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ScienceDirect
Cryobiology 54 (2007) 281–289
CRYOBIOLOGY
www.elsevier.com/locate/ycryo

The effect of osmotic stress on the cell volume, metaphase II spindle and developmental potential of *in vitro* matured porcine oocytes ☆

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Received 30 January 2007; accepted 29 March 2007
Available online 4 April 2007

Abstract

Porcine animal models are used to advance our understanding of human physiology. Current research is also directed at methods to produce transgenic pigs. Cryobanking gametes and embryos can facilitate the preservation of valuable genotypes, yet cryopreserving oocytes from pigs has proven very challenging. The current study was designed to understand the effects of anisotonic solutions on *in vitro* matured porcine oocytes as a first step toward designing improved cryopreservation procedures. We hypothesized that the proportion of oocytes demonstrating a normal spindle apparatus and *in vitro* developmental potential would be proportional to the solution osmolality. Oocytes were incubated for 10 min at 38 °C in various hypo- or hypertonic solutions, and an isotonic control solution and then assessed for these two parameters. Our results support the hypothesis, with an increasing proportion of spindles showing a disrupted structure as the levels of anisotonic exposure diverge from isotonic. Only about half of the oocytes maintained developmental potential after exposure to anisotonic solutions compared to untreated controls. Oocyte volume displayed a linear response to anisotonic solutions as expected, with an estimated relative osmotically inactive cell volume of 0.178. The results from this study provide initial biophysical data to characterize porcine oocytes. The results from future experiments designed to determine the membrane permeability to various cryoprotectants will allow predictive modeling of optimal cryopreservation parameters and provide a basis for designing improved cryopreservation procedures.

Keywords: Porcine; Oocyte; Cryopreservation; Osmotic; Spindle; Blastocyst; Vitrification

Pigs are an important animal model for human physiology and pathology [26,67]. In some instances, they are a preferred model compared to other organisms [61]. As with mice, transgenic technology is playing an increasingly important role in the development of genetically modified pigs [50] for diverse areas in biotechnology [11,28,30]. Preservation of rare genotypes is important, but often costly [12]. Germplasm cryopreservation can provide a cost-effective alternative to the maintenance of breeding colonies for the preservation of rare genotypes [29,32] and can also provides a means to prevent the loss of such rare genotypes in the event of an unexpected catastrophe [65]. The value and feasibility of genome resource banking for the preservation of rare genotypes has been recognized for many years [66]. Having frozen oocyte banks could also improve research endeavors by providing freer access to oocytes and reduce the effects of seasonal variation in oocyte quality. In fact, it has been argued that ''any improvements in the swine IVP

☆ Some of the data in this report was presented at the 42nd annual meeting of the Society for Cryobiology, 24–27 July 2005, Minneapolis, MN, USA. This project was supported in part by the Cryobiology Research Institute, The University of Missouri's Research Animal Diagnostic Laboratory (RADIL), and a grant from the National Institutes of Health (U42-RR018877).
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doi:10.1016/j.cryobiol.2007.03.005

(*in vitro* production) system would revolutionize not only the reproductive management of swine, but also increase the use of pigs for biotechnological and biomedical applications'' [64].

Cryopreserving gametes and embryos from pigs has proven much more challenging compared to several other species [9,14,51]. Porcine oocytes are very sensitive to cooling below physiologic temperatures [31,70]. The detrimental effects of cooling mammalian oocytes are manifested in many ways, including damage to the metaphase II spindle [3,33,71], lipid phase changes in the oolemma [4,22] and developmental potential [5,34,70]. Because of the chilling sensitivity, rapid cooling methods have been the focus of attempts to preserve porcine oocytes [17,41]; however, damage to the MII spindle and developmental potential remains a problem [55,69]. In order to prevent intracellular ice formation at high cooling rates, a condition which is nearly always lethal [35], high concentrations of permeable and non-permeable solutes need to be employed as cryoprotectants [15]. Exposing cells to solutions containing high concentrations of solutes can be damaging due to the direct chemical toxicity of these compounds and also as a result of the osmotic effects caused by the exposure. Fortunately, osmotic effects can be controlled by modifying the manner by which cells are exposed to the compounds. More specifically, high concentrations of permeable solutes can be loaded into cells in a stepwise manner, resulting in a reduced osmotic effect during each step [47,48]. There are numerous reports in the literature describing the benefit of a stepwise approach to addition and/or removal of high concentrations of solutes compared to a single-step exposure [6,21,24,44,46,54]. Thus, the optimal procedure for loading and unloading cryoprotectants will balance the chemical and osmotic effects by adding the cryoprotectants as quickly as possible (minimizing the chemical effects) but using a stepwise procedure to reduce the osmotic effects. Even though most reports on oocyte vitrification describe stepwise cryoprotectant (CPA) addition and removal procedures, the procedures employed are rarely designed to specifically take account of the osmotic tolerance and cell permeability characteristics of the cells. When these properties are taken into account, they can be utilized in a proactive manner to design theoretically optimal procedures for addition and removal of cryoprotectants [18]. Such an approach was successfully demonstrated several years ago with human sperm by Gao and colleagues [21].

