transport across the cell membrane at 38.5°C,

Table 5
Total cell numbers, number of cells in the ICM and TE, and rate of apoptotic cells recorded in Day 8 blastocysts derived from oocytes vitrified/warmed after the 25°C and 38.5°C equilibration protocols.

	Day 8 blastocysts									
	n		TCN ±SEM		ICM cell number ±SEM		TE cell number ±SEM		AR± SEM	
	Blast	Adv	Blast	Adv	Blast	Adv	Blast	Adv	Blast	Adv
Control	31	29	95.2 ± 2.2[1]	155.7 ± 5.6[a,2]	12.5 ± 0.7[a,1]	24.3 ± 1.0[a,2]	82.7 ± 2.1[1]	131.45 ± 5.2[a,2]	6.8 ± 0.4[a,1]	6.0 ± 0.5[a,1]
VIT25	11	9	87.8 ± 3.2[1]	180.5 ± 5.4[b,2]	8.9 ± 0.5[b,1]	18.5 ± 2.9[b,2]	79.0 ± 3.1[1]	162.0 ± 6.2[b,2]	19.9 ± 3.1[b,1]	15.3 ± 1.4[b,2]
VIT38.5	10	12	92.0 ± 4.5[1]	162.8 ± 8.1[ab,2]	10.8 ± 1.9[ab,1]	20.5 ± 2.3[ab,2]	81.1 ± 4.2[1]	142.3 ± 7.9[ab,2]	13.3 ± 1.3[c,1]	10.3 ± 1.0[c,1]

Unless indicated otherwise, data are given as mean ± SEM. [a,b,c] Values within columns with different superscripts differ significantly ($P < 0.05$); [1,2] Values within rows with different superscripts differ significantly ($P < 0.05$). Blast: early and non-expanded blastocysts (intermediate stage blastocysts); Adv: expanded, hatching, and hatched blastocysts. TCN: total cell number; ICM: inner cell mass; TE: trophectoderm; AR: apoptosis rate. Control: embryos derived from fresh, non-vitrified oocytes. VIT25: embryos derived from oocytes exposed to the equilibration solution at 25°C for 5 min 30 s and vitrified/warmed. VIT38.5: embryos derived from oocytes exposed to the equilibration solution at 38.5°C for 2 min 30 s and vitrified/warmed.

equilibration of the oocyte occurs earlier than at 25°C and less time is required for CPA addition. If we bear in mind that CPA exposure at 38.5°C had fewer deleterious effects on the meiotic spindle and oocyte apoptosis, CPA exposure at 38.5°C for 2 min 30 s may have allowed the oocyte to achieve the minimum intracellular glass-forming conditions so as not to fully impair the ability of vitrified/warmed oocytes to be successfully fertilized and develop into viable embryos.

It has been well established that for embryo survival and post-implantation development, blastocyst cells need to be optimally allocated to the ICM and TE. Studies have shown that a defined minimum number of ICM cells correlates strongly with normal fetal development and an adequate ICM:TE cell ratio seems essential for embryo viability and pregnancy [56]. Moreover, excessive allocation of cells to the TE possibly leads to early pregnancy loss [56,57]. Our blastocyst kinetics experiments revealed a clear trend towards a higher hatching ability of blastocysts derived from oocytes vitrified/warmed in the 38.5°C protocol. Further, blastocysts in the 38.5°C vitrification group featured similar TCN, ICM, and TE cell counts as in the non-vitrified groups, regardless of blastocyst stage. In contrast, higher TE and apoptotic cell numbers and lower ICM cell counts were observed in blastocysts derived from oocytes vitrified at 25°C. While in preimplantation embryos apoptosis serves to eliminate compromised cells, a high incidence of apoptosis results in a morphologically abnormal embryo [58]. This may explain why the lower apoptosis rate observed here in blastocysts derived from oocytes vitrified/warmed in the 38.5°C protocol gave rise to higher ($P < 0.1$) percentages of advanced blastocysts.

