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Modeling the Probability of MII Spindle Disruption in Bovine Oocytes as a Function of Total Osmolality Using Logistic Regression and Its Application toward Improved CPA Addition and Removal Procedures

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ABSTRACT

During cryopreservation, cells undergo volume excursions as a result of exposure to anisosmotic solutions containing cryoprotective agents. Cryopreservation methods can be engineered to maintain cell volumes within their osmotic tolerance limits. In this study we investigated the osmotic tolerance limits of the bovine oocyte oolemma and metaphase II spindle. Oocytes were exposed to solutions (75–3000 milliosmolal) for 10 min, then cultured for 1 h. Oolemma integrity was assessed visually prior to fixation. The structure of the metaphase II spindle was assessed using immunocytochemistry and laser scanning confocal microscopy. Logistic regression was used to model the probability of spindle disruption as a function of solution osmolality. Few cells experienced oolemma rupture or spindle disorganization between 200 and 1800 milliosmolal. Higher levels of osmotic stress caused rupture of the oolemma and disruption of the spindle structure. These results can be applied to improve methods for the addition and removal of cryoprotective agents that will protect the spindle from osmotic damage.

INTRODUCTION

RESEARCH conducted over the past two decades has demonstrated the susceptibility of mammalian oocytes to freezing injury.[1] Upon final maturation, oocytes from most mammalian species are arrested at the metaphase stage of the second meiotic division, with the metaphase II (MII) spindle present in the ooplasm.[2] There are numerous reports in the literature which document the detrimental effects of cold exposure on the spindle structure in oocytes from various organisms including human,[3] rhesus macaque,[4] bovine,[5] ovine,[6] caprine,[7] porcine,[8] and murine.[9] In addition to chilling, exposure to cryoprotective agents (CPAs) such as dimethylsulfoxide (DMSO), 1,2-propanediol (PG), and ethylene glycol (EG) has been shown to disrupt the spindle structure in mouse, bovine, and human oocytes.[10–14] In the mouse oocyte it has been shown that this disruption is repairable to a high degree after return to normal culture conditions.[15] Unfortunately, this is not the case for all species.[5,16] This characteristic may partially explain the ability to cryopreserve mouse oocytes at higher rates than oocytes from other species.[17] This difference makes the mouse a relatively poor model for mammalian oocyte cryobiology. The fidelity of chromosome reduction is essential to the developmental potential of mammalian oocytes. For example, aneuploidy results in developmental failure in the majority of instances in humans,[18] and aneuploid embryos have been shown to result from cryopreservation of mature mouse oocytes.[19,20] Therefore, it is es-

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sential to maintain the integrity of this structure during cryopreservation to preserve the developmental viability of the oocyte.

Damage resulting in loss of viability can occur at any stage in the cryopreservation process, and often the cells are susceptible to different types of damage during the various stages.[21] Damage as a result of exposure to the CPAs can occur from the chemical nature of the compounds (true chemical toxicity) as well as from the anisosmotic nature of the solutions.[22] In most instances, CPAs need to be present at high concentrations, typically greater than 1 molar, to afford their protection. Thus the concentration of solutes in these solutions (typically $>$ 1500 milliosmolal [mOsm]) is much higher than in physiologic medium (approximately 290 mOsm). As a consequence of exposure to such a solution, the cell will initially undergo exosmosis and a concomitant reduction in volume as a result of the difference between the intra- and extracellular total solute concentration. However, with a permeating CPA such as DMSO, the cell volume will eventually increase as the CPA enters the cell and water follows to maintain its chemical potential. A similar volume change occurs during CPA removal, but with initial cell swelling as water first enters the cell and subsequently CPA and water exits. The magnitude of the volume excursions is dependent upon the permeability of the cell membrane to water and the CPA and their activation energies.[23]

Many cell types have limited tolerance for changes in cell volume, with increasing volume changes causing additional loss of cell viability.[24–28] Thus, knowing the tolerable volume excursions (often referred to as the "osmotic tolerance limits") is essential for optimizing cryopreservation procedures. Many investigations into cell cryopreservation use methods for CPA addition and removal that are empirically derived, best guess approaches. However, one can define the osmotic tolerance limits by assessing cell viability in relation to cell volume changes[24–27,29,30] and use this information to design optimal methods for CPA addition and removal. Using such a fundamental approach, significant improvements in the cryopreservation of important cell types has been achieved.[31,32]