In order to use this approach, biophysical characteristics of cells need to be known. The current study was designed to characterize the relationship between cell volume and extracellular osmolality, as well as the osmotic tolerance of *in vitro* matured porcine oocytes as a first step toward developing optimal cryopreservation methods. Having this information will provide a foundation upon which CPA addition and removal procedures can be designed by setting osmotic tolerance limits within which the cells should be maintained during CPA addition and removal.

Materials and methods

Experimental design

This study was designed to determine the effect of exposing mature porcine oocytes to solutions with varying osmolalities on: (1) the cell volume; (2) the morphology of the metaphase II (MII) spindle; (3) the *in vitro* developmental ability as measured by *in vitro* fertilization and development to the blastocyst stage.

To determine the change in cell volume in response to the various solutions (see below for details of the solution compositions), photographs of the cells were taken after a 10 min incubation period, and the total cell volume was estimated using computer image analysis techniques (Fovea Pro®, Reindeer Graphics, Asheville NC, USA, and Photoshop®, Adobe Systems, Inc. San Jose, CA, USA; see Fig. 1 for details). Only cells maintaining near spherical geometry were used for these calculations. The cell volume was expected to be a linear function of the inverse of the relative osmolality, and linear regression was used to make this assessment.

To determine the effect of the treatment on the morphology of the MII spindle, individual oocytes were randomly assigned to one of the osmotic treatments and incubated in these solutions for 10 min. The treatments were conducted at 38 ± 1 °C to avoid any confounding effects of sub-physiologic temperatures on the MII spindle [31].

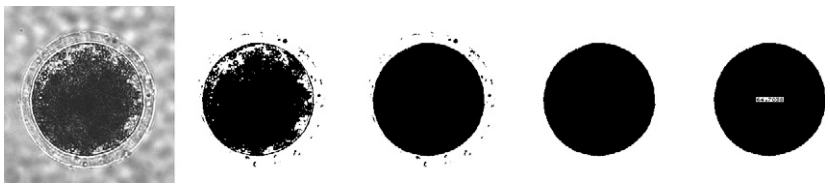

Fig. 1. The steps in the procedure used in image analysis are shown from left to right. The first panel shows an original photograph. A threshold process, with the oolemma intensity chosen as the threshold level, was applied to the original image, isolating pixels based upon their gray scale value. The result is shown in the second panel. The next process involved filling in areas that were completely surrounded by black pixels (panel 3). Finally, all objects smaller than a minimum size (chosen as 4000 pixels) were rejected from the image, leaving only the area originally occupied by the oocyte (panel 4). The radius of a circle containing an equivalent area was computed by the software, as shown. The volume of the oocyte was calculated using this value, assuming spherical geometry.

The treatment solutions were prepared as described below and had osmolalities of 35, 75, 150, 225, 290, 700, 1100, 1500, 1900, 2300, 2700, and 3150 milliosmolal (mOsm). The cells were then returned to an isotonic solution for 10 min and allowed to recover their normal cell volume, and then put into culture medium (porcine maturation medium without gonadotropins [16]) in a culture incubator at 38.5 ± 0.5 °C with a 5% CO_2/air atmosphere for 1 h prior to fixation. A complete randomized design was used for this experiment, with individual oocytes constituting the experimental units. The treatments were replicated within a day and also across several days. The spindle was scored as normal or abnormal based upon the spindle morphology in untreated porcine oocytes. The spindle of porcine oocytes is nearly spherical in shape, often with the pole-to-pole distance being slightly shorter than the distance across the metaphase plate. Spindles displaying any morphological differences to such a shape, including scattering of the chromosomes from the metaphase plate, were scored as abnormal. Logistic regression was used to analyze the data and determine the relationship between osmotic treatment and spindle morphology. Between 16 and 28 oocytes were assessed per treatment group (265 oocytes total). Approximately 10% of the spindles were not able to be assessed due to the spindle being obscured by the intracellular lipids, which was merely a reflection of the orientation of the cell on the microscope slide.

