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Original Article

Embryology

Preclinical validation of fast oocyte vitrification and warming protocols with comparable efficiencies to a standard method

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ABSTRACT

STUDY QUESTION: Can fast vitrification (FV) and fast warming (FW) protocols effectively reduce oocyte exposure times to cryoprotectants while maintaining survival, developmental potential, and laboratory efficiency compared to a standard protocol?

SUMMARY ANSWER: FV and FW protocols significantly reduce exposure times compared to a standard protocol without compromising oocyte integrity or developmental potential in the animal models tested, offering a more efficient workflow for IVF laboratories.

WHAT IS KNOWN ALREADY: Vitrification is a widely used oocyte cryopreservation technique in IVF centers, but current protocols are time-consuming and labor-intensive. Additionally, prolonged exposure to cryoprotectants under non-physiological conditions, such as high osmolality and room temperature, raises concerns about oocyte viability and developmental potential.

STUDY DESIGN, SIZE, DURATION: This preclinical study involved oocytes from two animal models (mouse and rabbit) and a subset of discarded human oocytes. Experimental stages included: (a) evaluation of meiotic spindle integrity; (b) assessment of survival and developmental rates post-warming and ICSI; (c) embryo transfers in mice to evaluate full-term developmental potential; and (d) development and validation of an *in silico* model for human oocytes.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Mouse and rabbit oocytes were randomly allocated to either a fast or standard vitrification (SV)/standard warming (SW) protocol. Survival and developmental rates were assessed post-warming. Meiotic spindle integrity and chromosomal alignment were analyzed using immunofluorescence. Full-term development was evaluated through embryo transfers in mice. Theoretical osmotic modeling was performed on human oocytes to predict their behavior under FV conditions, with empirical validation using discarded human oocytes.

MAIN RESULTS AND THE ROLE OF CHANCE: The FV/FW protocol significantly reduced the time required for vitrification compared to a SV/SW protocol, while maintaining comparable oocyte survival, meiotic spindle integrity, and developmental rates in animal models. In mouse oocytes, the FV/FW protocol achieved the highest survival rate (n = 249 oocytes; 97.2%) not statistically significantly different from the SV/SW protocol (n = 224 oocytes, 94.2%), but significantly higher ($P = 0.008$) than the SV/FW group (n = 229 oocytes, 91.7%). After ICSI, the SV/SW group reached 83.4% blastocyst rates, followed by the FV/FW group at 80.9%, and the SV/FW group at 75.9%, all comparable to the fresh oocyte control group (n = 123) with 86.4%. Embryo development after ICSI resulted in blastocyst formation rates of 80.9% for the FV/FW protocol compared to 83.4% in the SV/SW and 86.4% in the SV/FW group. Embryo transfer outcomes in mice demonstrated no statistically significant adverse effects on implantation or full-term development, with live birth rates of 38.7% (FV/FW) compared to 47.8% (SV/SW) and 43.2% (SV/FW). Survival rates of rabbit metaphase II oocytes ranged between 90% and 100% across all protocols, while blastocyst developmental rates were higher in the FV/FW group (28.6%) compared to the SV/SW group (22.2%) and the SV/FW group (13.6%). The mathematical *in silico* osmotic model predicted favorable responses for human oocytes, which were confirmed experimentally in discarded human oocytes with survival rates of 94.1% (n = 101) for the SV/FW protocol and 97.1% (n = 103) for the FV/FW protocol.

LARGE SCALE DATA: NA

LIMITATIONS, REASONS FOR CAUTION: This study was preclinical and involved animal models and discarded human oocytes. Further clinical trials are required to confirm the safety and efficacy of FV protocols in routine IVF clinical practice.

WIDER IMPLICATIONS OF THE FINDINGS: FV and FW protocols offer a promising alternative to conventional methods, enhancing laboratory workflow efficiency and reducing oocyte exposure to potentially harmful cryoprotectants. These findings lay the foundation for translational research and future clinical applications in clinical IVF settings.

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Keywords: preclinical / validations / vitrification / warming / fast / protocols / oocytes

Introduction

The ability to cryopreserve oocytes (Kuleshova *et al.*, 1999) represents a significant advancement in fertility preservation. Oocyte cryopreservation is now a viable option during IVF treatments, for women wishing to delay childbearing, or undergoing gonadotoxic treatments for cancer. It is also widely used for research purposes and oocyte banking programs (Cobo *et al.*, 2010; Cobo and Diaz, 2011; Cobo *et al.*, 2021). Among the available cryopreservation techniques, vitrification is currently the preferred method due to its effectiveness in preventing intracellular ice formation, thereby significantly enhancing oocyte survival (Kuwayama *et al.*, 2005; Kuwayama, 2007).

In 2012, a consensus established a competency value for oocyte vitrification, setting a survival rate of 70% as the standard, with 85% defined as a benchmark for optimal performance (Alpha Scientists in Reproductive Medicine, 2012). One year later, the vitrification technique was no longer considered experimental and was recognized as an established laboratory procedure (ASRM, 2013; Practice Committees of the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology. Mature oocyte cryopreservation: a guideline, 2013). While oocyte vitrification has achieved considerable success globally—leading to numerous live births with no evidence of increased adverse obstetric or perinatal outcomes (Noyes *et al.*, 2009; Levi Setti *et al.*, 2013; Cobo *et al.*, 2014)—there is still growing interest in further optimizing the process to enhance efficiency, clinical outcomes, and laboratory workflows.

Current vitrification protocols typically involve the sequential exposure of cells to increasing concentrations of cryoprotectants (CPAs), making this a time-consuming process, particularly in busy laboratories where the trend to work with vitrified samples has increased the demand for this procedure in recent years (Kuwayama *et al.*, 2005; Cobo *et al.*, 2021). Additionally, during preparation for cooling, oocytes are exposed to sub-optimal conditions, including room temperature, cryopreservation solutions with high osmolality and potentially toxic CPAs. Hence, reducing exposure times to CPAs could lead to improved outcomes and at the same time streamline laboratory workflows.

Initial studies using murine models explored the potential of a one-step exposure to vitrification solution (VS) for as little as 15 s (Nakagata, 1989; Shaw *et al.*, 1992). Subsequent experiments extended this approach to human oocytes, however, progress was limited by the slower cooling and warming rates used at the time. Despite this, authors reported satisfactory survival, fertilization, and embryo developmental rates and demonstrated that human oocytes could tolerate such a brief exposure regimen (Hunter *et al.*, 1995). More recently, through a simulation of the osmotic behavior of the human oocyte, it was predicted that the ice-forming tendency of the cytosol after only 1 min of exposure to equilibration solution (ES) should be equal to that expected after an equilibration period of 9–15 min that is typical in current standard protocols (Gallardo *et al.*, 2019). These results were confirmed with high survival rates in both human oocytes and zygotes that were exposed for just 1 min to ES and 1 min to the VS and vitrified in an open cryodevice (Gallardo *et al.*, 2019). Efforts to shorten the rehydration phase after warming have also been reported recently in human blastocysts—reducing the warming time from 10 min to 1 min without compromising survival or clinical outcomes (Manns *et al.*, 2021, 2022; Liebermann *et al.*, 2024a)—as well as with discarded immature human oocytes (Liebermann *et al.*, 2024b). The studies involving oocytes have assessed only survival rates and their ability to resume meiosis after warming (Liebermann *et al.*, 2024b), while fertilization and developmental potential with the fast protocols have yet to be investigated.

