Effect of slow freeze versus vitrification on the oocyte: an animal model

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Objective: To determine whether there is a deleterious effect on dynamic events in the nucleus and cytoplasm of oocytes by using different cryopreservation protocols in an animal model.
Design: Prospective study.
Setting: University hospitals.
Patient(s): Not applicable.
Intervention(s): Immunostaining and confocal laser scanning microscope techniques were used.
Main Outcome Measure(s): The spindle and chromosomal configurations, as well as dynamic changes of the cortical granules (CGs) and mitochondria in different cryogroups.
Result(s): After thawing/warming of bovine oocytes, CGs became more dispersed in the cytoplasm, particularly in the DMSO group. A significant reduction in normal spindle and chromosomal configurations was observed in all three cryogroups, particularly in the propylene glycol (PROH) group, when compared with the fresh group. Global DNA methylation levels were significantly reduced in the slow and DMSO groups, as compared with the fresh group; however, methylation levels were significantly increased in the PROH group. The proportion of severely apoptotic oocytes was dramatically increased in all three cryogroups, compared with the fresh group.
Conclusion(s): Overall, results demonstrate that using DMSO as the cryoprotectant is better for preserving the cellular and nuclear integrity of the oocyte. The PROH method makes the oocyte more vulnerable to increased DNA methylation, which may be associated with imprinting gene alteration. This study adds to the increasing body of evidence that cryopreservation protocols vary in their impact upon the oocyte. (Fertil Steril® 2012;98:752–60. ©2012 by American Society for Reproductive Medicine.)
Key Words: Slow freeze, vitrification, CGs, mitochondria, spindle, DNA methylation, apoptosis

Oocyte cryopreservation holds great potential in human assisted reproductive technology (ART) as a tool for keeping surplus oocytes and preserving female fertility. Oocyte survival rates after cryopreservation are affected by both morphological and biophysical factors (1). Successful oocyte cryopreservation has been achieved in several species, including mouse (2), goat (3), human (4), swine (5), cattle (6, 7), and buffalo (8). For more than two decades, however, attempts to cryopreserve human oocytes have been discouraged by inadequate methodologies, consequently resulting in poor clinical outcomes or uncertain usability (9–11).

This lack of success is due to the fact that the metaphase II (MII) spindle can be irreversibly disrupted by low-temperature storage (12–14). Oocyte viability may be jeopardized by the formation of intracellular ice crystals,

Received March 22, 2012; revised and accepted May 23, 2012; published online July 3, 2012.
W.H. has nothing to disclose. D.M. has nothing to disclose. J.Q. has nothing to disclose. H.L.F. has nothing to disclose.
This work was selected as an oral presentation at 2011 American Society for Reproductive Medicine Annual Meeting, which is supported by grants from the Overseas Outstanding Young Scientist Project (NSFC31128013/C120205; to H.L.F. and J.Q.); and the Academic Research Project (GRTR-156) from Ferring Pharmaceuticals, New Jersey (to H.L.F.), and from MERCK (to H.L.F.).
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Fertility and Sterility® Vol. 98, No. 3, September 2012 0015-0282/$36.00

doi:10.1016/j.fertnstert.2012.05.037

osmotic shock, or the replacement of intracellular water with cryoprotectants (15). Different cryopreservation protocols vary in oocyte survival, fertilization, and embryo development rates. Previous experience with oocyte cryopreservation demonstrates that slow freezing methods result in very low survival and pregnancy rates after thawing (16–18), while vitrification methods increase survival and pregnancy rates in the patients who underwent IVF treatment (19–22).

The two protocols have been widely used clinically (23), but no detailed information is known about their ability to preserve the cellular organization of the oocyte (11), particularly with respect to the possible deleterious effects of different cryoprotectants on the dynamic events occurring within the nucleus and cytoplasm of oocytes.

MATERIALS AND METHODS

In Vitro Culture

A total of 6,000 bovine cumulus-oocyte complexes (COCs) were purchased from Applied Reproductive Technology. Experiments were designed according to current, commonly used ovarian stimulation protocols and gonadotropin combinations in human ART clinics. The basic maturation medium was composed of tissue culture medium 199 (TCM-199; Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma), 25 mM sodium pyruvate, 50 IU/mL penicillin, 50 μg/mL streptomycin, 75 mIU/mL Bravelle, and 75 mIU/mL Menopur (Ferring). In vitro–matured (IVM) oocytes were washed 3 times in the holding medium (TCM-199 supplemented with 10% FBS) and then cultured in 4-well microplates with 500 μL basic maturation medium under 500 μL mineral oil for 24 hours in a humidified 38.5°C incubator with 5% CO_2. After in vitro IVM, COCs were washed for twice in a petri dish containing Hepes-buffered TCM-199 supplemented with 10% FBS, and cumulus cells were partially removed from COCs during gently pipetting for washing. After washing, COCs were randomly allotted into different cryopreservation methods and cryoprotectants: slow freezing, DMSO vitrification, and propylene glycol (PROH) vitrification, respectively. For each group, IVM fresh was set as the control.