To determine the effect of the treatment on the *in vitro* developmental potential, oocytes were randomly assigned to one of the osmotic treatments and incubated in these solutions for 10 min, also at 38 ± 1 °C. The treatment levels for this experiment were 75, 150, 225, 290, 460, 650, 880, 1410, and 2080 mOsm. The cells were then returned to an isotonic solution for 10 min and allowed to recover their normal cell volume, and then put into culture medium (porcine maturation medium without gonadotropins) in a culture incubator at 38.5 ± 0.5 °C with a 5% CO_2/air atmosphere for 1 h. After this time, the cells were subjected to *in vitro* fertilization (IVF; see below for more details). A complete randomized design was used for this experiment, with groups of 10–25 oocytes constituting the experimental units (exact numbers depending upon the total number of acceptable quality oocytes on each day). The experiment was independently replicated on 7 days. Development was carried out to day 6 (the day of the experiment was day 0) and the proportion of oocytes undergoing development to blastocysts was determined. Analysis of variance with a Tukey correction for multiple comparisons was used to determine the relationship between osmotic treatment and developmental potential [63].

For all statistical tests, the α-value was chosen to be 0.05. Assignment of the experimental units to the treatments was conducted by randomizing the treatment order on each day with a random number table [10]. Statistical analyses were performed using the SAS system (Cary, NC USA).

Chemicals

Unless otherwise stated, the chemicals used in these experiments were purchased from Sigma–Aldrich (St. Louis, MO USA).

Source of oocytes, in vitro maturation (IVM), and in vitro fertilization

Oocytes were purchased commercially (BOMED, Inc. Madison, WI, USA). The oocytes were shipped in a battery-powered and insulated incubator which held the oocytes at a temperature of 38.5 ± 0.5 °C during shipment to our laboratory on the day of collection from abattoir-derived ovaries. Immediately upon receipt, the tube containing the oocytes was transferred to a culture incubator which also was maintained at 38.5 ± 0.5 °C. Oocytes were shipped in maturation medium for 20–22 h, and then transferred to maturation medium without gonadotropins in a culture incubator at 38.5 ± 0.5 °C with a 5% CO_2/air atmosphere for an additional 20–22 h after reaching our laboratory. Each of these media was supplied by BOMED. After the final maturation time, the oocytes were incubated with approximately 500 μl of flushing and holding medium (FHM: [42]) containing hyaluronidase at a final concentration of 1 mg/ml in a 1.5 ml Eppendorf tube. The cells were vortexed for 30–60 s to remove the cumulus cells and transferred through 3 dishes containing ~2 ml of Hepes-buffered Tyrode Albumin Lactate Pyruvate (TALP-Hepes) medium [7] containing 3 mg/ml of bovine serum albumin (fraction V). The cells were inspected and any deteriorated oocytes or oocytes having undergone parthenogenetic activation (as assessed by cleavage) were discarded. The oocytes were then randomly assigned to the treatments. After the treatments and post-treatment holding periods, the oocytes were transferred to 50 μl drops of modified Tris-buffered medium (mTBM) and an equal volume of frozen and thawed porcine spermatozoa was added; final sperm concentration was $\sim 0.5 \times 10^6$ total sperm per ml. The sperm and oocytes were allowed to incubate for 5–6 h. After the IVF procedure, the oocytes were removed from the mTBM drop, washed through 3 volumes (~2 ml each) of TALP-Hepes, and transferred to North Carolina State University-23 (NCSU-23) medium for long-term culture in an incubator at 38.5 ± 0.5 °C with a 5% CO_2/air atmosphere. All oocyte manipulations were carried out in a laboratory room with an air temperature of 25 °C, and all manipulations were processed in a manner to maintain the cells at 38.5 ± 0.5 °C. The details of the maturation and fertilization procedures, including recipes for the solutions, can be found in [16].

