BIOLOGY OF REPRODUCTION **54**, 1059–1069 (1996)

Development into Blastocysts of Bovine Oocytes Cryopreserved by Ultra-Rapid Cooling[1]

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ABSTRACT

The objective of the research described was to devise an efficient procedure to cryopreserve in vitro-matured bovine oocytes, using in vitro fertilization (IVF) and development of resultant zygotes into blastocysts as criteria of oocyte survival. Oocytes at metaphase II were found to be extremely sensitive to chilling. Cooling them to 0°C for as little as 5 sec significantly decreased their capability to cleave and develop further after IVF; after 80 sec at 0°C, only ~10% of chilled oocytes developed into blastocysts. Oocytes were also adversely affected by brief exposures to 4 M and 5.5 M ethylene glycol (EG) solutions supplemented with sucrose; after being suspended in either of these EG solutions in plastic straws and plunged directly into liquid nitrogen (LN_2), few of the oocytes were fertilized and developed. To "outrace" chilling injury, oocytes contained in < 1 µl of EG solution were placed onto electron microscope grids and plunged directly into N_2 slush or LN_2. After such ultra-rapidly cooled oocytes were warmed, 30% of them cleaved after IVF, and half of these developed into blastocysts—survival rates equivalent to those for oocytes that had been exposed to EG without any cooling. This method offers promise as a novel way to cryopreserve bovine oocytes.

INTRODUCTION

The first mammalian oocytes to be successfully frozen and thawed were those of the mouse and the hamster [1–3]. The procedures used were very similar to that first described for the freezing and thawing of zygotes and cleavage-stage embryos [4]. Approximately 10 yr later, these same procedures were used to freeze human oocytes, resulting in pregnancies and the birth of a child [5, 6]. Despite those successes, it has proven much more difficult to cryopreserve oocytes than late-stage embryos. Many of the causes of damage and poor fertilization rates have been elucidated; these have been described in several recent reviews [7–9]. With mice, it has been possible to circumvent much damage to oocytes by vitrification [10]. This nonequilibrium approach to cryopreservation, first described for mouse embryos and later applied to several other species (for review see [11, 12]), is based on the dehydration of embryos by brief exposure to a concentrated solution of a cryoprotective additive (CPA) before plunging the samples directly into liquid nitrogen (LN_2) to achieve high cooling rates and solidification without crystallization.

Successful application of these procedures to oocytes of domestic animals has been more difficult to achieve. Yet cryopreservation of these oocytes, especially those of cattle, would yield numerous benefits. By alleviating logistical problems, it would substantially increase the efficiency of in vitro fertilization (IVF) and would facilitate fundamental studies of both cryobiology and fertilization itself. Although pregnancies and live calves have been derived from cryopreserved bovine oocytes [13–15], survival of oocytes as judged by development into blastocysts has been low, usually amounting to < 3% of all oocytes treated [13, 14, 16–22]. In some studies, in vitro-matured oocytes were found to be more resistant to freezing than immature ones [13, 17]. Recently, Schellander et al. [20] examined the freezing sensitivity of immature as well as mature oocytes frozen in three CPAs (glycerol, propylene glycol, and dimethylsulfoxide [DMSO]) and tested three sugars (sucrose, trehalose, and lactose) as osmotic buffers to recover frozen-thawed oocytes from the cryoprotectants after thawing. Of a total of > 3100 oocytes frozen, only ~50 developed into 6/8-cell embryos, regardless of the treatment; none were reported to have developed further. Otoi et al. [15, 18, 19] also investigated the effects of many cryobiological variables, including different CPAs, sugars, and cooling rates, on the fertilization and development of bovine oocytes. Although one pregnancy was produced, the survival obtained in these very exhaustive studies was low, and only 0.6–2.7% of the cryopreserved oocytes developed into blastocysts.

Concluding that conventional approaches to the cryopreservation of bovine oocytes are unlikely to be successful, we set out to devise a method to cryopreserve them by extremely rapid cooling. The rationale was similar to that recently adopted by Steponkus et al. [23] and Mazur et al. [24] to cryopreserve embryos of *Drosophila*, which are exceedingly sensitive to chilling injury. Both groups developed very effective methods to cryopreserve these insect embryos by using very rapid cooling to traverse the damaging temperature zone as rapidly as possible. We modeled our experiments with bovine oocytes on the procedures shown to be successful with *Drosophila* [23–25].