The primary aim of this study was to perform a preclinical validation of fast protocols for the vitrification and warming of oocytes. To assess the efficacy of these new methods, oocytes from two different animal models (mouse and rabbit) were used in initial experiments. Meiotic spindles were analyzed using confocal microscopy, and survival and developmental rates post-ICSI were compared between the fast vitrification (FV) and fast warming (FW) protocols and a standard method used as a control. Additionally, validations included embryo transfers in mice to assess implantation and full-term developmental rates across groups. Next, a theoretical model was developed to simulate the osmotic behavior of human oocytes under the new CPA exposure regimes. These predictions were validated using a subset of discarded human oocytes, with survival rates assessed to provide additional evidence supporting the feasibility of the new protocols before their translation into clinical practice.

Materials and methods

Animals

All procedures involving mice were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the Barcelona Science Park (IACUC-PCB, reference number: 10133). Hybrid (B6/CBA) and outbred CD1 females of 5–6 weeks of age (25–30 g), and male mice from the same genetic strains of 8–10 weeks of age (25–30 g) were purchased from Janvier Laboratories (France). Procedures involving rabbits were reviewed and approved by the Universitat Politècnica de València ethical committee (reference number: 2021/VSC/PEA/0270). New Zealand White (NZW) females of 5–6 months of age (3200–3500 g) and male rabbits from the same genetic background of 6–10 months of age (3300–3600 g) were obtained from the Universitat Politècnica de València experimental farms.

Ethical approval and informed consent for discarded human oocytes

The study protocol involving the use of discarded human oocytes was reviewed and approved by the Ethics Committee for Clinical Research IIS La Fé (Internal Code: 2310-VLC-181-AC). Informed

consent was obtained from all participants, and the protocol adhered to Spanish national guidelines and regulations. After denudation, human oocytes found at immature germinal vesicle (GV) or metaphase I (MI) stage were excluded from conventional ICSI cycles and used for experiments. Discarded oocytes were kept in culture in a commercial medium (Genea, Australia) at 37°C and a humidified atmosphere with 6% CO_2 and 5% O_2. The maturation progress of the discarded oocytes was monitored for up to a maximum of 48 h. Oocytes exhibiting a first polar body were considered mature and were then used for vitrification and warming with the corresponding assigned protocols.

Collection of mouse oocytes, zygotes, and sperm

For the collection of mouse oocytes, hybrid B6CBAF1 females were induced to superovulate by intraperitoneal injection of 7.5 IU of pregnant mare serum gonadotropin (PMSG) followed 48 h later by 7.5 IU of hCG. Cumulus–oocyte complexes were released from the oviducts by 14 h after hCG administration and treated with 80 IU hyaluronidase (Sigma-Aldrich, United States) until cumulus cells dispersed. Once denuded, oocytes with good morphology were selected, washed thoroughly, and kept in culture medium (CM) (KSOM, MerkMillipore, United States) under oil (Hypure heavy, Kitazato, Japan) at 37°C, in an atmosphere with 7% CO_2 and 7% O_2, until use. Spermatozoa were collected from cauda epididymis and then diluted and incubated in CM (KSOM, MerkMillipore, United States) at 37°C, in an atmosphere with 7% CO_2 and 7% O_2, until use.

Collection of rabbit oocytes and sperm

Rabbit oocytes were collected from nulliparous NZW females as described by Viudes-de-Castro *et al.* (2017). Briefly, females were superovulated with a combination of FSH (Corifollitropin alfa, 3 μg, Elonva, Merck Sharp & Dohme S.A., Spain) and 7.5 IU of hCG. Seventy-two hours later, ovulation was induced by intramuscular administration of 1 μg/ml of buserelin acetate (Aventis Pharma, S.A., Spain). Cumulus-oocyte complexes were collected 14–15 h after inducing ovulation by flushing the dissected oviducts with HEPES-buffered human tubal fluid (HTF) medium supplemented with 10% (v/v) fetal bovine serum (FBS). Afterwards, oocytes were exposed to 80 IU hyaluronidase at 37°C for 3–5 min and pipetted until cumulus cells were removed. Denuded oocytes showing a first polar body were selected, washed thoroughly, and cultured in KSOM supplemented with 10% FBS under oil at 38.3°C, in an atmosphere with 7% CO_2 and 7% O_2, until use. Semen was collected from adult males with proven fertility using an artificial vagina. Only ejaculates exhibiting a white color with more than a 70% motility rate and less than 15% abnormal sperm were considered for experiments. After collection and assessment, semen was washed by centrifugation at 300*g* for 5 min and prepared by swim-up in HEPES-buffered HTF medium supplemented with 10% FBS for a minimum of 30 min at 37°C.

Vitrification and warming protocols

All vitrification and warming protocols were carried out using commercial solutions (Kitazato, Japan) and an open device (Cryotop®, Kitazato, Japan). The same protocols were applied across the different species. The standard vitrification (SV) method was conducted at room temperature, following the manufacturer's instructions. Briefly, samples were gradually exposed to buffer solution (BS)/ES for 12–15 min, then transferred to VS1 for 30 s and VS2 for an additional 30 s. Afterward, the samples were quickly loaded onto the leaf of a Cryotop and immediately plunged into liquid nitrogen.

FV was also conducted at room temperature. Samples were first exposed to ES for only 1 min, then transferred and exposed to VS1 and VS2 for 30 s each, loaded on a Cryotop, and quickly plunged into liquid nitrogen.

For warming using the standard warming (SW) method, one device at a time was initially submerged in thawing solution (TS) for 1 min at 37°C, until the cells detached from the Cryotop. Afterward, samples were transferred to dilution solution (DS) for 3 min, followed by warming solution 1 (WS1) for 5 min and WS2 for 1 min, at room temperature. Finally, the samples were thoroughly washed and cultured for 1–2 h before further use.

In the FW protocol, Cryotops were quickly submerged in TS for 1 min at 37°C, and the warmed samples were directly transferred to CM, thoroughly washed, and cultured under optimal conditions until further use.

ICSI and embryo culture

All manipulations were performed on a heated stage calibrated to 37°C (Okolab) of an Olympus IX73 inverted microscope, using TransferMan 4 m (Eppendorf, Germany) micromanipulators. Mouse and rabbit oocytes were inseminated with sperm from the corresponding species by ICSI, utilizing a piezo-driven unit (PiezoXpert, Eppendorf, Germany). After injection, mouse oocytes were cultured in KSOM supplemented with 5 mg/ml human serum albumin (HSA) under oil at 37.3°C for 5 days, while rabbit oocytes were cultured in KSOM supplemented with 10% FBS at 38.3°C for 7 days, in both cases in an atmosphere of 7% CO_2 and 7% O_2 in air.