Treatment solutions

Dulbecco's phosphate-buffered saline (DPBS) supplemented with glucose and pyruvate (Gibco #14287-080) constituted the base isotonic solution (~290 mOsm).

Hypotonic solutions were made by diluting the DPBS with embryo culture grade water. Hypertonic solutions were made by supplementing the DPBS with sucrose. The osmolality of the solutions was assessed using a vapor pressure osmometer (VAPRO 5520, Wescor Inc., Logan, UT, USA) and were made within ±10 mOsm of the target osmolality. A 5 ml aliquot of these solutions was supplemented with bovine serum albumin (fraction V) at a final concentration of 1 mg/ml and sterile filtered before use. A volume of two ml of each solution was pipetted into a 35 mm Petri dish (Falcon 35-1008) and covered with sterile, embryo culture grade mineral oil, and held on a slide warmer to maintain the temperature at 38.5 ± 0.5 °C during the treatment.

MII spindle staining and analysis

We have published our technique for immunostaining the MII spindle of mammalian oocytes in great detail [40]. Briefly, after the treatment and recovery period, the oocytes were fixed in cold methanol (−20 °C) for 5–6 min and held in a blocking buffer for at least 24 h. A primary antibody (clone DM1-A) targeting α-tubulin was used by mixing the stock solution with blocking buffer at a final dilution of 1:9000. The cells were incubated in ~2 ml of this solution at 25 °C for ~60 min, or overnight at 4 °C. The cells were washed with ~3 ml of blocking buffer at 25 °C for ~60 min. The cells were then transferred to ~2 ml of blocking buffer containing the secondary antibody (donkey anti-mouse antibody conjugated with Texas Red®, purchased from Jackson Immunoresearch, West Grove, PA, USA) which was diluted by adding 5 μl–7.5 ml of blocking buffer. This incubation also proceeded at 25 °C for ~60 min, or overnight at 4 °C. A final wash was conducted with ~3 ml of blocking buffer at 25 °C for 1–2 h. Cells were then mounted on glass slides with ProLong antifade compound containing 1 μl of SYTOX green DNA stain per ml of antifade. Each of these compounds was purchased from Molecular Probes (Invitrogen; Carlsbad, CA, USA). The antifade was allowed to set overnight. The spindle morphology was assessed using laser scanning confocal microscopy with a BIORAD 1024 confocal microscope equipped with a Krypton–Argon laser, at the University of Missouri's Molecular Cytology Core Facility.

Results

Cell volume and osmolality relationship

The volume of cells equilibrated in solutions with concentrations of 225, 290, 700, or 1100 mOsm was estimated as described above. The average volume of 31 oocytes held in the isotonic solution was $7.75 \pm 0.98 \times 10^5$ μm^3 (mean ± standard deviation). When the relationship between equilibrated cell volume and solution concentration was assessed, the cells were found to behave as ideal osmometers, with a linear change in volume as a function of the inverse of the relative osmolality (Fig. 2; $R = 0.95$). In this instance, the linear relationship (usually referred to as a Boyle–van't Hoff relationship) is given by Eq. 1

$$Y = 0.824X + 0.178. \quad (1)$$

Extrapolation of this relationship to infinite osmolality suggests that the osmotically inactive cell volume (V_b) is 17.8% of the isotonic volume (95% confidence interval: 0.12–0.24).

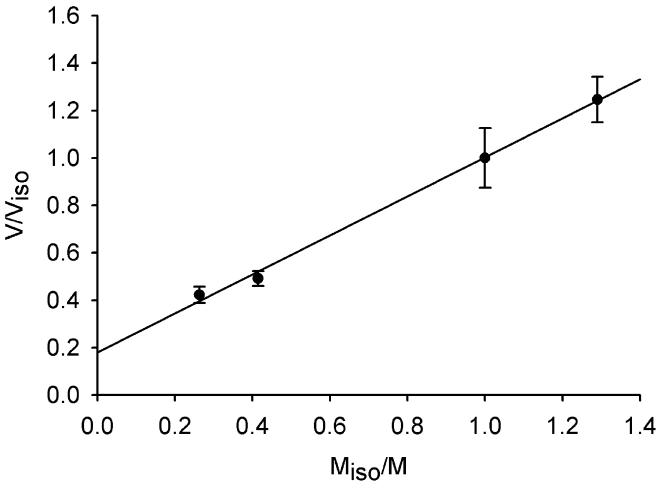

Fig. 2. The relationship between the cell volume of *in vitro* matured porcine oocytes and the solution osmolality (Miso/M) is shown. Data points represent means ± SEM.