Accepted January 9, 1996.
Received September 28, 1995.
[1]Research supported by Canadian Natural Sciences and Engineering Research Council. A. Martino was supported by a fellowship from the Spanish Ministry of Science and Education (Dirección General de Investigación Científica y Técnica).
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In this paper, we will refer to crystallization of solutions as freezing or ice formation, and to ice-free solidification as vitrification. We will assume, without proof, that samples subjected to cooling at very high rates to low subzero temperatures will have vitrified. Correspondingly, we will refer to thawing (of ice) in frozen samples vs. warming of vitrified samples. In addition, we will refer to the cooling of plastic straws plunged directly into LN_2 as rapid cooling and that of grids plunged into LN_2 as ultra-rapid cooling.

MATERIALS AND METHODS

Chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and media from GIBCO (Grand Island, NY), unless otherwise stated. All of the manipulation procedures of oocytes, sperm, and embryos were conducted in a room the temperature of which was monitored and maintained at +35°C.

Collection of Oocytes and In Vitro Maturation (IVM)

Ovaries from mature Holstein cows were obtained at a local slaughterhouse and transported to the laboratory in PBS at 35°C. The collection of oocytes was performed in a warm hood, adapting the methodology described by Xu et al. [26] except that the collection medium was supplemented with 2% steer serum (SS) obtained from Cansera Inc. (Rexdale, ON, Canada). Cumulus-oocyte-complexes (COCs) having three or more complete layers of cumulus cells and fully grown oocytes with homogeneous cytoplasm were selected for IVM. Groups of 25 selected COCs were transferred to individual 100-μl droplets of tissue culture medium (TCM) 199 (Earle's salts) supplemented with 25 mM HEPES, sodium pyruvate (274 mg/L), glutamine (146 mg/L), penicillin (50 000 U/L), streptomycin (50 000 mg/L), and 10% SS. COCs were cultured for 22–22.5 h at 39°C in a humidified atmosphere of 5% CO_2 in air.

IVF

Fertilization was effected in 100-μl droplets of IVF-Tyrode's albumin lactate pyruvate (TALP) medium supplemented with 20 μg/ml of heparin and bovine oviductal epithelial cell (BOEC) explants [26]. At the end of maturation, COCs were vortexed for 90 sec in 5 ml of modified HEPES-buffered Chatot-Ziomek-Bavister (CZB) medium (H-CZB described previously [27]) to remove most of the cumulus cells. Oocytes were observed with a stereomicroscope, and those 10% to 15% of each day's total showing signs of cytoplasmic degeneration were excluded from the experiment. For a given day's experiment, the remaining oocytes were assigned to the controls and different experimental treatments without selection. Control oocytes were maintained at 35°C in a culture dish in H-CZB medium while other sets were subjected to experimental manipulation. After oocytes had been cooled, exposed to cryoprotectant, or cryopreserved in LN_2, they were washed with HEPES-TALP medium. Twenty-five oocytes from a given set were then introduced into separate 100-μl fertilization droplets. In this way, all oocytes treated on one day were subjected to identical conditions of maturation, fertilization, and culture.

Semen from a single bull was used for all experiments; several of his ejaculates, frozen in whole-milk extender at a commercial artificial insemination center, were used. After thawing, sperm were washed in HEPES-TALP medium; motile sperm were selected after 1 h of "swim-up" at 37°C in the same medium; 100 000 spermatozoa were added to each 100-μl fertilization droplet; and sperm and oocytes were cocultured in 5% CO_2 in air at 39°C. Fertilization rates were determined from representative samples of presumptive zygotes removed for fixation and staining at 18 h post-insemination.

In Vitro Culture (IVC)

After 22 h of culture with sperm, presumptive zygotes were vortexed for 30 sec in TCM199 to remove remaining cumulus cells. The medium was the same as the IVM medium except that it was supplemented with a two-times-higher sodium pyruvate concentration and with BSA (fraction V, 3.5 g/L) and did not contain HEPES buffer. Embryos were cocultured with BOEC explants in the same medium in 100-μl droplets for 7 days in 5% CO_2 in air at 39°C.