Immunofluorescence analysis

For analysis of the meiotic spindle structure and chromosome alignment and distribution, mouse and rabbit oocytes were fixed following the same protocol, as described previously (Costa-Borges *et al.*, 2020). Briefly, oocytes were fixed for 30 min at 37°C in a microtubule stabilizing buffer (MTSB-XF). A triple-labeling protocol was then used for the detection of microtubules, microfilaments, and chromatin by fluorescence microscopy (Messinger and Albertini, 1991). Fixed oocytes were first incubated overnight at 4°C in a mixture of mouse monoclonal anti-α/β-tubulin antibodies, and then in a mixture of secondary antibodies (chicken anti-mouse IgG) conjugated to Alexa Fluor 488 and of AlexaFluor 594 phalloidin for 3 h at 37°C. Finally, all oocytes were washed for 10 min at 37°C in Phosphase Buffered Saline (PBS) blocking solution, incubated in Hoechst 33258 for 10 min at room temperature, and put on a 1:1 PBS-glycerol mounting solution droplet on a glass slide. For the analysis of total cell counts, mouse and rabbit blastocysts were fixed in 4% (v/v) paraformaldehyde (PFA) for 20 min at room temperature and then permeabilized in 2.5% Triton-X100 for 30 min at 37°C. Afterwards, they were incubated in 10 mg/ml solution of bisbenzimide (Hoechst 33342, Sigma-Aldrich) for 10 min at room temperature, and rabbit blastocysts were directly mounted on a glass slide in a drop of mounting solution. Mouse blastocysts were additionally incubated in a TUNEL apoptosis-detection solution (*in situ* cell death detection kit, TMR red, Roche, Switzerland) for 60 min at 37°C, prior to mounting. Stained oocytes or blastocysts were examined using an epifluorescence microscope (Nikon E1000) fitted with specific filters for Hoechst, Fluorescein, and Texas Red and a 50 W mercury lamp. Digital images were acquired with E1000 Nikon software.

Embryo transfers

Mouse blastocysts graded as good quality (GQ), characterized by an expanded blastocoel cavity, a thinned zona pellucida, uniform

trophectoderm cells, and a compacted inner cell mass, from different experimental groups were selected and transferred using a surgical procedure in groups of 8–10, into the uterus of synchronized pseudo-pregnant CD1 recipients, previously mated with vasectomized mice. Following embryo transfers, recipients were allowed to deliver spontaneously. Born pups were raised by the surrogates until weaning.

Simulation of cellular volume changes in human MII oocytes

Previously estimated permeability parameters of human oocytes to ethylene glycol (EG) and dimethyl sulfoxide (DMSO) (Van den Abbeel *et al.*, 2007) CPAs were used to simulate the osmotic response of oocytes to different preparation protocols. A two-parameter (2P) transport formalism was employed to assess the flow of water and solutes across the cell membrane using coupled linear ordinary differential equations, assuming no interaction between water and permeable solutes at the membrane (Kleinhans, 1998), as detailed in our previous work (Gallardo *et al.*, 2019). Three different protocols were simulated, as follows: (i) SV/SW protocol: gradual, stepwise exposure to ES (PREQ1: 3 min of pre-equilibration in 1:1 BS + ES; PREQ2: 3 min of pre-equilibration in 1:2 BS + ES), then 6 min in ES and 1 min in VS. SW protocol in TS for 1 min, followed by 3 min in DS and 5 min in WS; (ii) SV/FW: preparation for cooling as in the previous group, but rehydration (warming) is shortened to 1 min in TS and transferred to CM; (iii) FV/FW: Both the preparation for cooling and washing after warming to 1 min each: 1 min in ES, 1 min in VS and 1 min in TS and transferred to CM. All steps of the protocol were simulated at 25°C, except for TS, which was simulated at 37°C. In the FW protocols, the osmotic behavior of the oocyte during the first minute in culture media was simulated and included in the graphs as 'CM', assuming an osmolarity of 300 mOsmoles/L, in order to understand their reaction after the FW.

In vitro oocyte osmotic behavior in human MII oocytes

To verify the accuracy of the model predictions, the *in-silico* dehydration profiles obtained with theoretical models were compared with *in vitro* observations of the osmotic behavior of human oocytes exposed to both standard and fast preparation protocols.

Statistical analysis

Sample sizes were calculated to detect a difference of 10% in survival rates with a statistical power of 85% and alpha 0.05. In embryo transfers simple sizes were estimated to detect a difference of 20% with a statistical power of 80% and alpha 0.05.

To analyze the categorical variables (e.g. survival rates, blastocyst formation, live births) a chi-squared test of independence was employed if the frequency of each group was >5 or a Fisher's exact test otherwise, with a significance level of $\alpha = 0.05$. For continuous Variables (e.g. cell counts), the normality of the data distribution was assessed using the Shapiro–Wilk test. If the data were found to have a normal distribution, a Student's *t*-test was used to compare the means of two groups, and the Mann–Whitney *U* test was used otherwise. In both cases, two-tailed hypothesis was used with a significance level of $\alpha = 0.05$ to determine statistical significance.

Results

Meiotic spindle morphology and chromosome distribution in mouse oocytes

In the first series of experiments, we aimed to evaluate the effects of the different vitrification protocols on the structural integrity of metaphase II (MII) spindle and chromosome alignment in oocytes. Results from the analysis across the different treatment groups are illustrated in Fig. 1 and shown in Supplementary Table S1). The fresh control (n = 23) and the vitrification/warming groups (n = 30–34) showed similar results with most oocytes displaying a normal barrel-shaped spindle (97.0–100%) and the chromosomes correctly aligned (94.1–100%) on the metaphase plate (Fig. 1). These findings suggest that neither standard nor the FV/FW protocols tested seem to adversely affect the meiotic spindle apparatus or chromosome distribution.

Oocyte survival and in vitro embryo developmental rates post-ICSI in mouse oocytes

Next, we assessed the survival and developmental potential of MII mouse oocytes subjected to the different vitrification and warming protocols alongside experimental controls. Overall results are detailed in Supplementary Table S2 and the methods followed are illustrated in Fig. 2. The FV/FW protocol achieved the highest survival rate (n = 249 oocytes; 97.2%), which was statistically comparable to the SV/SW protocol (n = 224 oocytes, 94.2%) and significantly higher ($P = 0.008$) than the SV/FW group (n = 229 oocytes, 91.7%). After ICSI, all groups showed similar ($P > 0.05$) two-cell formation rates (98.4–99.4%) and comparable results to the control group using fresh oocytes (n = 118, 100%). For blastocyst formation, the SV/SW group reached 83.4%, followed by the FV/FW group at 80.9%, and the SV/FW group at 75.9%, all comparable to the fresh oocyte control group (n = 123) with 86.4%. Blastocyst quality was assessed based both on morphological criteria and by performing total cell counts in the blastocysts after nuclear staining. Results showed no differences between the control group (n = 49; 130.5 ± 29.1) and the SV/SW (n = 96; 127.3 ± 28.1), SV/FW (n = 91; 130.7 ± 30.5) and FV/FW (n = 112; 120.7 ± 30.4) experimental groups (see Supplementary Table S2 and Fig. 2). Overall, all protocols tested effectively preserved oocyte viability and potential to develop *in vitro* up to the blastocyst stage after warming and ICSI.