MII spindle morphology

As the osmolality of the solution in which the oocytes were equilibrated diverged from isotonic, an increasing proportion of oocytes displayed an abnormal MII spindle morphology (Figs. 3 and 4; $P < 0.05$). The majority of oocytes were able to maintain a normal spindle structure when equilibrated in solutions ranging from 150 to 1900 mOsm (Fig. 4); concentrations beyond that range were more detrimental to the spindle structure. Two common anomalies in the spindle configuration were seen, as demonstrated by the images in panels E and F from Fig. 3. Reduced microtubule density with fibers projecting away from the chromosomes and into the cytoplasm was common (E). It was also common to see spindles which had an apparently normal density of microtubule fibers but an increase in the number of microtubule organizing centers, suggesting that the pericentriolar material was displaced from an organized focal point at the spindle poles (F). Apparently, the dislocation of the material did not affect its ability to act as a microtubule organizing center, as microtubules were focused at these different locations. Activation occurred to some oocytes (panel D), and was more common in the oocytes having been exposed to extreme hypertonic solutions. Only 10–15% of the oocytes were activated by treatments ranging from 35 to 2300 mOsm (except for oocytes exposed to 1500 mOsm,

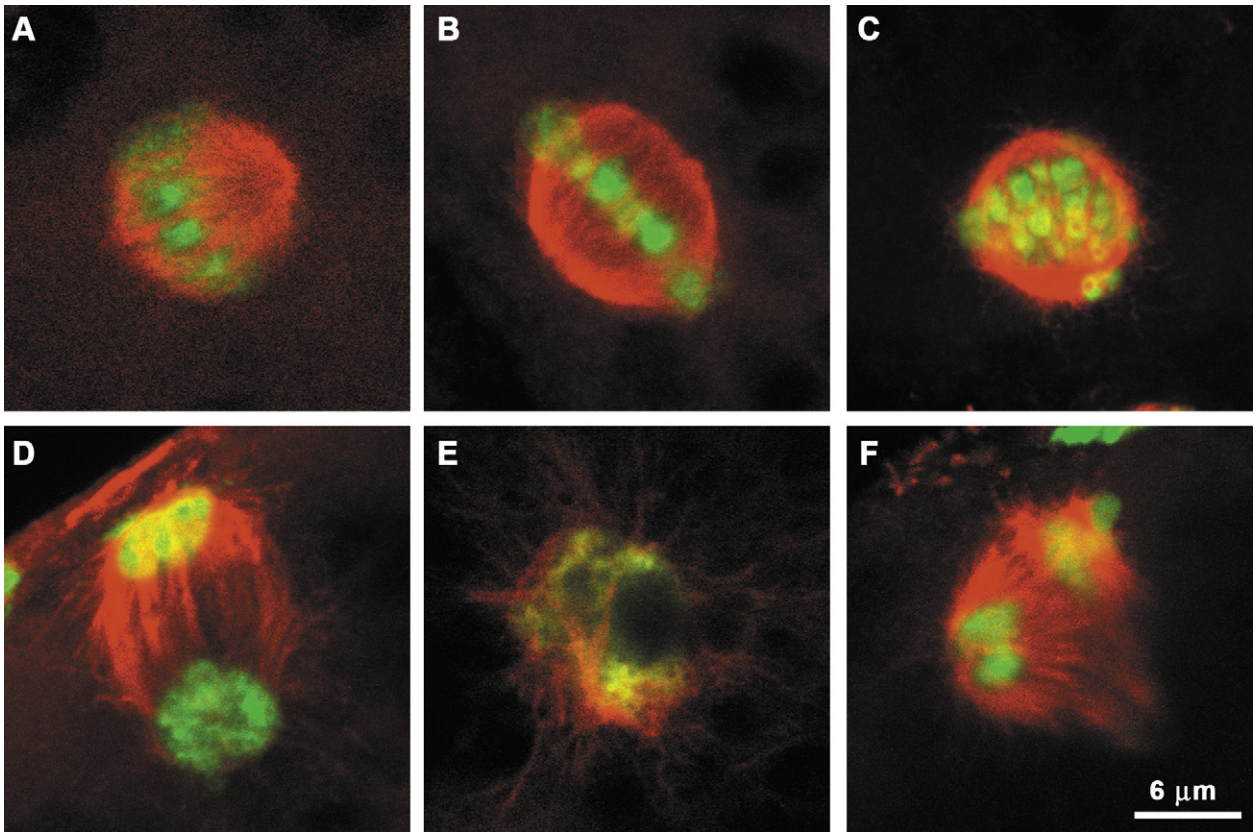

Fig. 3. Various morphologies of MII spindles are shown. Normal spindle morphology is represented by the images in panels (A) and (B), with near spherical shaped spindles and chromosomes aligned along the metaphase plate. Panel (C) shows a spindle which has chromosomes