In preliminary investigations, it was determined that the MII spindle is more sensitive to anisosmotic exposure than the oolemma. Thus, the goal of the present study was to define the osmotic tolerance limits of bovine oocytes as measured by the maintenance of a morphologically normal MII spindle. To achieve our goal, we designed an experiment to test the hypothesis that the proportion of oocytes which maintain a normal MII spindle structure is related to the concentration of hypo- or hyperosmotic non-permeating solute solutions to which they are exposed. We then developed a probability model for spindle disruption using the logistic regression analysis. Defining this relationship will allow the design of methods for CPA addition and removal which will reduce osmotic damage to the MII spindle in bovine oocytes and should lead to improvements in bovine oocyte cryopreservation.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise indicated.

Source and handling of oocytes

In vitro matured bovine oocytes were obtained commercially (BOMED, Inc., Madison, WI). Care was taken to keep all of the solutions near 39°C throughout the experiment. Upon completion of maturation (22–24 h), the cumulus oocyte complexes (COCs) were transferred to a HEPES buffered Tyrodes Lactate solution (TL-HEPES) containing 3 mg per mL, bovine serum albumin (BSA)[33] supplemented with hyaluronidase at a concentration of 5 mg ($\sim$5000 units) per mL. The COCs were held in this solution for 3 min, and subsequently vortexed for an additional 2 min to remove the cumulus cells. After vortexing, the oocytes were washed through three, 2-mL solutions of TL-HEPES. After the final wash, oocytes with a size nearly filling the space within the zona pellucida and possessing dark, evenly granulated cytoplasm were selected for inclusion into the

experiment. During spindle assessment, maturation to the MII stage was confirmed by the presence of chromatin from the first polar body. Upon selection for inclusion into the experiment, the oocytes were segregated from the remaining oocytes and held in TL-HEPES until the experiment commenced (within 10 min). The remaining oocytes were placed into a 200-μL drop of bovine IVF medium,[34] which had been equilibrated overnight in an incubator at 5% CO_2 and 39°C, and returned to the incubator for the duration of the experiment. These oocytes were subsequently fixed and used as positive controls for the staining assay.

Anisosmotic solutions

Anisosmotic solutions consisted of sucrose as the extracellular, impermeable osmolyte supplemented with 0.23 mM sodium pyruvate, dissolved in commercial embryo culture grade water. Solution osmolality was determined using a vapor pressure osmometer (VAPRO 5520, Wescor Inc., Logan, UT). Solutions with osmolalities of 75, 150, 200, 600, 900, 1200, 1500, 1800, 2100, 2400, 2700, and 3000 ($\pm$5) mOsm were used. An isotonic treatment of 280 ($\pm$5) mOsm was also included (TL-HEPES). All solutions contained BSA at a concentration of 3 mg per mL.

Oocyte fixation and immunocytochemistry

Oocytes were fixed for 6–7 min in cold methanol (approximately -20°C) containing 5mM EGTA.[35] They were then transferred to an immunocytochemical blocking buffer (PBS + 0.2% Triton X-100, 0.1% saponin, 130 mM glycine, 3mM sodium azide, 2% BSA, and 5% horse serum (horse serum was obtained from Gibco BRL, Grand Island, NY) and held up to 7 days at 4°C prior to staining. The remaining procedures were conducted at room temperature unless otherwise noted. The cells were transferred to a solution of the blocking buffer containing an anti-α-tubulin monoclonal antibody (clone DM1A) at a final concentration of $\sim$2.5 μg/mL, and incubated overnight at 4°C. They were subsequently washed in approximately 2 mL of blocking buffer without antibody for 2 h, and then transferred to approximately 2 mL of blocking buffer which contained the secondary antibody (donkey anti-mouse IgG conjugated to Texas-Red®, Jackson Immunoresearch, West Grove, PA) at a final concentration of $\sim$1 μg/ml and held for 1–2 h. Finally, the cells were transferred to approximately 2 mL of blocking buffer without antibody and washed for 30–60 min. Upon completion of the final wash, the oocytes were mounted on microscope slides using ProLong® antifade reagent which contained SYTOX® green (1 μM final concentration) to counterstain the chromosomes (Molecular Probes, Eugene, OR).