Implantation and term developmental rates in mouse oocytes

To further assess the safety of the test protocols, blastocysts generated from oocytes vitrified using the different protocols were subjected to a second round of vitrification using the same protocol as that used for oocytes so that they could be transferred to synchronized female recipients previously mated with vasectomized males. Prior to these transfer attempts, experiments were carried out to assess the feasibility of using the FV and FW protocols separately in blastocysts generated by ICSI from fresh oocytes. Results confirmed the efficacy of the protocols, evaluating blastocyst survival and re-expansion, and the apoptotic cell index at 24 h post-warming (see Supplementary Table S3 and Supplementary Fig. S1).

After embryo transfer, recipients were allowed to deliver naturally, and the pups born were weaned and monitored until adulthood. A comparative overview of the outcome and representative photos are shown in Fig. 3. The parameters evaluated included the number of blastocysts transferred, the number of recipients

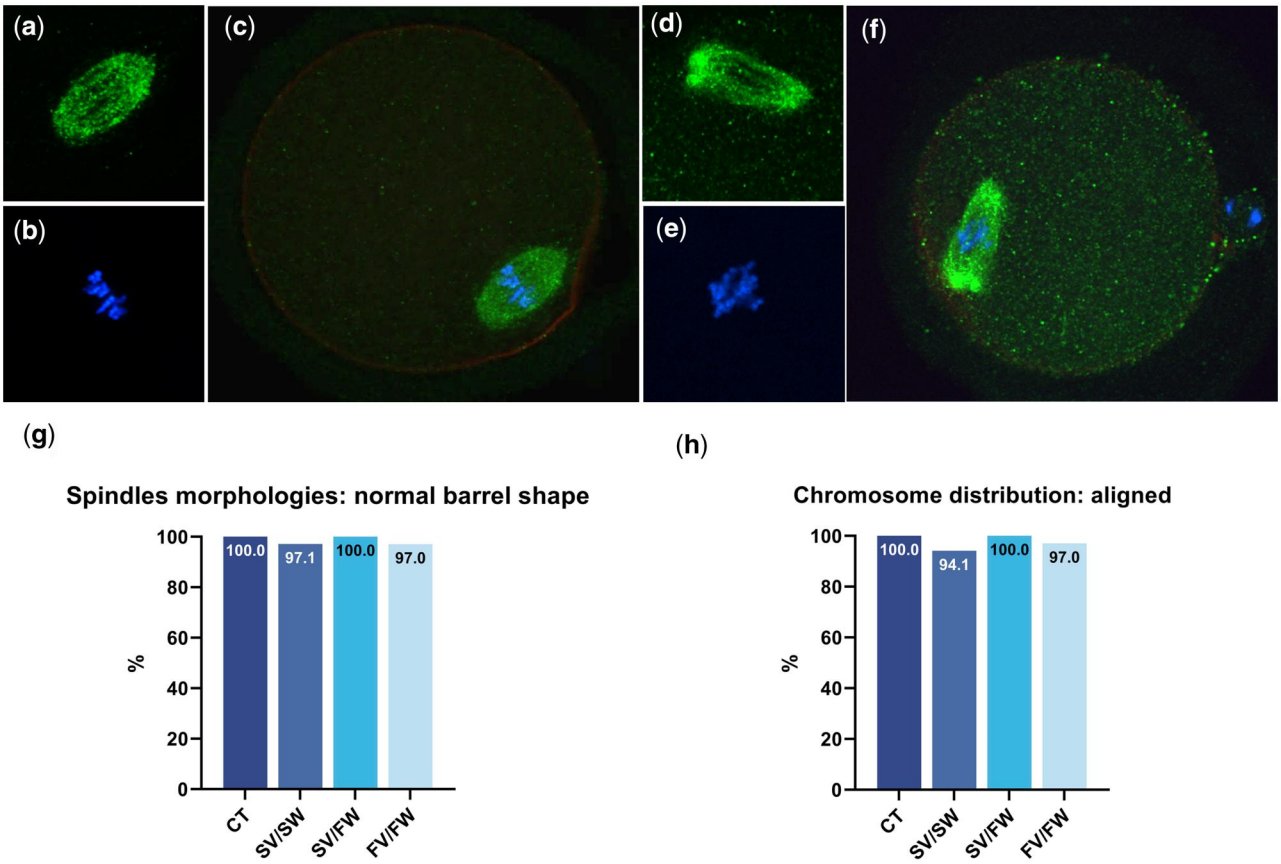

Figure 1. Representative images of the confocal analysis performed on meiotic spindles and chromosome alignment of mouse oocytes subjected to the fast vitrification and warming protocol and results comparisons. Images of a normal barrel-shaped spindle (**a**) and the correctly aligned chromosomes in the metaphase II plate (**b**), both displayed individually and once merged (**c**). An abnormal spindle (**d**) with misaligned chromosomes (**e**), both separately and merged (**f**). Graphs of meiotic spindle morphology (**g**) and chromosome alignment (**h**) comparing the results obtained with the different vitrification and warming protocols, alongside the corresponding controls. Microtubules are shown in green, chromosomes in blue, and microfilaments in red. CT: control; SV: standard vitrification; SW: standard warming; FV: fast vitrification; FW: fast warming.

used, the number of pregnancies established, and the number of live pups born. Nine embryo transfers were performed in the SV/SW, SV/FW, and FV/FW groups, while five transfers were performed in the control group with blastocysts derived from fresh oocytes vitrified with SV/SW. The control group produced 23 live pups from 47 blastocysts transferred, achieving a live birth rate of 48.9%. The SV/SW group yielded the highest number of live pups—44 out of 92 blastocysts transferred, with a live birth rate of 47.8%. The SV/FW group had a live birth rate of 43.2%, with 41 pups born from 95 blastocysts, while the FV/FW group resulted in a live birth rate of 38.7%, with 36 pups born out of the 93 blastocysts transferred. Statistical analysis revealed no significant differences between groups. All pups developed into adulthood without any observable health issues or evident abnormalities.

Oocyte survival and in vitro embryo developmental rates post-ICSI in rabbit

Overall results of survival and *in vitro* developmental rates of rabbit MII oocytes subjected to various vitrification and warming protocols are illustrated in Fig. 4 and shown in Supplementary Table S4. Survival rates ranged from 90% to 100%, with no statistically significant differences among the groups. After insemination by ICSI, 87% of the fresh oocytes in the control group cleaved, while the two-cell developmental rates in the vitrified groups were 63.0% for the SF/SW group, 77.3% for the SV/FW group, and 76.2% for the FV/FW group. In terms of blastocyst development, the control group with fresh oocytes achieved a 47.8% development rate. By comparison, the SV/FW group showed a significantly lower rate of 13.6%. The blastocyst developmental rates for the SV/SW and FV/FW groups were 22.2% and 28.6%, respectively. The average total cell count in blastocysts ranged from 331.7 ± 166.7 to 356.7 ± 96.5 across these groups, which was comparable to the control group's count of 207.8 ± 106.3 (see Fig. 4 and Supplementary Table S4).