dislocated from the metaphase plate, with some located near one of the spindle poles. Panel (D) shows a spindle after oocyte activation, with the chromosomes in a late-anaphase configuration, being located at opposite sides of the spindle. The images in panels (E) and (F) show abnormal spindle configurations. The spindle in panel (E) was highly disrupted, with reduced microtubule density as well as displaced chromatin. The spindle in panel (F) shows the development of multiple spindle poles after treatment, with chromosomes being displaced toward these multiple poles.

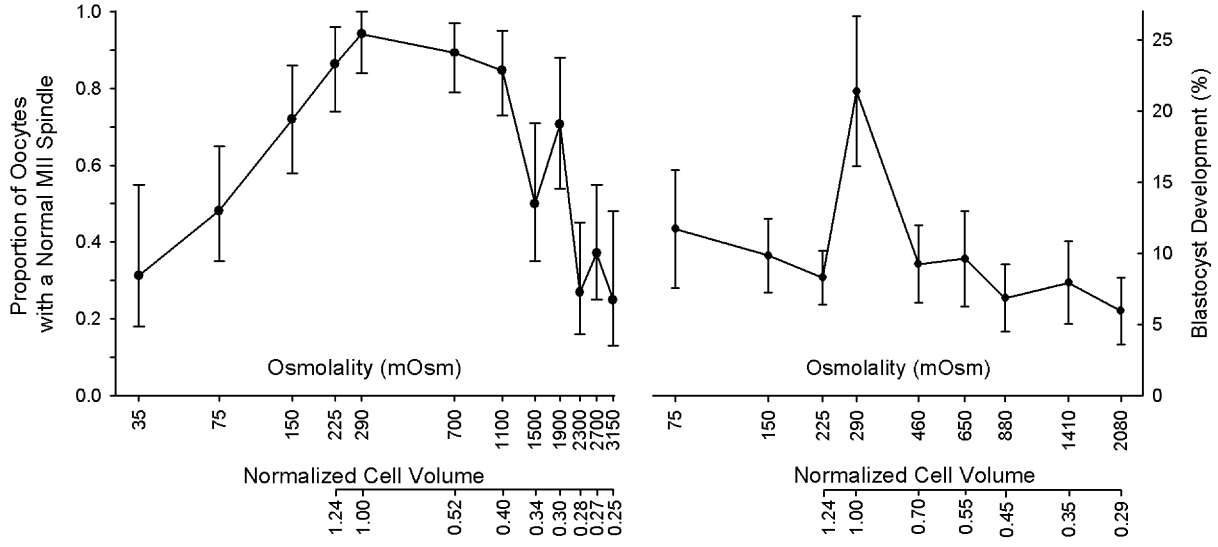

Fig. 4. The proportions of oocytes having a normal spindle configuration (with exact 95% confidence intervals; left panel) and *in vitro* developmental potential (±SEM; right panel) after equilibration with solutions of various osmolalities are shown. The respective cell volumes have been added as a second *X*-axis for comparison. The cell volume response does not follow the expected Boyle–van't Hoff relationship as expected in mammalian oocytes at hypotonic osmolalities, as the cell is inhibited from swelling due to the presence of the zona pellucida. Thus the volume data for the hypotonic treatments beyond 225 mOsm were not included on this scale. We have plotted the *X*-axis as a logarithmic scale to make the data easier to visualize, and not due to an assumption about logarithmic relationship between the variables.

where about one third of them were activated). However, 2700 and 3150 mOsm solutions activated 26% and 63% of the oocytes, respectively. Spindles with a telophase appearance were classified as abnormal spindles, as they deviated from the MII configuration.