Cooling Oocytes

In vitro-matured oocytes were separated into six groups: five groups were cooled to 0°C for 5, 10, 20, 40, or 80 sec, and a control group was maintained at 35°C. For cooling, an ethanol bath in a programmable freezer (BioCool IV; FTS Systems, Stone Ridge, NY) was used to maintain 5-ml plastic tubes containing 0.5-ml volumes of H-CZB + 10% SS at 0°C. The temperature in a replicate tube in the freezer was recorded with a 35-gauge copper-constantan thermocouple connected to a millivolt recorder with a rapid response time (Model RD 02; Omega Engineering Inc., Stamford, CT); it varied from −0.4° to 0.0°C. Ten to fifteen microliters of H-CZB medium at 35°C containing 30–60 oocytes was loaded into precision glass capillaries and was expelled directly into the tube containing the 0.5 ml of cooled medium. At the end of the cooling period, each tube containing oocytes was rapidly transferred into a 41.5°C water bath located beside the freezer, and an additional 3.5 ml of medium at 41°C was immediately added to the tube. Tubes were kept for 1 min in the water bath and then maintained in the 35°C room pending IVF. With this method, oocytes were first cooled rapidly to 0°C and then warmed back to 30–35°C at very high rates. The temperature, as recorded with a thermocouple placed in a capillary subjected to the same sequence, warmed from 0°C to 27°C in $<$ 1 sec and to 33–35°C within 1 min.

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Solutions of CPAs

Solutions of CPAs were prepared in H-CZB medium supplemented with 10% SS, since the pH of solutions of organic buffers, e.g., HEPES, does not change during freezing as does that of inorganic buffers, e.g., phosphate buffer. Two different solutions were made with ethylene glycol (EG; Fisher Scientific, Unionville, ON, Canada), one consisting of 5.5 M EG + 1.0 M sucrose (identified below as EG5.5), and the other consisting of 4 M EG + 0.5 M sucrose (identified as EG4). To dilute the CPAs, sucrose solutions also prepared in H-CZB were used.

Oocyte Cryopreservation

Two cryopreservation experiments were conducted. In the first, three groups of oocytes suspended in EG5.5 were cooled either rapidly or ultra-rapidly, as described below, while a fourth group was held in H-CZB at 35°C as controls; this experiment was repeated a total of three times. In the second experiment, one group of oocytes was suspended in EG4; half of these were cryopreserved in LN_2 while the remainder were used as CPA controls. A second group was suspended in EG5.5; half of these were cryopreserved and the others used as CPA controls. A third group was held for the entire time in H-CZB at 35°C as controls. This experiment was repeated a total of four times.

Two different kinds of sample containers or holders were used to cryopreserve the oocytes: straws and grids. Plastic 0.25-ml straws (#A201; IMV, L'Aigle, France) were partially filled with a column of 200 µl of 0.5 M sucrose and then with a second column of 20 µl of EG5.5 separated from the first column by an 8-mm-long air bubble. At 35°C, oocytes in groups of 10–15 were exposed to 3 ml of EG5.5 for about 5–8 sec and immediately pipetted into the CPA column in the straws. A third 25-µl column of 0.5 M sucrose was aspirated into the straws, which were heat-sealed and immediately plunged into LN_2. To prevent cracking of the straw, first the bottom of the straw containing the two small columns was plunged into LN_2, and after a few seconds, the entire straw was lowered into the LN_2. The time that elapsed from the beginning of the CPA exposure to the plunging of the straws into LN_2 was 30 sec.

Electron microscope copper grids (GQ 400-C, 3.05 mm in diameter, 0.037 mm thick; Marivac Limited, Halifax, Nova Scotia, Canada) were used to obtain very high cooling rates. Oocytes in groups of 10–15 were exposed to 3 ml of the CPA solution (EG5.5 or EG4) for ~20 sec. With the aid of a fine pipette, they were then transferred onto the grid in a very small volume (< 1 µl). To further reduce the volume, the underside of the grid was blotted on a millicell membrane (Millipore, Bedford, MA), leaving the oocytes exposed (see Fig. 1). Grids, handled with a watchmaker's forceps, were immediately plunged into LN_2 or into N_2 slush, depending on the treatment. N_2 slush was prepared by connecting a vacuum pump to a glass vacuum desiccator that contained a polystyrene plastic cup filled with ~250 ml LN_2. Grids frozen in N_2 slush were then transferred into LN_2. The total time that elapsed from the beginning of the CPA exposure to plunging of the grids into LN_2 or into N_2 slush was 30 sec.