At this point we should mention that most human oocytes are vitrified by long exposure (up to 8–15 min) to equilibration media and using similar CPA concentrations to those used in our study at room temperature (25°C) [14,59–62]. This protocol has proved to be the best strategy for the cryopreservation of all human embryo developmental stages, especially of mature oocytes, as it significantly improves cryosurvival and clinical outcomes when compared to slow freezing, and has also allowed for the creation of oocyte banks [63]. Our worse results using a longer equilibration time at 25°C in bovine oocytes compared with the vast amount of data supporting this vitrification protocol for human oocytes, illustrates that factors other than CPA exposure times and temperatures may have an impact on oocyte cryosurvival (for a review, see Ref. [3]). Beside other features, we propose that the different outcomes observed for bovine and human oocytes vitrified using the same procedure might be related to differences in oocyte membrane permeability or the accumulation of lipid vesicles in the ooplasm [64]. In effect, it is well-known that the presence of these intracytoplasmic lipid droplets is an obstacle for the successful cryopreservation of bovine oocytes [65]. Nevertheless, shorter protocols for the vitrification of human oocytes have been recently proposed [66,67]. This trend allows for more efficient IVF workflow while minimizing the impacts of CPA toxicity.

5. Conclusion

In conclusion, in this study we validate new CPA equilibration procedures at two different temperatures as a first step to optimize vitrification/warming methods for bovine IVM oocytes. Based on *in silico* data on the biophysical permeability of oocytes and *in vitro* osmotic observations, we here propose a shorter vitrification/warming protocol for MII bovine oocytes. Using a standardized combination of vitrification solutions, exposure to the equilibration solution was constrained to 5 min 30 s or 2 min 30 s when ES exposure was done at 25°C or 38.5°C, respectively. After validating oocyte morphofunctionality and embryo development, our results indicate that limiting the exposure time to ES to 2 min 30 s at 38.5°C seems to improve the quality of vitrified/warmed oocytes improving blastocyst rates and quality by protecting spindle integrity and reducing DNA fragmentation. These data also indicate that toxic damage at a given temperature may be reduced by minimizing the duration of CPA addition and removal while maintaining cell volumes within osmotic tolerance limits. Our findings also pave the way for using a similar approach to improve the vitrification of other mammalian oocytes.

Funding

This study was founded by Spanish Ministry of Science and Innovation (Project PID2020-116531RB-I00) and the Generalitat de Catalunya (Project No. 2017 SGR 1229). Scholarship to Mrs. Tania García-Martínez (2019 FI_B2 00055).

Availability of data and materials

All data generated or analyzed during this study are included in the published article.

CRediT authorship contribution statement

Tania García-Martínez: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, All authors have read and agreed to the published version of the manuscript. **Iris Martínez-Rodero:** Investigation, Methodology, All authors have read and agreed to the published version of the manuscript. **Joan Roncero-Carol:** Investigation, Methodology, All authors have read and agreed to the published version of the manuscript. **Iván Yánez-Ortiz:** Methodology, All authors have read and agreed to the published version of the manuscript. **Adam Z. Higgins:** Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing – review & editing, All authors have read and agreed to the published version of the manuscript. **Teresa Mogas:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

Not applicable.

References

[1] Prentice JR, Anzar M. Cryopreservation of Mammalian oocyte for conservation of animal genetics. Vet Med Int 2010;2011.
[2] Mogas T. Update on the vitrification of bovine oocytes and invitro-produced embryos. Reprod Fertil Dev 2018;31:105–17.
[3] Dujickova L, Makarevich AV, Olexikova L, Kubovicova E, Strejcek F. Methodological approaches for vitrification of bovine oocytes. Zygote 2020:1–11.
[4] Fahy GM. Cryoprotectant toxicity neutralization. Cryobiology 2010;60:S45–53.
[5] Agca Y, Liu J, Critser ES, McGrath JJ, Critser JK. Temperature-dependent osmotic behavior of germinal vesicle and metaphase II stage bovine oocytes in the presence of Me2SO in relationship to cryobiology. Mol Reprod Dev 1999;53:59–67.
[6] Aman RR, Parks JE. Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. Biol Reprod 1994;50:103–10.
[7] Dujíčková L, Makarevich AV, Olexiková L, Kubovičová E, Strejček F. Methodological approaches for vitrification of bovine oocytes. Zygote 2021;29:1–11.
[8] Ruffing NA, Steponkus PL, Pitt RE, Parks JE. Osmometric behavior, hydraulic