In vitro blastocyst development

The success of the embryo production system used in the present study (ranging from 10% to 50% blastocyst development across replicates for the control treatment) is consistent with other reports using a similar maturation and fertilization system [59]; (reviewed in [13]). Anisotonic exposure also affected oocyte developmental potential, as shown in Fig. 4. On average, 21% of the oocytes were able to develop to the blastocyst stage in culture after *in vitro* maturation without any anisotonic exposure. Treating oocytes with hypo- or hypertonic solutions caused about one-half of the oocytes to lose the ability to develop to blastocysts. However, as the level of hypotonic stress increased, the proportion of oocytes which lost the ability to develop to blastocysts in culture was not statistically different. While treatment with hypertonic solutions resulted in a reduction of the average developmental potential in an apparent concentration-dependent manner, the differences were not statistically significant.

Discussion

Developing effective methods to cryopreserve mammalian oocytes has proven very challenging, with those from domestic species, notably cattle and pigs, being especially sensitive to cryopreservation procedures. Many characteristics make mammalian oocytes particularly susceptible to cryoinjury, including the large amount of cytoplasmic lipids in some taxa including swine [36,60], large volume [62], and the presence of the MII spindle [57]. Due to the inability of these cells to proliferate in culture like cells from standard cell lines, as well as the relatively small number of oocytes obtainable from a given individual, the proportion that survives cryopreservation is important. The present study was designed as a first step in characterizing the fundamental biophysical properties of porcine oocytes that are important for cryopreservation. We determined the isotonic cell volume and relative cell water volume, as well as the effect on the cells of exposure to anisotonic conditions. We have shown that the MII spindle structure can tolerate a fairly wide range of osmotic conditions. Developmental potential, on the other hand, was reduced by about 50% across all anisotonic conditions tested. This difference suggests that the MII spindle is less sensitive to osmotic stress in comparison to other cellular components in *in vitro* matured porcine oocytes.

Similar to other studies with mammalian oocytes [43,45,52,58], this study showed that porcine oocytes act as ideal osmometers with a linear relationship between the cell volume and the inverse of the relative solution osmolality across the range of solutions tested. The estimate for V_b in the present study (17.8%) is similar to that for oocytes from other mammals (*e.g.*, V_b ranged from 19% to 25% in the studies cited above).

The Boyle–van't Hoff relationship can be used to relate the effects of solution osmolality on cell volume. The results from this study show that a high proportion (~80%) of oocytes can maintain a normal spindle structure after exposure to solutions with osmolalities ranging from 150 to 1100 mOsm. The estimated cell volume range, as a proportion of the isotonic volume, is 1.77–0.4. On the contrary, developmental potential was reduced to about half that of the control oocytes in all of the solutions tested for that part of the study (75–2080 mOsm). The estimated cell volume range (relative to the isotonic volume) is 3.36–0.29. In these instances, the upper cell volume is an estimate based upon the Boyle–van't Hoff relationship. However, mammalian oocytes are confined within a glycoprotein matrix known as the zona pellucida. The zona restricts the amount of cell swelling; hydrostatic pressure will develop to compensate for the osmotic pressure difference. Using the same image analysis technique described in Fig. 1, we determined that the cells could only swell to an average of ~1.54 times the isotonic volume in the 150 mOsm treatment solution, which is less than the expected volume using the Boyle–van't Hoff estimation (~1.77). The discrepancy would be even greater at the 75 mOsm treatment level. This restriction prevents the use of these data points to estimate the relationship between cell volume and solution osmolality. At hypertonic conditions >1100 mOsm, aspherical shrinkage also restricted our ability to estimate the cell volume. However, when volume estimation techniques that do not rely upon cell shape have been applied to other cell types that do not have a restriction on cell swelling, a linear relationship between volume and osmolality has been shown to exist [1,20,49,68], suggesting that it is acceptable to extrapolate beyond the osmotic range used to estimate the relationship between cell volume and solution osmolality in this study.

Previous investigations have established that mammalian oocytes from other taxa are also sensitive to anisotonic exposure. The osmotic tolerance of porcine oocytes as measured by the MII spindle structure was more similar to oocytes from cattle than humans [38,39], with a high proportion of porcine and bovine oocytes maintaining a normal spindle structure at osmolalities ranging from ~200 to 2000 mOsm. Given the unusually high incidence of aneuploidy in humans [25], it is not surprising that the spindle would be more sensitive to cellular perturbations in human oocytes compared to those from other taxa.