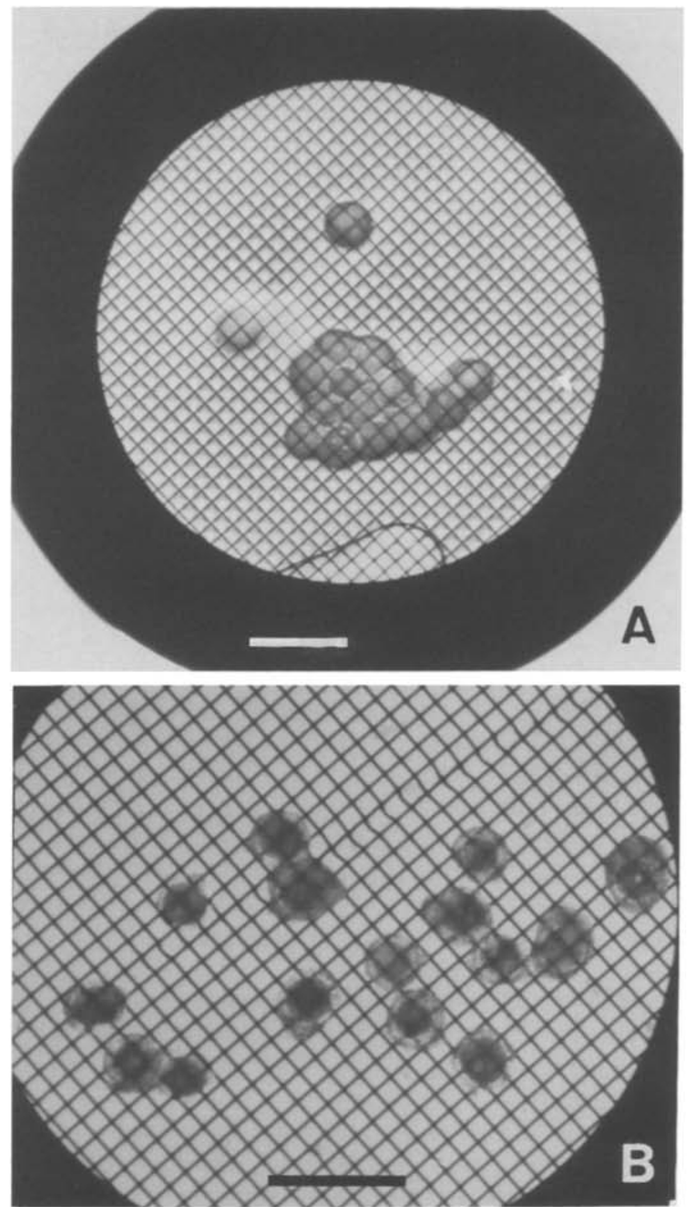

FIG. 1. Photographs of the electron microscope grids used to cryopreserve the oocytes. **A**) Oocytes immediately before being cooled in LN_2. **B**) Oocytes after warming, during the CPA dilution. Scale bar = 400 µm.

Oocyte Warming

Straws were warmed in a water bath at 37°C for 15 sec and then immediately shaken vigorously to mix the columns of fluid within them. The contents were expelled, and the oocytes were transferred into 0.5 M sucrose. One minute

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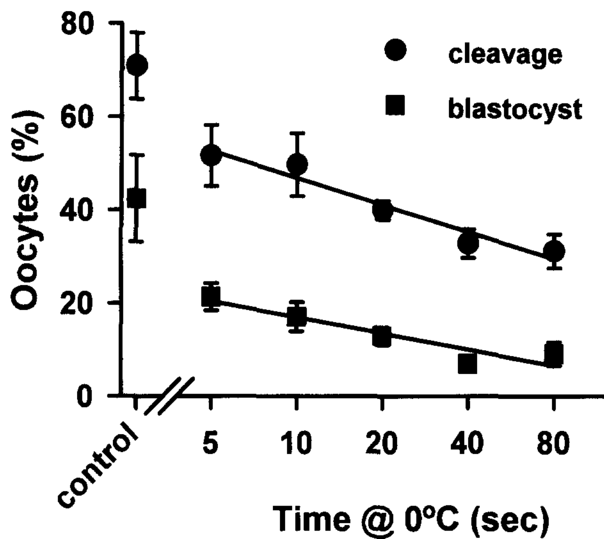

FIG. 2. Effect of cooling in vitro-matured oocytes at 0°C for short times on their subsequent development after IVF. Time has been plotted on a $\log_{10}$ scale on the x-axis. Development was calculated as a percentage of the total oocytes (n) that were chilled before IVF. Each point represents data pooled from three replicates and shows the mean ± SE.

later, the oocytes were transferred into 0.25 M sucrose solution for 1 min, into 0.125 M sucrose for 1 min, and finally into H-CZB for at least 5 min. The oocytes were washed in HEPES-TALP before undergoing IVF.