Assessment of the oolemma and MII spindle

Lysis of the oolemma was evident by a change in the color of the cytoplasm and loss of a sharply defined membrane boundary inside of the zona pellucida. Spindle assessment was performed using laser scanning confocal microscopy (Radiance 2000 system with LaserSharp 2000™ software, BioRad Inc., Hercules, CA). The Krypton Argon laser was used to excite the fluorophores. An HQ 515/30 and an E580LP emission filter were utilized for the green and red fluorophores, respectively. Spindles with only two anastral poles and chromosomes confined to the metaphase plate, having a size, shape, and estimated fluorescence intensity similar to the control oocytes' spindles were scored as normal. All other spindle morphologies were scored as abnormal. Oocytes with the tubulin and chromatin staining displaying the structure of the telophase stage were scored as having been activated by the treatment. Even though the spindle in activated oocytes is normal from a functional standpoint, it is abnormal from the standpoint of preserving the cell in its pre-treated state. Hence we classified activated oocytes as having an abnormal spindle, since the spindle was not in the MII configuration.

Experimental design

A completely randomized design was used for this study.[36] The first six replicates contained solutions with osmolalities between 75 and 2400 mOsm. Seven additional replicates were performed which included treatment lev-

els of 2700 and 3000 mOsm since some oocytes from the initial replicates were able to tolerate exposure to 2400 mOsm as assessed by their normal oolemma and spindle structure. After selecting oocytes of acceptable quality for inclusion into the experiment as described above, individual oocytes were randomly allocated to the experimental treatments for each replicate. Oocytes were transferred to, and held in the treatment solution for 10 min, then returned to TL-HEPES for 10 min, which allowed the cells to return to their isosmotic volume. Subsequently, the cells were placed in an incubator with an atmosphere of 5% CO_2 and air in bovine IVF medium at 39°C. The oocytes remained in culture for approximately 60 min prior to fixation. Prior to fixation, the integrity of the oolemma was recorded as intact or lysed as described above. The structure of the MII spindle was scored as normal or abnormal after immunofluorescence analysis.

Statistical analysis and model development

Spindles scored as normal or abnormal were coded as 0 or 1, respectively. These data were analyzed by logistic regression[37] using the SAS system (SAS Institute, Cary, NC). The hypo- and hypertonic treatments were analyzed independently. An α value of 0.05 was used.

The logistic function used to relate the probability of the event being modeled and the experimental variable is described by:

$$\pi = \frac{e^{\beta_0+\beta_1 x}}{1 + e^{\beta_0+\beta_0 x}}; \quad (1)$$

where π is the probability of the event occurrence, in this case the loss of normal spindle morphology, β_0 is the coefficient for the model constant, β_1 the coefficient for the experimental parameter, and x is the value of the experimental variable, extracellular concentration in mOsm in this instance. The coefficients are determined by the logistic regression procedure using maximum likelihood estimation.

Oocyte water and CPA volume and intracellular CPA concentration changes were modeled using two coupled nonlinear differential equations as first described by Kedem and Katchalsky[38]:

$$\frac{dV_{w+s}}{dt} = -L_p ART[(M_n^e - M_n^i) + \sigma(m_{CPA}^e - m_{CPA}^i)]; \quad (2)$$

and

$$\frac{dm_{CPA}^i}{dt} = \frac{(1 + \overline{V}_{CPA} m_{CPA}^i)^2}{(V - V_b)} \left\{ \left[\overline{m}_{CPA}(1 - \sigma) - \frac{m_{CPA}^i}{1 + \overline{V}_{CPA} m_{CPA}^i} \right] \frac{dV_{w+s}}{dt} + AP_{CPA}[m_{CPA}^e - m_{CPA}^i] \right\}; \quad (3)$$

where $0 \le \sigma \le 1 - \frac{P_{CPA}\overline{V}_{CPA}}{L_p RT}$. (4)