conductivity, and incidence of intracellular ice formation in bovine oocytes at different developmental stages. Cryobiology 1993;30:562–80.
[9] Agca Y, Liu J, Peter AT, Critser ES, Critser JK. Effect of developmental stage on bovine oocyte plasma membrane water and cryoprotectant permeability characteristics. Mol Reprod Dev 1998;49:408–15.
[10] Agca Y, Liu J, Rutledge JJ, Critser ES, Critser JK. Effect of osmotic stress on the developmental competence of germinal vesicle and metaphase II stage bovine cumulus oocyte complexes and its relevance to cryopreservation. Mol Reprod Dev 2000;55:212–9.
[11] Wang X, Al Naib A, Sun DW, Lonergan P. Membrane permeability characteristics of bovine oocytes and development of a step-wise cryoprotectant adding and diluting protocol. Cryobiology 2010;61:58–65.
[12] Jin B, Kawai Y, Hara T, Takeda S, Seki S, Nakata Y, et al. Pathway for the movement of water and cryoprotectants in bovine oocytes and embryos. Biol Reprod 2011;85:834–47.
[13] Martins RD, Costa EP, Chagas JSC, Ignácio FS, Torres CAA, C M. Effects of vitrification of immature bovine oocytes on in vitro maturation. Anim Reprod Sci 2005;2:128–34.
[14] Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. Theriogenology 2007;67:73–80.
[15] Wang L, Liu J, Zhou GB, Hou YP, Li JJ, Zhu SE. Quantitative investigations on the effects of exposure durations to the combined cryoprotective agents on mouse oocyte vitrification procedures. Biol Reprod 2011;85:884–94.
[16] Rizos D, Ward F, Boland MP, Lonergan P. Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification. Theriogenology 2001;56:1–16.
[17] Kleinhans FW. Membrane permeability modeling: kedem-Katchalsky vs a two-parameter formalism. Cryobiology 1998;37:271–89.
[18] Dormand JR, Prince PJ. A family of embedded Runge-Kutta formulae. J Comput Appl Math 1980;6:19–26.
[19] Shampine LF, Reichelt MW. The MATLAB ODE suite. SIAM J Sci Comput 1997;18:1–22.
[20] Lagarias JC, Reeds JA, Wright MH, Wright PE. Convergence properties of the nelder–mead simplex method in low dimensions. SIAM J Optim 1998;9:112–47.
[21] Garcia-Martinez T, Vendrell-Flotats M, Martinez-Rodero I, Ordonez-Leon EA, Alvarez-Rodriguez M, Lopez-Bejar M, et al. Glutathione ethyl ester protects in vitro-maturing bovine oocytes against oxidative stress induced by subsequent vitrification/warming. Int J Mol Sci 2020:21.
[22] Arcarons N, Vendrell-Flotats M, Yeste M, Mercade E, Lopez-Bejar M, Mogas T. Cryoprotectant role of exopolysaccharide of Pseudomonas sp. ID1 in the vitrification of IVM cow oocytes. Reprod Fertil Dev 2019;31:1507–19.
[23] Chian RC, Kuwayama M, Tan L, Tan J, Kato O, Nagai T. High survival rate of bovine oocytes matured in vitro following vitrification. J Reprod Dev 2004;50:685–96.
[24] Morato R, Izquierdo D, Paramio MT, Mogas T. Cryotops versus open-pulled straws (OPS) as carriers for the cryopreservation of bovine oocytes: effects on spindle and chromosome configuration and embryo development. Cryobiology 2008;57:137–41.
[25] Vendrell-Flotats M, Garcia-Martinez T, Martinez-Rodero I, Lopez-Bejar M, LaMarre J, Yeste M, et al. In vitro maturation with leukemia inhibitory factor prior to the vitrification of bovine oocytes improves their embryo developmental potential and gene expression in oocytes and embryos. Int J Mol Sci 2020:21.
[26] Martinez-Rodero I, Garcia-Martinez T, Ordonez-Leon EA, Vendrell-Flotats M, Olegario Hidalgo C, Esmoris J, et al. A shorter equilibration period improves post-warming outcomes after vitrification and in straw dilution of in vitro-produced bovine embryos. Biology 2021;10.
[27] Gutierrez-Anez JC, Lucas-Hahn A, Hadeler KG, Aldag P, Niemann H. Melatonin enhances in vitro developmental competence of cumulus-oocyte complexes collected by ovum pick-up in prepubertal and adult dairy cattle. Theriogenology 2021;161:285–93.
[28] Spricigo JF, Morato R, Arcarons N, Yeste M, Dode MA, Lopez-Bejar M, et al. Assessment of the effect of adding L-carnitine and/or resveratrol to maturation medium before vitrification on in vitro-matured calf oocytes. Theriogenology 2017;89:47–57.
[29] Raju R, Bryant SJ, Wilkinson BL, Bryant G. The need for novel cryoprotectants and cryopreservation protocols: insights into the importance of biophysical investigation and cell permeability. Biochim Biophys Acta Gen Subj 2021;1865:129749.
[30] Azari M, Kafi M, Ebrahimi B, Fatehi R, Jamalzadeh M. Oocyte maturation, embryo development and gene expression following two different methods of bovine cumulus-oocyte complexes vitrification. Vet Res Commun 2017;41:49–56.
[31] Zhou XL, Al Naib A, Sun DW, Lonergan P. Bovine oocyte vitrification using the Cryotop method: effect of cumulus cells and vitrification protocol on survival and subsequent development. Cryobiology 2010;61:66–72.
[32] Ortiz-Escribano N, Smits K, Piepers S, Van den Abbeel E, Woelders H, Van Soom A. Role of cumulus cells during vitrification and fertilization of mature bovine oocytes: effects on survival, fertilization, and blastocyst development. Theriogenology 2016;86:635–41.
[33] Arcarons N, Vendrell-Flotats M, Yeste M, Mercadé E, Mogas T. Spindle configuration of in vitro matured bovine oocytes vitrified and warmed in media supplemented with a biopolymer produced by an Antarctic bacterium. Anim Reprod 2017;14:972.