Grids with oocytes suspended in EG5.5 were warmed by being transferred as rapidly as possible directly from LN_2 into a culture dish containing 4 ml of 0.5 M sucrose at 37°C. After 5 sec, the grids were transferred to a second dish of 0.5 M sucrose to complete a 1-min exposure. The next steps (0.25 and 0.125 M sucrose into isotonic H-CZB) were the same as those described for the straws. The same procedure was used for oocytes vitrified in EG4 except that the first step was done in 0.25 M sucrose. Rather surprisingly, most of the oocytes adhered to the grids during the entire thawing-dilution procedure (see Fig. 1B). Oocytes were carefully recovered from the grids by being pipetted, while still held in the isotonic medium, before being subjected to IVF.

CPA Controls

In the second experiment, to determine the effect of exposure to the CPA solution at 35°C, oocytes were pipetted directly into EG5.5 or EG4, transferred to grids, and washed out of the CPAs by use of the same procedure as described above for the cryopreserved oocytes. Unhandled controls were maintained in H-CZB at 35°C during treatment of the experimental groups, which usually required about 1–2 h.

Evaluation of Survival

About 23 h after the cryopreserved oocytes had been warmed, morphological survival of presumptive zygotes, based on cytoplasmic appearance and membrane integrity, was evaluated by stereoscopic examination at ×40 magnification immediately before they were transferred into culture droplets.

Cleavage and blastocyst development were evaluated at 44 h and at 192 h (8 days), respectively, after insemination and calculated as a percentage of the total number of oocytes assigned to each treatment. In some experiments, embryos were cultured for 3 additional days to record the number of hatched blastocysts on Days 8, 9, 10, and 11. The hatching rate was calculated as a percentage of all blastocysts present on Day 8 that hatched completely from their zonae on each day. Some recently hatched blastocysts (< 8 h after hatching) were collected for cell counts.

Cytological Procedures

To evaluate fertilization, presumptive zygotes were vigorously shaken in tubes containing 3% sodium citrate to remove cumulus cells and then fixed in acetic acid-ethanol (1:3) for 48 h. Fixed presumptive zygotes were mounted on microscope slides, stained with 1% aceto-lacmoid, and observed under a phase-contrast microscope. Oocytes were considered penetrated when at least one spermatozoon was detected in the ooplasm. Penetrated oocytes were classified as follows: 2PN—oocytes with 2 fully developed, synchronous pronuclei and a sperm tail clearly visible in the ooplasm; PS—polyspermic oocytes penetrated by 2 or more spermatozoa; Abnormal—oocytes penetrated by a single spermatozoon showing any abnormality in the male or female pronucleus (lack of decondensation of the sperm head or lack of female pronucleus formation, delay or blockage of pronucleus formation, asynchronous development of the two pronuclei). To determine the average number of cells per embryo, blastocysts were fixed in acetic acid-methanol (1:3), spread on glass slides, and stained for counting [28].

Statistical Analyses

Regression analyses were performed to evaluate the relationship between the length of exposure at 0°C and the subsequent rates of cleavage and development into blastocysts. Percentages of morphological survival, cleavage, and development to the blastocyst stage were compared by one-way ANOVA following arcsin transformation of the proportions. In each treatment, the mean numbers of cells in hatched blastocysts were compared two by two by means of the Student's t-test. Differences were considered significant when $p < 0.05$.

RESULTS

Effect and Kinetics of Oocyte Cooling

The development into blastocysts of bovine oocytes cooled for only 5 sec to 0°C was only half that of the controls

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(21.3% vs. 40.4%, Fig. 2). Development measured both at 44 h (cleavage) and after 8 days (blastocyst formation) decreased progressively as a function of the duration of chilling (Fig. 2). In both cases, there was a high negative correlation between development and the length of exposure at 0°C (r^2 = 0.95 and 0.88, respectively). Development to the blastocyst stage was proportionally more affected than was cleavage.