Membrane permeability model and in vitro osmotic behavior in human MII oocytes

The simulation of the volumetric excursion of the human oocytes subjected to the control protocol and the two experimental protocols is shown in Fig. 5. In the SV/SW protocol, during the dehydration phase, the oocyte is exposed gradually to ES, for a total duration of 12 min. During the two pre-equilibration steps, and the exposure to ES, it is possible to observe a similar pattern characterized by a rapid shrinking of the cytoplasm followed by a gradual swelling back toward its isotonic volume, as the cytoplasm regains osmotic equilibrium with the CPA solution. The minimum relative volume attained in this phase is 61.8% at the pre-equilibration phase 1 ($t = 49$ s). At the end of the equilibration stage ($t = 12$ min), the oocyte is exposed to VS for 1 min, where a fast shrinking due to water loss is predicted, reaching a minimum volume of 50.7% of its isotonic state. During warming in TS at 37 °C, the volume of the oocyte increases up to a maximum of 94.3% of original volume (Fig. 5). In the next solution, DS, the oocyte's volume steadily decreases, reaching a minimum at the

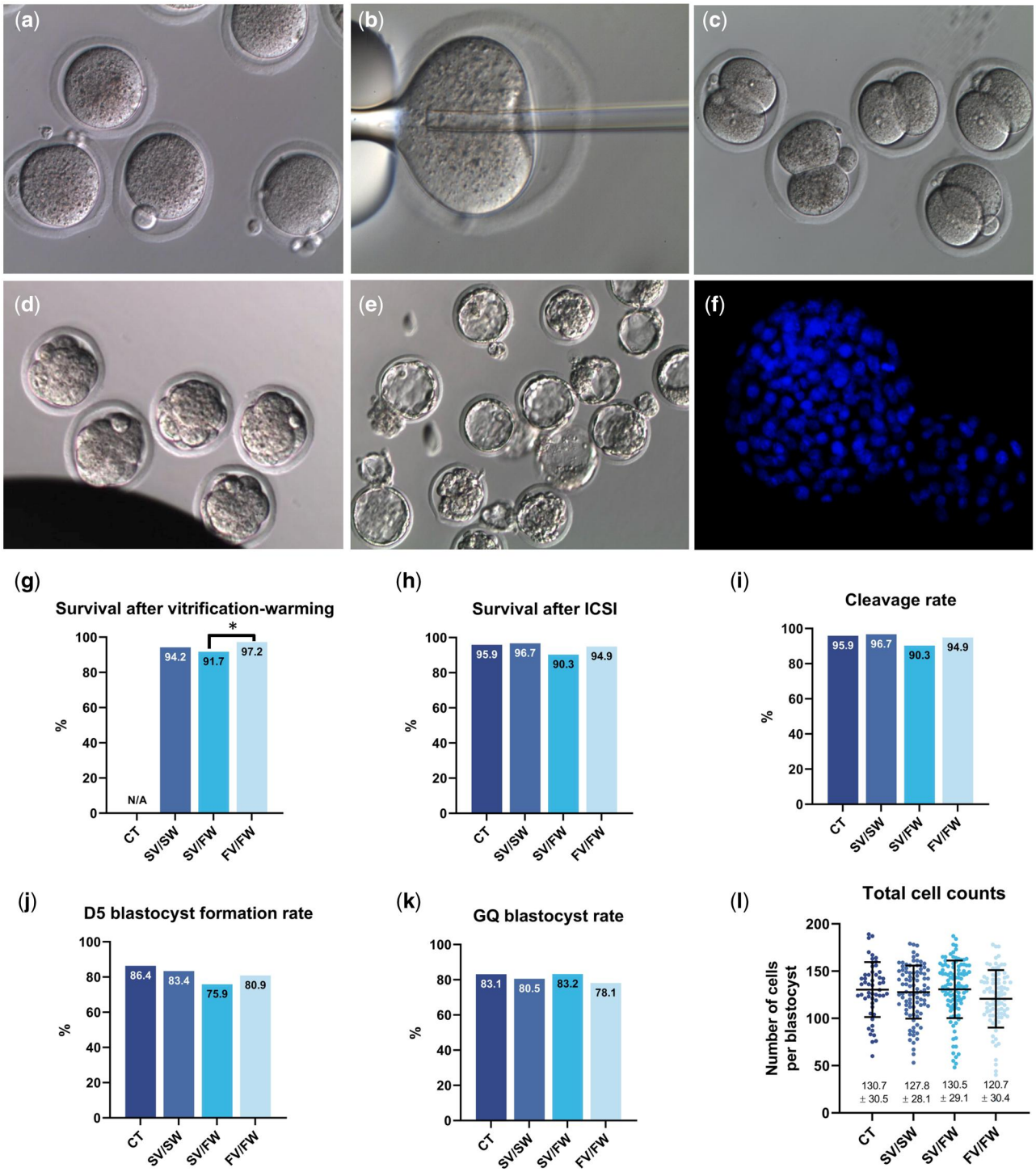

Figure 2. Representative images of the evaluations conducted to assess the survival and in vitro developmental potential of mouse oocytes, comparing outcomes across different experimental groups. Images of mouse oocytes assessed for survival 2 h post-warming (**a**), followed by insemination by ICSI (**b**), with subsequent evaluation of embryo development stages: two-cell (**c**), morula (**d**), and blastocyst formation rates (**e**). An image of a blastocyst (**f**) generated with the fast vitrification and warming protocol is shown with nuclei stained in blue to assess the total cell number. Graphs display comparative analyses of survival after warming (**g**), ICSI success (**h**), two-cell formation (**i**), morula development (**j**), blastocyst formation rates (**k**), and the average cell count per blastocyst (**l**) across different vitrification and warming protocols, and their respective controls. CT: control; SV: standard vitrification; SW: standard warming; FV: fast vitrification; FW: fast warming.

end of the exposure of 51.4% of original volume. When moved to WS, the oocyte gradually recovers its relative volume, finishing this stage at a maximum of 91.9% of original volume. It would be expected that the oocyte regains its full isotonic volume at a later stage when cultured in standard culture media. In the SV/FW protocol, all steps up to TS are identical. The relative volume of the oocyte after 1 min in TS is at 87.0% (Fig. 5). Skipping the 3 min of DS and placing the oocytes directly in CM results in over-swelling to 118.2%. In the FV/FW protocol, the oocyte Is directly exposed to full strength ES for 1 min, shrinking rapidly to a