[34] Lawson A, Ahmad H, Sambanis A. Cytotoxicity effects of cryoprotectants as single-component and cocktail vitrification solutions. Cryobiology 2011;62:115–22.
[35] Mukherjee IN, Song YC, Sambanis A. Cryoprotectant delivery and removal from murine insulinomas at vitrification-relevant concentrations. Cryobiology 2007;55:10–8.
[36] Mullen SF, Li M, Li Y, Chen ZJ, Critser JK. Human oocyte vitrification: the permeability of metaphase II oocytes to water and ethylene glycol and the appliance toward vitrification. Fertil Steril 2008;89:1812–25.
[37] Paynter SJ, Fuller BJ, Shaw RW. Temperature dependence of Kedem-Katchalsky membrane transport coefficients for mature mouse oocytes in the presence of ethylene glycol. Cryobiology 1999;39:169–76.
[38] Newton H, Pegg DE, Barrass R, Gosden RG. Osmotically inactive volume, hydraulic conductivity, and permeability to dimethyl sulphoxide of human mature oocytes. J Reprod Fertil 1999;117:27–33.
[39] Paynter SJ, Cooper A, Gregory L, Fuller BJ, Shaw RW. Permeability characteristics of human oocytes in the presence of the cryoprotectant dimethylsulphoxide. Hum Reprod 1999;14:2338–42.
[40] Zhao G, Zhang Z, Zhang Y, Chen Z, Niu D, Cao Y, et al. A microfluidic perfusion approach for on-chip characterization of the transport properties of human oocytes. Lab Chip 2017;17:1297–305.
[41] Mazur P, Schneider U. Osmotic responses of preimplantation mouse and bovine embryos and their cryobiological implications. Cell Biophys 1986;8:259–85.
[42] Sydykov B, Oldenhof H, de Oliveira Barros L, Sieme H, Wolkers WF. Membrane permeabilization of phosphatidylcholine liposomes induced by cryopreservation and vitrification solutions. Biochim Biophys Acta Biomembr 2018;1860:467–74.
[43] Paynter SJ. A rational approach to oocyte cryopreservation. Reprod Biomed Online 2005;10:578–86.
[44] Almeida PA, Bolton VN. The effect of temperature fluctuations on the cytoskeletal organisation and chromosomal constitution of the human oocyte. Zygote 1995;3:357–65.
[45] Pickering SJ, Johnson MH. The influence of cooling on the organization of the meiotic spindle of the mouse oocyte. Human Reproduction 1987;2:207–16.
[46] Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. Fertil Steril 1990;54:102–8.
[47] Arcarons N, Morato R, Spricigo JF, Ferraz MA, Mogas T. Spindle configuration and developmental competence of in vitro-matured bovine oocytes exposed to NaCl or sucrose prior to Cryotop vitrification. Reprod Fertil Dev 2015;28(10):1560–9.
[48] Chaves DF, Campelo IS, Silva MM, Bhat MH, Teixeira DI, Melo LM, et al. The use of antifreeze protein type III for vitrification of in vitro matured bovine oocytes. Cryobiology 2016;73:324–8.
[49] Lawson A, Mukherjee IN, Sambanis A. Mathematical modeling of cryoprotectant addition and removal for the cryopreservation of engineered or natural tissues. Cryobiology 2012;64:1–11.
[50] Best BP. Cryoprotectant toxicity: facts, issues, and questions. Rejuvenation Res 2015;18:422–36.
[51] Kirchner B, Reiher M. The secret of dimethyl sulfoxide-water mixtures. A quantum chemical study of 1DMSO-nwater clusters. J Am Chem Soc 2002;124:6206–15.
[52] Damien M, Luciano AA, Peluso JJ. Propanediol alters intracellular pH and developmental potential of mouse zygotes independently of volume change. Human Reproduction 1990;5:212–6.
[53] Gardner DK, Sheehan CB, Rienzi L, Katz-Jaffe M, Larman MG. Analysis of oocyte physiology to improve cryopreservation procedures. Theriogenology 2007;67:64–72.
[54] Karran G, Legge M. Non-enzymatic formation of formaldehyde in mouse oocyte freezing mixtures. Human Reproduction 1996;11:2681–6.
[55] Davidson AF, Glasscock C, McClanahan DR, Benson JD, Higgins AZ. Toxicity minimized cryoprotectant addition and removal procedures for adherent endothelial cells. PLoS One 2015;10:e0142828.
[56] Van Soom A, Boerjan M, Ysebaert MT, De Kruif A. Cell allocation to the inner cell mass and the trophectoderm in bovine embryos cultured in two different media. Mol Reprod Dev 1996;45:171–82.
[57] Loureiro B, Bonilla L, Block J, Fear JM, Bonilla AQ, Hansen PJ. Colony-stimulating factor 2 (CSF-2) improves development and posttransfer survival of bovine embryos produced in vitro. Endocrinology 2009;150:5046–54.
[58] Hardy K, Handyside AH, Winston RM. The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Development 1989;107:597–604.
[59] Cobo A, García-Velasco JA, Coello A, Domingo J, Pellicer A, Remohí J. Oocyte vitrification as an efficient option for elective fertility preservation. Fertil Steril 2016;105. 755-64.e8.
[60] Solé M, Santaló J, Boada M, Clúa E, Rodríguez I, Martinez F, et al. How does vitrification affect oocyte viability in oocyte donation cycles? A prospective study to compare outcomes achieved with fresh versus vitrified sibling oocytes, vol. 28; 2013. p. 2087–92.
[61] Rienzi L, Romano S, Albricci L, Maggiulli R, Capalbo A, Baroni E, et al. Embryo development of fresh 'versus' vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study, vol. 25; 2010. p. 66–73.
[62] De Munck N, Verheyen G, Van Landuyt L, Stoop D, Van de Velde H. Survival and post-warming in vitro competence of human oocytes after high security