As in the present study with porcine oocytes, a detrimental effect of osmotic stress on the developmental potential of MII bovine oocytes has also been shown [2]. The results from these studies are qualitatively similar. In the study by Agca and colleagues [2], the developmental potential of bovine oocytes was reduced by approximately 50% across the osmotic range used in the present study; how-

ever, a concentration effect was stronger with bovine oocytes in comparison to porcine oocytes. Anisotonic exposure has also been shown to negatively affect porcine spermatozoa and embryos. Boar sperm were determined to be very sensitive to anisotonic conditions, with 100% motility loss beyond a range of 220–320 mOsm [23]. An assessment of the osmotic tolerance of porcine blastocysts showed that both the morphology and cellular actin filament organization were disrupted by anisotonic exposure [37]. Morphological damage occurred in ~40–50% of the blastocysts, and damage to the actin filaments occurred in ~60–70% of the blastocysts at the extreme osmolalities (75 and 2400 mOsm).

Tolerance to anisotonic conditions is an important consideration for the development of cryopreservation procedures due to the near-universal requirement for cryoprotective compounds [19]. Permeating cryoprotectants (CPA), such as ethylene glycol (EG), dimethylsulfoxide (Me_2SO), and non-permeating CPA such as sucrose are typically used in combination and in multi-molar concentrations. Very high concentrations of CPA (~6–7 M) are typically needed to achieve vitrification [15], which has become the standard approach for the cryopreservation of cattle and swine oocytes due to their sensitivity to prolonged exposure to cold temperatures [34]. It has been shown that just exposing porcine oocytes to a vitrification solution can be damaging. In one study [55], the proportion of oocytes with normal spindles was about 85% relative to untreated oocytes, whereas the proportion able to develop to blastocysts was reduced by about half compared to controls. These investigators used a 3-step CPA exposure procedure for their work, and the greatest degree of osmotic stress with their method would have been approximately equivalent to 1900 mOsm. On the contrary, a higher degree of spindle damage (~50% reduction compared to controls) and reduction of developmental potential (~15% of the control level) was reported by another group [56] after exposure to a different vitrification procedure. In this second report, the level of osmotic stress was higher (~3500 mOsm) due to greater CPA concentrations as well as the use of a 2-step addition procedure (each group used a 5-step dilution procedure). The results from our study reported here are consistent with the results from these previous reports, where intermediate levels of osmotic stress resulted in a high proportion of cells maintaining a normal spindle structure and about 50% developmental potential. The procedures used in the second report [56] exceeded the range of osmotic stress we imposed upon cells which underwent fertilization and development, which could explain the greater reduction in developmental potential than that seen in our study. Furthermore, a combination of osmotic stress and chemical toxicity of the cryoprotectants could account for the greater reduction in developmental potential seen in the report by Shi and colleagues [56].

Methods to cryopreserve oocytes have evolved to include a very short exposure to the final vitrification solution due to reports suggesting that oocytes can survive exposure to high solute concentrations for only brief periods of time [8,53]. The results from such studies are usually interpreted to mean that the cells were damaged by the chemical toxicity of the solutions. In fact, results from such studies are often equivocal, as the chemical effects are confounded by osmotic effects. Several investigations into oocyte cryopreservation suggest that the osmotic effects may actually be more harmful than the chemical effects, as stepwise CPA addition and removal procedures are often better than a single-step exposure [6,27,54]. This is fortunate because the osmotic effects can easily be controlled. Appropriate CPA addition and removal procedures can be designed *a priori* if the fundamental biophysical knowledge of the cells is taken into account. An illustrative example using human spermatozoa has been reported [21]. This approach requires an understanding of the cell's permeability to water and cryoprotectants, knowledge which is presently unavailable for porcine oocytes. Future studies will be directed at assessing the osmotic tolerance over a more narrow osmotic range near isotonic conditions to determine the range of osmolalities which a high proportion of porcine oocytes can tolerate, as well as determining the membrane permeability of porcine oocytes to water and permeable CPA. With this information, fundamental principles will be applied to develop and test optimal methods to vitrify porcine oocytes in an effort to improve the survival of these important cell types.

Acknowledgments

We thank Hongsheng Men for his assistance with *in vitro* fertilization and culture experiments, and Sara Brown and Alan Young for general laboratory assistance.

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