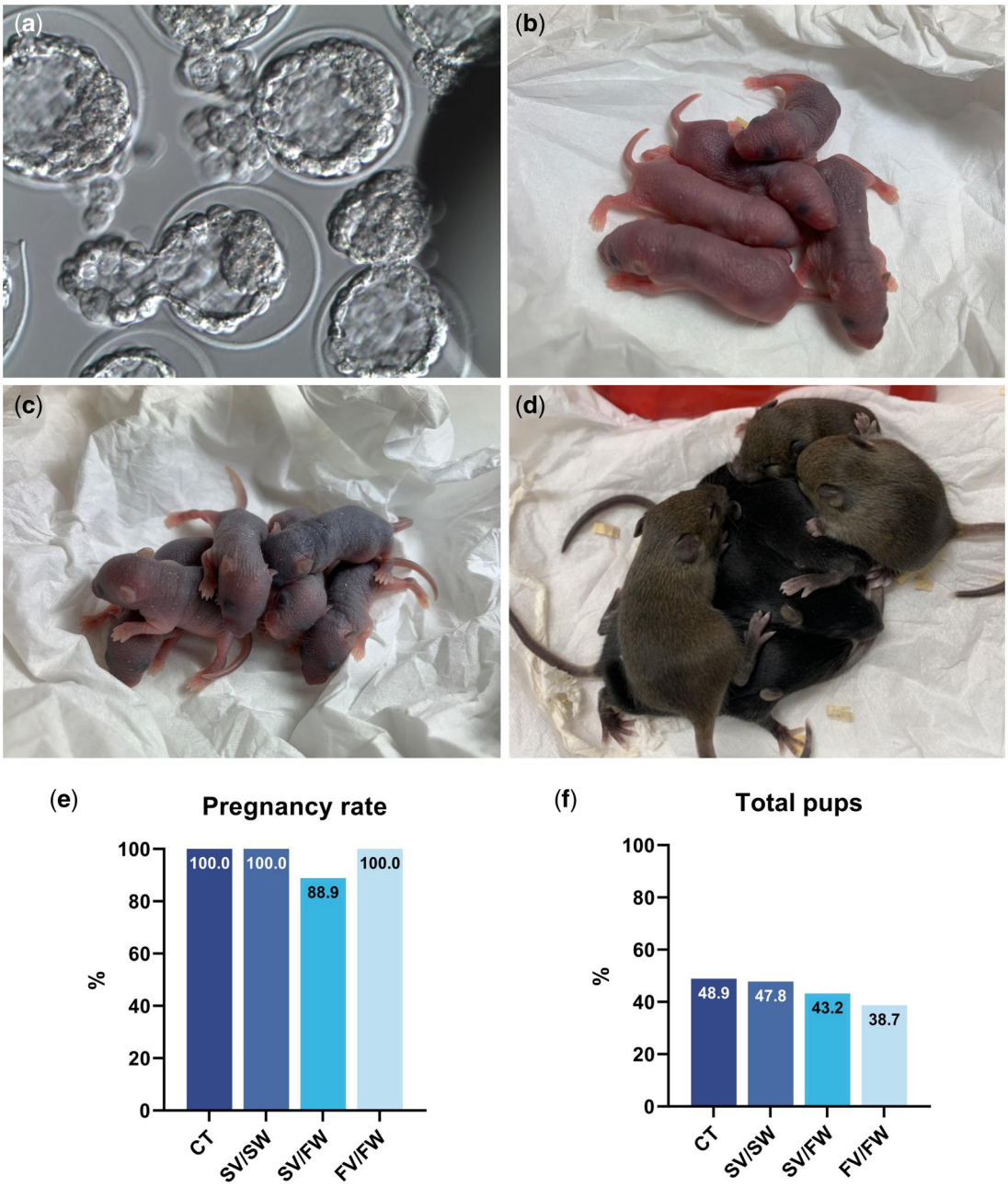

Figure 3. Representative images of the evaluation of mouse blastocyst potential to develop to term and the outcomes after embryo transfer, and comparison of outcomes across the different experimental groups. (**a**) Blastocysts vitrified using the FV/FW protocols. (**b**) Mice generated from these transferred blastocysts at 2-, 5-, and 15-day post-birth (**c–d**). Comparative graphs illustrate pregnancy rates (**e**) and total pup numbers (**f**) across experimental groups in the mouse model, along with overall results. CT: control; SV: standard vitrification; SW: standard warming; FV: fast vitrification; FW: fast warming.

minimum volume of 47.4% at $t = 28$ s, and recovering to 51.9% of relative volume prior to the subsequent exposure to VS, where it undergoes further dehydration, and consequently, shrinkage to 37.6% of its isotonic volume, recovering to 41.0% at the time of cooling (Fig. 5a). During FW in TS, the oocyte is predicted to recover only 55.3% of its isotonic volume at $t = 2$ min 11 s (11 s in TS). When transferred to CM, the volume is expected to achieve 67.1% at the end of 1 min exposure (Fig. 5).

Survival rates in human MII oocytes

To optimize sample use and given that SV and SW protocols are well established for human oocytes, survival rates in these experiments were evaluated at 2- and 24-h post-warming, specifically comparing the SV/FW and FV/FW protocols. In the SV/FW group (n = 101 oocytes), the survival rate was 97% at 2 h and 94.1% at 24 h post-warming. In the FV/FW group, which comprised 103 oocytes, the survival rate at 2 h was 100%, and 97.1% at 24-h post-warming. Statistical analysis indicated that the survival rates at the two time points were comparable ($P > 0.05$) between the two vitrification and warming protocols. This finding suggests that both methods were effective in preserving oocyte viability after maturation. Overall, this study demonstrates that both the SV/FW and FV/FW protocols yield similar survival rates for MII human oocytes.