V_{w+s} is the volume of water plus solvent, L_p is the cytoplasmic membrane water permeability, A is the cell surface area, R is the gas constant, T is the absolute temperature, M_n^i is the osmolality of internal salts, M_n^e is the osmolality of external salts, σ is the reflection coefficient, m_{CPA}^i is the molality of the internal CPA, m_{CPA}^e is the molality of the external CPA, $\overline{V}_{CPA}$ is the partial molar volume of CPA, V is the cell volume, V_b is the osmotically inactive cell volume, $\overline{m}_{CPA} = (m_{CPA}^e - m_{CPA}^i)/\ln(m_{CPA}^e/m_{CPA}^i)$, and P_{CPA} is the CPA permeability.

The equilibrated cell water and CPA volumes were equated to the total cell volumes by the addition of V_b, and then related to the solution osmolality using the Boyle van't Hoff relationship[39] as described by:

$$V = \left(\frac{M_{iso}}{M}\right)(V_{iso} - V_b) + V_b; \quad (5)$$

where V is the normalized cell volume, V_{iso} is the isosmotic cell volume, M_{iso} is the isotonic osmolality, and M is the solution osmolality. The appropriate cryobiological and biophysical parameters for mature bovine oocytes at 22°C have been previously reported.[23,40,41]

Finally, the osmolality was related to the probability of spindle disruption using equation [1]. Since all of the oocytes at the treatment levels of 2700 and 3000 mOsm showed a disrupted spindle, the analysis for the hypertonic data were restricted to the treatments between 280 and 2700 mOsm.

RESULTS

The effects of the treatments on the oolemma and spindle are shown in Table 1. A high proportion (91%) of oocytes maintained oolemma integrity after exposure to 150 through 2400 mOsm. However, many oocytes exposed to 150 and 2400 mOsm had a disrupted spindle, some (15%) as a result of oocyte activation. Nearly all of the oocytes exposed to 75, 2700, and 3000 mOsm exhibited abnormal spindles, most of these concomitant with oolemma lysis. In contrast, all of the cells in the isotonic treatment maintained an intact oolemma and had normal spindle morphologies.

Spindle structures representing those seen in bovine oocytes after anisosmotic exposure are shown in Figure 1. An example of a spindle with a normal structure after having been exposed to the isotonic treatment is shown in panel A. Only three of the 82 oocytes (4%) with spindles having a normal microtubule pattern showed evidence of chromosome displacement from the metaphase plate (panel B). Two were from the 2100 mOsm treatment and one was from the 600 mOsm treatment. In all three cases, the staining pattern suggested that there was a single sister chromatid located at each pole (arrows).

An interesting phenomenon occurred in two of the oocytes exposed to the 150 mOsm solution. Several clusters of chromosomes were present which were not confined to the metaphase plate, and each had an associated "mini-spindle" (panel C). We did not observe this occurrence in oocytes exposed to any of the other treatments. Occasionally, bovine spindles developed a third anastral pole after exposure to the anisosmotic treatment, as seen in panel D. When this occurred, some chromosomes became displaced from the metaphase plate and migrated toward the third pole as seen in this panel. Of the 39 abnormal spindles seen in the oocytes, 10 (26%) exhibited the pattern of a telophase spindle (panel E), where the sister chromatids had moved to opposite sides of the spindle and a spindle midbody was evident. When oolemma lysis occurred, microtubule staining was absent in 100% of the oocytes, as seen in panel F.

The solution concentration was a significant predictor of the loss of spindle morphology for both the hypo- and hyperosmotic treatment levels, with the p-value of the −2 Log Likelihood test statistic being less than 0.0001. Interpretation of the model suggests that the odds of spindle disruption increases by a factor of 1.016 to 1.052, with 95% confidence, for each 1 mOsm decrease in the hyposmotic range, and increases by a factor of 1.001 to 1.003, with 95% confidence, for each 1 mOsm increase in the hyperosmotic range. Using the logit equation [1], the probability of bovine oocytes experiencing irreversible disruption of the MII spindle as a function of the osmolality can be modeled as:

$$\pi = \frac{e^{(6.3588 - 0.0344x)}}{1 + e^{(6.3588 - 0.0344x)}} \quad (6)$$

Table 1. The Effects of Osmotic Stress on Bovine Oocytes

Sample size	*Osmolality (mOsm)*	*Number of cells with ruptured oolemma*	*Number activated*[a]	*Number with a normal spindle*	*Percent with a normal spindle*
13	75	10	0	1	8
13	150	3	2	2	15
13	200	2	1	8	62
13	280	0	0	13	100
13	600	0	0	11	85
13	900	1	1	10	77
13	1200	1	1	9	69
13	1500	0	0	10	77
13	1800	0	1	9	69
13	2100	0	2	6	46
13	2400	5	2	3	23
7	2700	7	0	0	0
7	3000	7	0	0	0

[a]Spindle having a telophase configuration.

in hypotonic solutions, and

$$\pi = \frac{e^{(-3.4282+0.00183x)}}{1 + e^{(-3.4282+0.00183x)}} \quad (7)$$

in hypertonic solutions. By taking the inverse of these values, the probability of a given oocyte maintaining a normal spindle structure as a function of the osmolality can be determined. Graphic representations of these calculations are presented in Figure 2 along with the experimentally determined probability at the solution concentrations used for this study. As shown in the plots, the probability decreases as the solution concentration diverges from isotonic. These models can be used as guides in the development of procedures for the addition and removal of CPAs for maintaining the integrity of the spindle structure at a chosen probability. For example, to prevent osmotic damage to the MII spindle with a probability of 0.9, one would have to use a CPA addition and removal procedure which maintains the cells within a volume range of 1.1 to 0.52 times the isotonic volume.

DISCUSSION

Due to the generally low success of slow cooling as an approach to cryopreserve bovine oocytes, investigators have turned to ultrarapid cooling approaches (>10,000°C min^{-1}) to overcome the difficulties inherent with these cells.[42] Two challenges associated with ultrarapid cooling are the potentially toxic effects of the high concentrations (e.g., 5M) of CPAs required with this approach, and the osmotic effects associated with their addition and removal. The results from this study demonstrate that damage to the oolemma and MII spindle in bovine oocytes occurs at a higher frequency as the solution concentrations diverge from isosmotic. However, the tolerance of the bovine oolemma and MII spindle to hypertonic conditions is relatively high, with significant proportions of oocytes maintaining a normal spindle structure up to an 1800 mOsm exposure. With such a broad tolerance to hyperosmotic stress, CPA addition can be performed in relatively few steps. Using such an approach, the exposure times to these compounds prior to long-term storage can be minimized, which should reduce the associated CPA toxicity.[43] Critically, since the MII spindle was shown to be fairly sensitive to osmotic swelling, multistep CPA removal procedures may need to be utilized, depending on the degree of dehydration prior to cryopreservation. A previous report form our group[44] analyzed the developmental potential of cumulus enclosed bovine oocytes after anisosmotic exposure, in a similar manner to the present investigation. In that study, fertilization and cleavage rates, normalized to the controls, remained high for treatments ranging from 75 to 2400 mOsm. However, when *in vitro* development to the blastocyst stage was assessed, the rates were significantly reduced as the treatment levels diverged from isotonic.

Potential mechanisms for osmotically induced spindle disruption

There are several possible mechanisms by which exposure to an anisosmotic solution could alter the structure and association of microtubules and chromosomes in the meiotic spindle. With high osmotic and hydrostatic pressure, the hydration state of macromolecules can be altered; in extreme cases leading to changes in conformation and activity.[45] The changes in solute concentrations within cells associated with volume changes may lead to alterations in protein-protein interactions due to changes in intracellular ionic strength. This effect is particularly applicable to microtubules, since the α- and β-tubulin molecules associate through non-covalent interactions, and changes in solute conditions have been shown to alter microtubule assembly characteristics.[46,47] Either of these mechanisms could also cause disruption of the kinetochores or microtubule organizing centers (MTOC), both of which are multi-protein complexes. Several proteins associated with the MTOC in mitotic spindles have been shown to be necessary for structural maintenance, such as TPX2[48] and NuMa[49] in Xenopus and asp[50] and dd4[51] in drosophila. It is likely that similar proteins are responsible for maintaining spindle pole organization in meiotic spindles, and their disrup-