All the oocytes looked normal shortly after exposure to 0°C. However, when they were transferred into the IVC droplets 22 h later, some of them had a light yellow cytoplasm clearly different from the dark-brown cytoplasm of the controls. This was observed mainly in the oocytes cooled for longer periods; although some of the yellowish oocytes did cleave, most did not, and none developed beyond the 2- to 4-cell stage.

Comparison of Oocytes Cryopreserved in Straws and on Grids

In this experiment, oocytes were cryopreserved in LN_2 either in straws or on grids, the controls consisting only of oocytes held in H-CZB at +35°C throughout the experimental treatment. Immediately after warming, most of the oocytes exhibited a normal appearance and were still surrounded by 1 or 2 layers of cumulus cells (Fig. 3A). It was difficult to evaluate their survival on the basis of morphology shortly after they were placed into culture. However, 22 h later, it was easy to observe signs of degeneration (uneven or contracted cytoplasm, or loss of a portion of cytoplasm in some oocytes) (Fig. 3B).

When judged after > 20 h in culture, the morphological survival of oocytes cryopreserved on grids (ranging from 51% to 72%) and straws (34%) was lower than that for the control (86%), although the differences were not significant (p = 0.053, Fig. 4A). Very high variability among replicates was observed in the percentage of morphological survival of oocytes cooled and warmed in straws (range = 6.9–73.3%). In addition, some of the oocytes cooled in straws had an even cytoplasm with no signs of degeneration, but with a yellowish color, as observed in the chilled oocytes. Very little development was obtained from oocytes cryopreserved in straws; 3% cleaved and < 1% developed into blastocysts. In contrast, although significantly lower than development of the controls, substantial development was achieved from oocytes cryopreserved on grids (Fig. 4, B and C). After IVF, 40% and 25% of oocytes on grids plunged in LN_2 and N_2 slush, respectively, cleaved, compared to 71% for the controls. The percentages that reached the blastocyst stage were 15% and 10% for those plunged into LN_2 and N_2 slush, respectively—significantly lower than 41% for the controls. The morphology of blastocysts derived from such oocytes cryopreserved on grids was normal and indistinguishable from that of the controls.