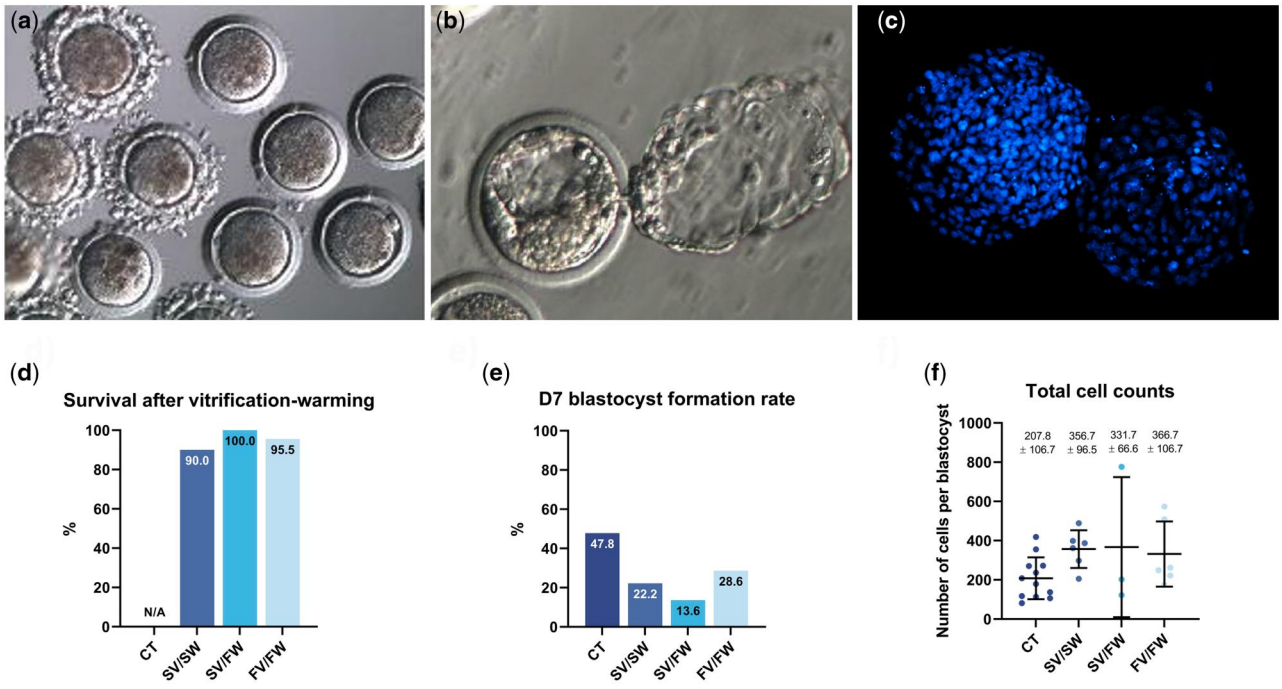

Figure 4. Representative images of evaluations conducted to assess the survival and in vitro developmental potential of rabbit oocytes, comparing outcomes across different experimental groups. Rabbit oocytes assessed for survival 2 h post-warming (**a**) and a Day 7 hatching blastocyst obtained from FV/FW after ICSI (b). An image of the same blastocyst with nuclei stained in blue to assess its total cell number (**c**). Graphs display comparative analyses of survival after warming (**d**), blastocyst formation rates (**e**), and the average cell count per blastocyst (**f**) across different vitrification and warming protocols, and their respective controls. CT: control; SV: standard vitrification; SW: standard warming; FV: fast vitrification; FW: fast warming.

Discussion

Vitrification has become the standard procedure for cryopreserving oocytes in most IVF centers (Cobo *et al.*, 2021). While several commercial protocols exist, they all share a common principle based on the sequential exposure of oocytes to increasing concentrations of CPAs (De Munck and Vajta, 2017). This process can be both time-consuming and labor-intensive for embryologists, especially in high-demand IVF laboratories. The extended exposure of oocytes to non-physiological conditions such as high osmolality, room temperature, and the high concentration of permeant CPAs, also raises concerns about the potential negative effects on oocyte viability and developmental potential (Chang *et al.*, 2022). Therefore, the development of faster vitrification and warming protocols is crucial to improve both the efficiency and laboratory workflow.

The results from our study offer valuable insights into the feasibility of reducing vitrification and warming times without compromising oocyte integrity or developmental potential in the animal models tested. Using both mouse and rabbit oocytes, we demonstrated that these protocols effectively maintained oocyte morphology, meiotic spindle integrity, and chromosome distribution, key factors for successful fertilization and embryo development. This concept of shortening exposure to VS and WS aligns with previous findings that demonstrated the feasibility of reducing time spent in the dehydration phase in human oocytes (Gallardo *et al.*, 2019; Liebermann *et al.*, 2024b; Schiewe *et al.*, 2024; Wozniak *et al.*, 2024) and the rehydration phase in cryopreserved human blastocysts without adverse effects on survival and/or clinical outcomes (Manns *et al.*, 2021; Liebermann *et al.*, 2024b). Unlike earlier studies, which primarily evaluated survival rates and meiotic resumption post-warming using discarded immature human oocytes (Liebermann *et al.*, 2024b), our research expands on these findings by assessing fertilization and developmental competence in animal models. This comprehensive approach is essential for fully understanding the impact of fast protocols before clinical application. To further evaluate the safety and efficacy of these protocols, we conducted blastocyst transfers in mice. The results showed developmental rates comparable to the standard method, with all mice developing to adulthood without health issues or signs of abnormalities. However, the offspring rates were lower in the FV/FW group compared to the control group using SV and SW protocols. This difference may reflect biological variability, sample size limitations, or procedural nuances, particularly associated with the fact the blastocysts used in transfer procedures were vitrified using the same fast protocol as in oocytes. Further research is warranted to investigate this trend. Additionally, we developed a theoretical model to simulate the osmotic response of human oocytes during exposure to the CPA solutions. Our model predicts that the FV protocol, which exposes oocytes for 1 min to ES and 1 min to VS, results in shrinkage to 37.6% of its isotonic volume, below the 50.7% attained in the conventional vitrification protocol. While a 50% shrinkage tolerance limit has been historically referenced as ideal (Newton *et al.*, 1999; Mullen *et al.*, 2004; Paynter *et al.*, 2005), the results from our murine and rabbit validation experiments support the notion that lower levels of shrinkage in oocytes during vitrification can be tolerated without compromising their viability. Also, although the intracellular osmolarity is predicted to be equivalent after standard and reduced exposure times, the fast protocol yields a lower intracellular free water content, which may provide more consistent results (Gallardo *et al.*, 2019). When the FV protocol is followed by FW, the rehydration profile does not show any signs of over-swelling for the simulated timeframe. On the other hand, SV followed by FW does show in the osmotic model a risk of over-swelling—attaining over 100% of its isotonic volume—when transferred to culture media. However,