closed system vitrification, vol. 30; 2013. p. 361–9. JJoar, genetics.
[63] Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM, et al. Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. Hum Reprod Update 2017;23:139–55.
[64] Hwang IS, Hochi S. Recent progress in cryopreservation of bovine oocytes. BioMed Res Int 2014;2014:570647.
[65] Ohata K, Ezoe K, Miki T, Kouraba S, Fujiwara N, Yabuuchi A, et al. Effects of fatty acid supplementation during vitrification and warming on the developmental competence of mouse, bovine and human oocytes and embryos. 2021.
[66] Karlsson JOM, Szurek EA, Higgins AZ, Lee SR, Eroglu A. Optimization of cryoprotectant loading into murine and human oocytes. Cryobiology 2014;68:18–28.
[67] Gallardo M, Saenz J, Risco R. Human oocytes and zygotes are ready for ultra-fast vitrification after 2 minutes of exposure to standard CPA solutions. Sci Rep 2019;9:15986.
[68] García-Martínez T, Mogas T, Mullen SF, Martínez-Rodero I, Gulieva RE, Higgins AZ. Effect of cryoprotectant concentration on bovine oocyte permeability and comparison of two membrane permeability modelling approaches. Sci Rep 2021;11:15387.
[69] Vian AM, Higgins AZ. Membrane permeability of the human granulocyte to water, dimethyl sulfoxide, glycerol, propylene glycol and ethylene glycol. Cryobiology 2014;68:35–42.