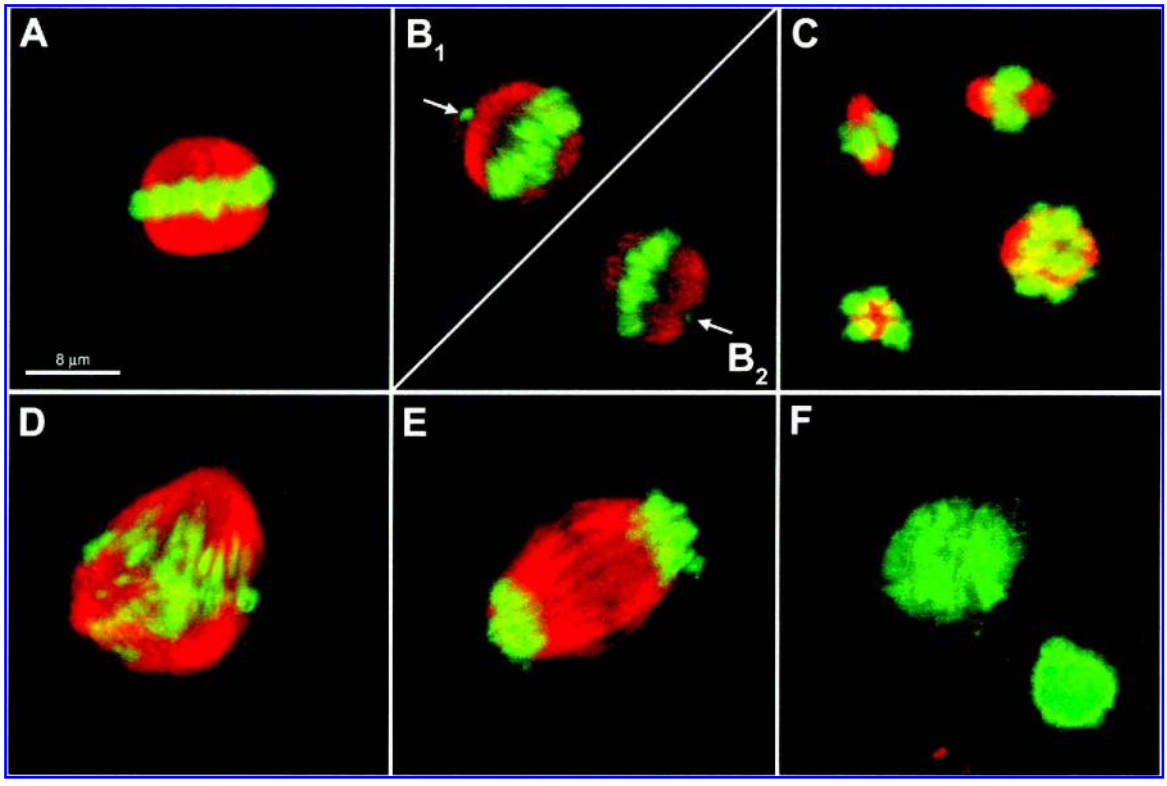

FIG. 1. Examples of metaphase II spindle configurations seen after anisosmotic exposure. A normal spindle configuration is shown in panel **A**, while various abnormal configurations are shown in the remaining panels. Note the arrows pointing to chromatin located at opposite poles of the spindle in panels $\mathbf{B_1}$ and $\mathbf{B_2}$. These images are of the same spindle, with two different focal planes being shown in the subpanels.