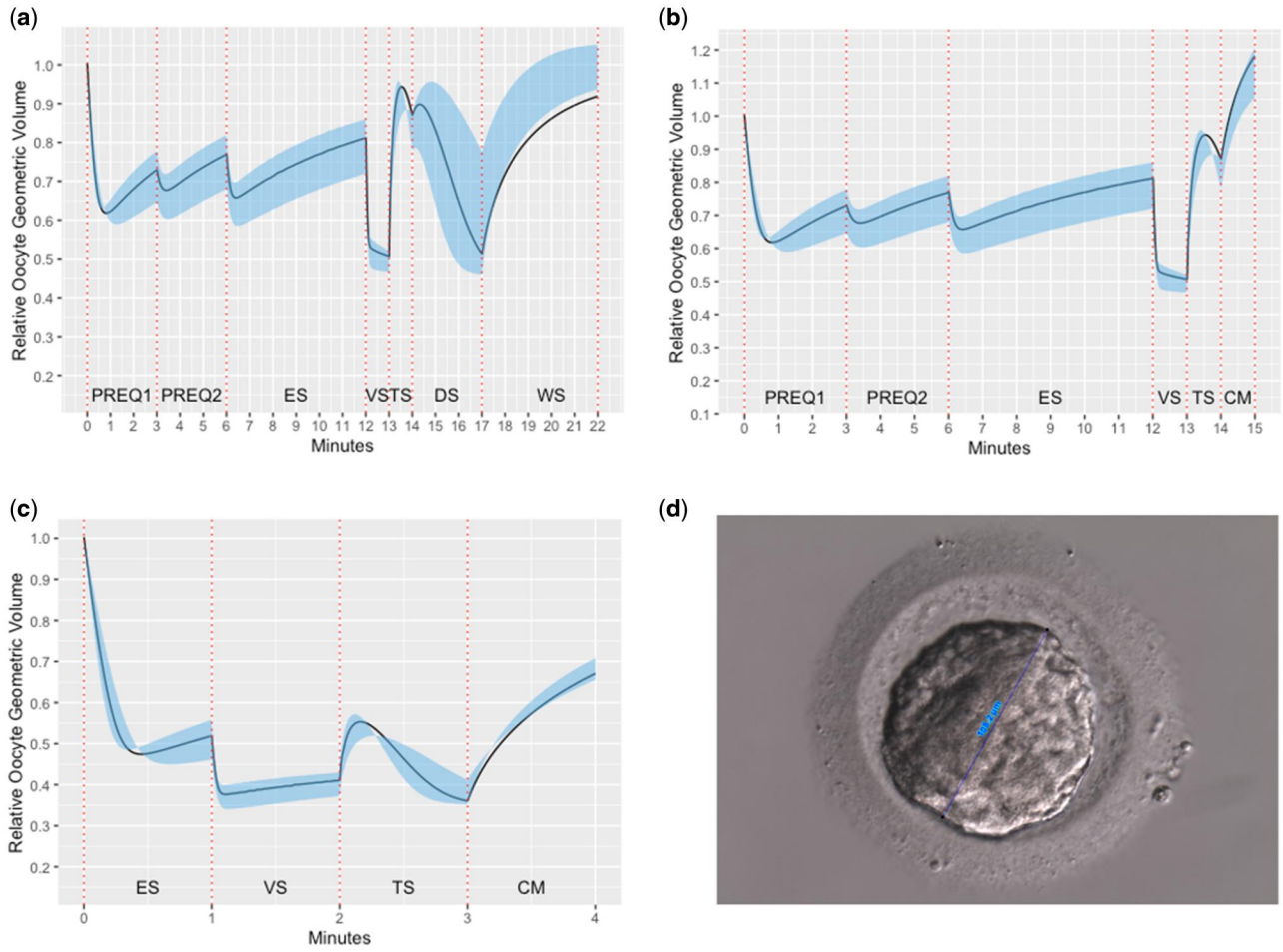

Figure 5. Simulations of the expected osmotic behavior of a human oocyte when exposed to three different cryoprotectant solution protocols (a–c). The *y*-axis in each graph (**a–c**) represents the relative geometric volume of the oocyte where shrinkage and swelling reflect the degree of dehydration, which correlates with osmolarity. The red dotted lines indicate the transition points between different cryoprotectant solutions. The black line shows the average expected values, while the blue ribbon represents the standard deviation. Note that in some regions of the graph, the expected values (black line) fall over the standard deviation range, due to the curvilinear relationship beween permeability to water and CPAs and the oocyte's osmotic behaviour. All simulations conducted at 25°C except for the TS step, which is at 37°C. Panel (**a**) illustrates the response under the standard vitrification and standard warming (SV/SW) protocol, panel (**b**) represents the standard vitrification and fast warming (SV/FW) protocol, and panel (**c**) depicts the fast vitrification and fast warming (FV/FW) protocol. Panel (**d**) illustrates the morphological appearance of a human oocyte after the 60 s exposure to the ES solution in the fast vitrification protocol, right before transfer to VS solution. PREQ1: pre-equilibration solution 1, (1:1 BS+ES); PREQ2: pre-equilibration solution 2, (1:2 BS+ES); ES: equilibration solution; VS: vitrification solution; TS: thawing solution; DS: dilution solution; WS: washing solution; CM: culture media.

again no detrimental effect was observed in the preclinical validation of this combination of protocols. This finding is consistent with previous research suggesting that oocytes can withstand significant osmotic changes if these alterations occur gradually and under physiological temperatures (McWilliams *et al.*, 1995; García-Martínez *et al.*, 2021). Although the osmotic shrinkage tolerance threshold for human oocytes remains to be fully established, our findings indicate that the reduced exposure times in fast protocols do not negatively impact oocyte survival.

Finally, while this study focused on oocytes, the application of rapid vitrification and warming protocols to human blastocysts warrants further investigation. Current blastocyst vitrification protocols often adapt oocyte methods empirically. Preliminary post-warming findings from shortened blastocyst FV and FW protocols suggest that these streamlined approaches hold potential for universal protocols across different developmental stages.

In conclusion, this study provides a comprehensive validation across different animal models, compelling evidence that rapid vitrification and warming protocols can preserve oocyte viability and developmental potential, while significantly reducing the time required for these procedures. Vitrification time was reduced from 12–15 min to 2 min and warming time from 10 min to 1 min. These fast protocols can not only enhance workflow efficiency in IVF laboratories but also minimize oocyte exposure to potentially harmful CPAs and non-physiological conditions. Both the theoretical model and experimental data support their feasibility; however, clinical trials with larger cohorts of sibling human oocytes to assess clinical outcomes such as fertilization, embryo development, clinical pregnancy, and live birth rates, as well as multi-center studies to evaluate reproducibility and feasibility in diverse patient populations, are essential to confirm their suitability for routine clinical practice. Further research is also needed to assess the effects of rapid vitrification on blastocyst

development, including embryos at various stages. Ultimately, these protocols could represent a significant advancement in oocyte cryopreservation, offering logistical benefits and improved outcomes for IVF patients.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Authors' roles

N.C.-B., A.F.-S.F., A.Cobo, A.C.-B., F.M.-J., and J.C. conceived and designed the study. N.C.-B., Q.M.-A., E.M., M.A., C.C., and F.M.-J. conducted the experiments in mouse and rabbit models. M.G. developed the mathematical *in silico* osmotic model. A.Coello and A. Cobo carried out experiments involving human oocytes. N.C.-B., Q.M.-A., E.M., and M.A. analyzed the data and performed the statistical analysis. N.C.-B. wrote the manuscript and prepared the figures. All authors reviewed and revised the manuscript before submission.

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Conflict of interest

N.C.-B. reports stock with Embryotools and stock options with Conceivable Life Sciences and Fertility. N.C.-B. is also salaried by Embryotools. A.C.-B. received support for attending meetings from Conceivable Life Sciences and holds patents and stock with Conceivable Life Sciences, and stock with Hope IVF Mexico and IVF 2.0. A.F.-S.F. has received travel support from Conceivable Life Sciences and has patents with them as well as stock options. A.C. is salaried by IVIRMA. J.C. is salaried by Conceivable Life Sciences, holds stock options with Conceivable Life Sciences, IVF 2.0 and Athea Science and has other financial/non-financial interests with Reproduction Healthcare and TMRW Life Science. The other authors have no conflicts of interest to declare.

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