Slow Freezing and Thawing Methods

IVM oocytes were washed twice with phosphate-buffered saline (PBS; Sigma) containing 10% FBS, which also served as the carrier medium for cryoprotectants. Oocytes were introduced to a series of solutions that include cryoprotectants (Propanediol, PROH; Sigma) at 0.5∼1.5 M and 0.3 M sucrose (Sigma). Exposure to the final concentration (1.5 M of PROH) was performed in a three-step (0.5, 1.0, and 1.5 M) manner in 5-minute intervals (12, 17, 23), at room temperature (23–28°C). Oocyte equilibration in the final concentration of PROH plus 0.3 M sucrose was allowed for another 5 minutes at room temperature.

Groups of 10–20 oocytes were loaded into 0.25 mL straws (IMV Technologies France) and subjected to slow freezing in a programmable freezer (Planer-III; Planer Freezer, Planer Co.). Briefly, straws were cooled to −7°C at a rate of −2°C/minute. Manual seeding was performed at −7°C; this temperature was maintained for 10 minutes to allow for uniform ice propagation. Cooling then proceeded at a rate of −3°C/minute to −30°C and then at a rate of −50°C/minute to −150°C (12, 17). Finally, straws were plunged into liquid nitrogen.

After storage in liquid nitrogen for at least 2 days, straws were warmed rapidly by plunging them into a 37°C water bath for 3 minutes. The contents of the straw were then drained into a sterile 4-well microplate (Nunc A/S). Dilution of the cryoprotectants was also performed in a three-step (1.0 and 0.5 M PROH plus 0.3 M sucrose, 0.3 M sucrose) manner.

Vitrification and Warming

Vitrification and equilibration solutions were prepared in the base medium (PBS + 20% FBS + 0.5 M sucrose). Oocytes were exposed to the final concentration of cryoprotectant in a three-step manner by equilibrating oocytes serially in wash solution (PBS + 20% FBS) for 2 minutes, then in equilibration solution (7.5% ethylene glycol [EG] + 7.5% DMSO or 7.5% EG + 7.5% PROH, respectively) for 8–10 minutes, before exposing them to the final vitrification solution (VS) either 15% EG + 15% DMSO (Sigma) or 15% EG + 15% PROH (Sigma) (4–6, 17). Groups of approximately three to five oocytes in VS (1∼3 μL; 15% EG + 15% DMSO or 15% EG + 15% PROH) were then loaded into 0.25 mL straws (IMV Technologies France) (7) with a top inclined cut. Straws were plunged into liquid nitrogen within 1 minute of loading and stored for at least 2 days.

For warming, straws were rapidly dipped into prewarmed thawing solution (1.0 M sucrose in base medium +20% FBS) at 37°C for 1 minute, and the vitrified-warmed oocytes were placed in a three-step warming solution (0.5 and 0.25 M sucrose and culture medium plus 20% FBS), with 3 minutes of equilibration in each step (4–6). Oocytes were then washed 2 times with culture medium plus 20% FBS for 3 minutes each.

Evaluation of Oocyte Viability

Oocytes were also assessed for viability based on oolemma integrity by propidium iodide (PI) and Hoechst staining. For these purposes, oocytes were stained with PI (50 μg/mL) and Hoechst 33342 (10 μg/mL) for 10 minutes and then washed and observed under fluorescence microscopy. Dead cells exuded red fluorescence (PI-positive), indicating a disruption of the cellular membrane, while viable cells showed blue fluorescence without red (PI-negative), indicating an intact cell membrane (24).

Assessment of Nuclear Maturation

The nuclear status of oocytes was evaluated by staining with PI (50 μg/mL). The meiotic stage of IVM oocytes was classified as described elsewhere (10): immature (germinal vesicle [GV] and GV breakdown stage), intermediate (mI and anaphase I), and matured (telophase I and mII). After IVM, oocytes were analyzed in this study; the total oocyte maturation rate was found to be 63.4% ± 2.1% after three rounds of IVM.