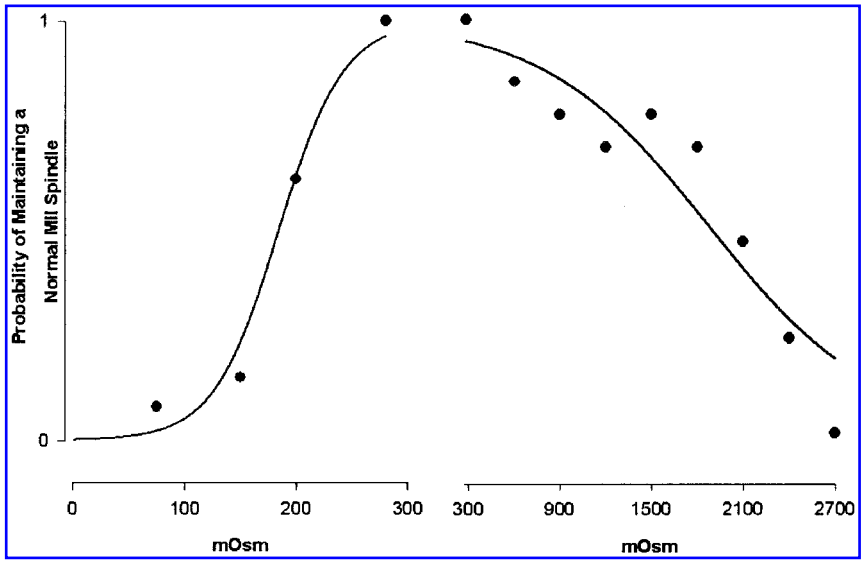

FIG. 2. The experimental datapoints plotted along with the logistic model (line) described by equations 5 and 6 for the hypotonic (left side) and hypertonic (right side) conditions respectively. This model describes the relationship between solution concentrations and the probability of maintaining a morphologically normal spindle.

tion would likely lead to disorganized MTOCs as seen in many of the anisosmotically treated spindles in the present study.

Maintaining the bonds between the spindle fiber proteins and the kinetochores as well as between the sister chromatids is critical for the proper function of the MII spindle during chromosome segregation.[52] It is interesting to note in our analysis that very few spindles with a normal microtubule pattern showed evidence of displacement of the chromatids from the metaphase plate (Table 1). In the few that showed such evidence, there was chromatin staining at both spindle poles (Fig. 1, panel B_1 and B_2), with the staining intensity suggesting that only one of the sister chromatids was present at each pole. Thus, it appeared that only the cohesin bonds between sister chromatids were affected. One common structural abnormality seen in this study, as well as in unpublished observations in our lab after exposing bovine oocytes to CPAs, is the appearance of an additional spindle pole with an associated focus of microtubule nucleating activity. When this occurs, several of the chromosomes appear to be "captured" by the microtubules associ-

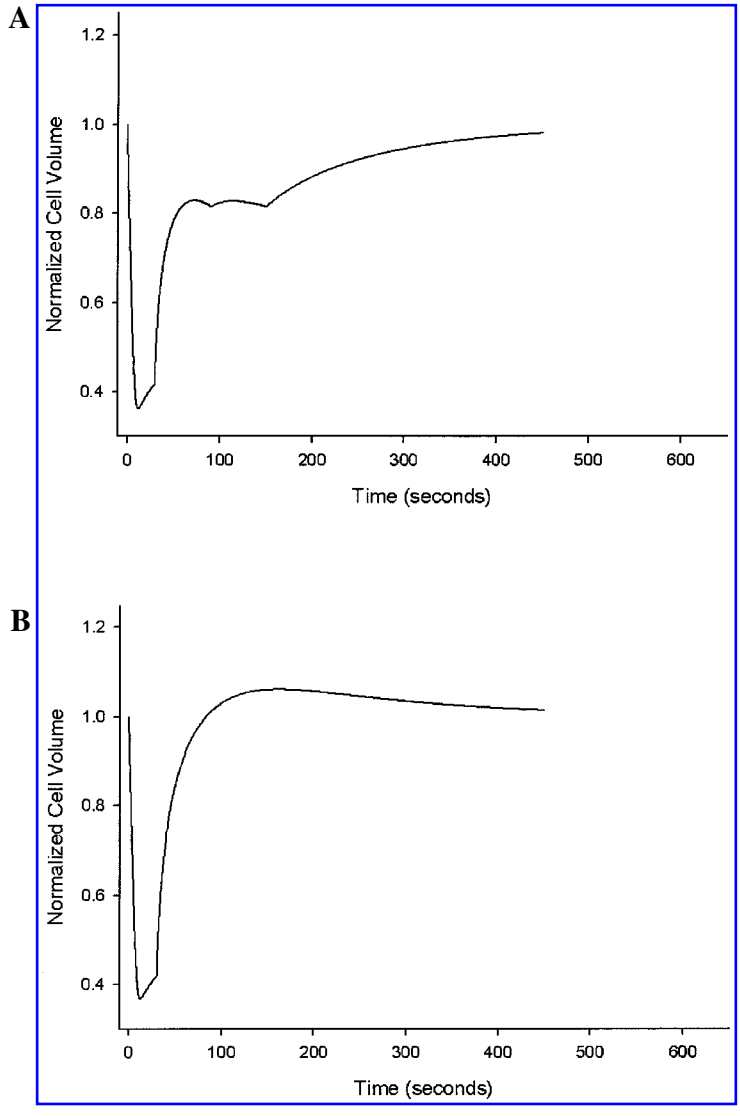

FIG. 3. A comparison of the volume changes associated with a CPA addition and removal method described by Martino et al.[54] (**A**) and a theoretically optimized method developed using the results from the current study (**B**). Note how rapidly the cell volume returns to near isotonic with the optimized method.

ated with this new pole, and are displaced toward this new MTOC. Thus it seems that maintaining the association of the proteins of the MTOCs may be more critical than maintaining the bonds between the kinetochores and spindle fibers for preserving the overall spindle structure.

Due to the variety of potential mechanisms of damage, it is not surprising that a recent report[53] documented a high degree of disruption of the MII spindle structure in bovine oocytes after exposure to a vitrification solution. In this report, the effects of a two-step addition and a three-step removal of a solution containing DMSO and EG resulted in disruption of the spindle structure in 31% of the cells (normalized to control). Because a multistep procedure was employed, the results from the present study suggest that the effects seen resulted from a combination of osmotic as well as chemical damage. In their report, when vitrification was also performed with these CPA addition and removal procedures, 67% of the oocytes (normalized to controls) had a disrupted spindle structure after thawing. These results highlight the many challenges to stabilizing the spindle structure when ultra-rapid cooling procedures are employed.

Modeling the probability of spindle disruption and designing theoretically optimal cryopreservation procedures

Recent studies have used various approaches for the ultra-rapid cryopreservation for mature bovine oocytes, with higher rates of success compared to slow cooling methods.[42,54] In one of the experiments in the report by Martino et al.,[54] the investigators applied a single step CPA addition of 4 M EG plus 0.5 M sucrose in H-CZB medium, and utilized a multi-step removal as one of the treatments, achieving an average blastocyst developmental rate of approximately 14% (development rate of the controls was approximately 42%). This rate was a significant improvement compared to a slow cooling cryopreservation treatment used in the same study. When the volume change associated with this procedure was modeled with the approach previously described by Gao et al.,[31] the greatest volume excursion would have occurred during the CPA addition, as it was performed in a single step. The probability of maintaining a normal spindle structure with this technique is approximately 0.88, according to the results from the present study. This high level is not surprising given the degree of tolerance of the MII spindle for hyperosmotic stress as we have shown. When the volume change associated with a single-step dilution procedure was modeled using the single step EG addition as discussed above, the cell volume returned to near isotonic, with little cell swelling beyond isotonic volume. These results suggest that a single-step CPA removal would be a more optimal procedure than the multistep CPA removal scheme utilized in the original publication, with the estimated probability of spindle maintenance remaining at 0.88. The estimated volume changes associated with each of these approaches are plotted in Figure 3. The top panel (A) shows the volume excursions associated with the original procedure described by Martino et al.,[54] while the bottom panel shows the volume changes associated with the theoretically improved procedure described above. While from an osmotic standpoint this method should be more optimal, there may be detrimental effects associated with the CPA concentration independent of the osmotic stress as discussed above. Future experiments will need to define optimal CPA types and concentrations to further improve ultra-rapid cryopreservation of bovine oocytes. Using the results from this study, optimal procedures for CPA addition and removal can be designed in future experiments which prevent excessive damage to the MII spindle and should help to maintain the developmental competence of the cells after freezing.

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