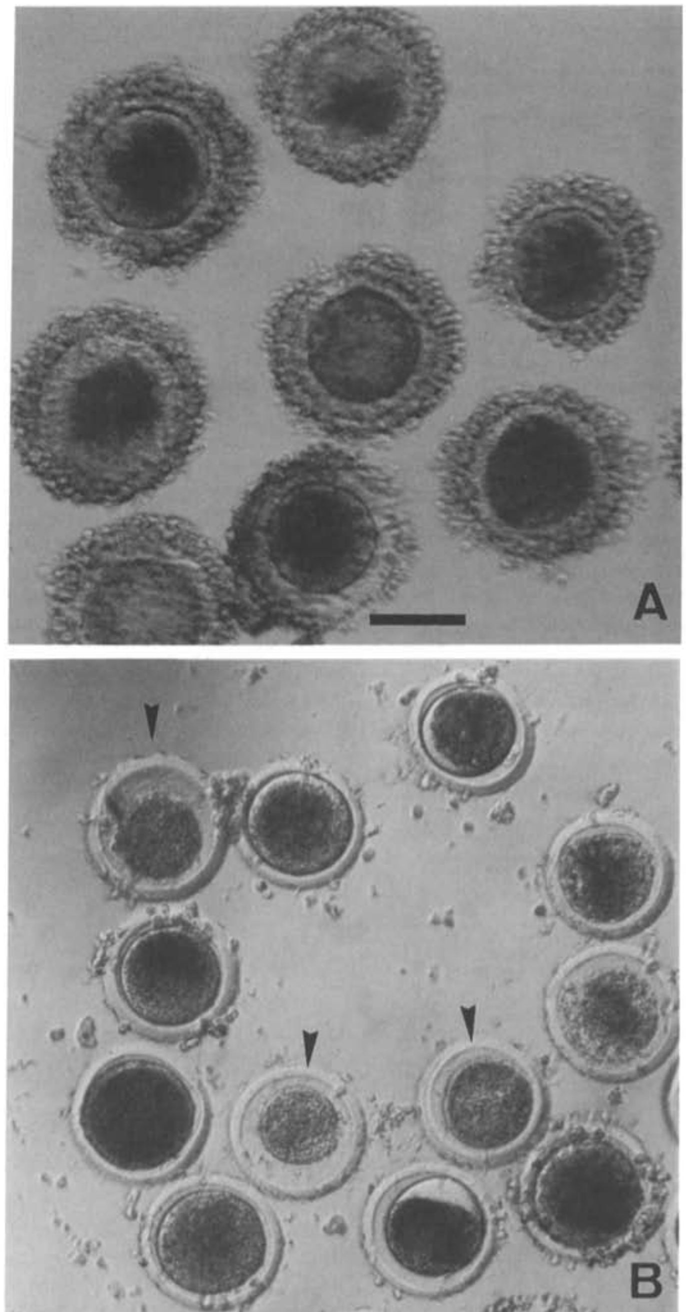

FIG. 3. Morphology of oocytes cryopreserved on grids. **A)** A group of oocytes a few minutes postwarming, after being pipetted off the grid into isotonic medium. **B)** A group of oocytes 22 h after warming, when they were transferred from the IVF droplets into the IVC droplets and the cumulus cells had been removed by vortexing. The most common manifestation of cell lysis was a contraction of the ooplasm, indicated by arrowheads. The bar is 100 μm.

Cryopreservation on Grids of Oocytes in EG5.5 and EG4

In the second cryopreservation experiment, two sets of oocytes exposed to each CPA were held at +35°C as two other sets were cryopreserved in LN_2; a fifth set was held in H-CZB at +35°C as overall controls. Again, when judged

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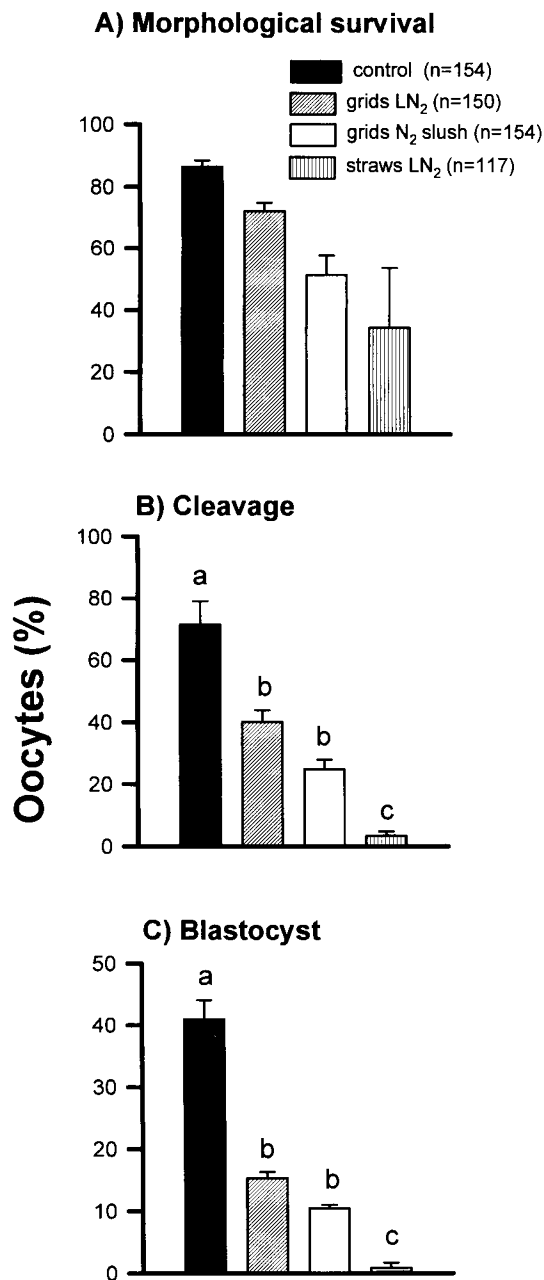

FIG. 4. Survival of oocytes suspended in EG5.5 and cryopreserved on grids (cooled in LN_2 or in N_2 slush) or in straws (cooled in LN_2), calculated as percentages of the total number (n) of oocytes cryopreserved. Different letters (a, b, c) indicate significant differences between treatments ($p < 0.05$). Data were examined by one-way ANOVA following arcsin transformation. Each bar represents data pooled from three replicates ± SE.

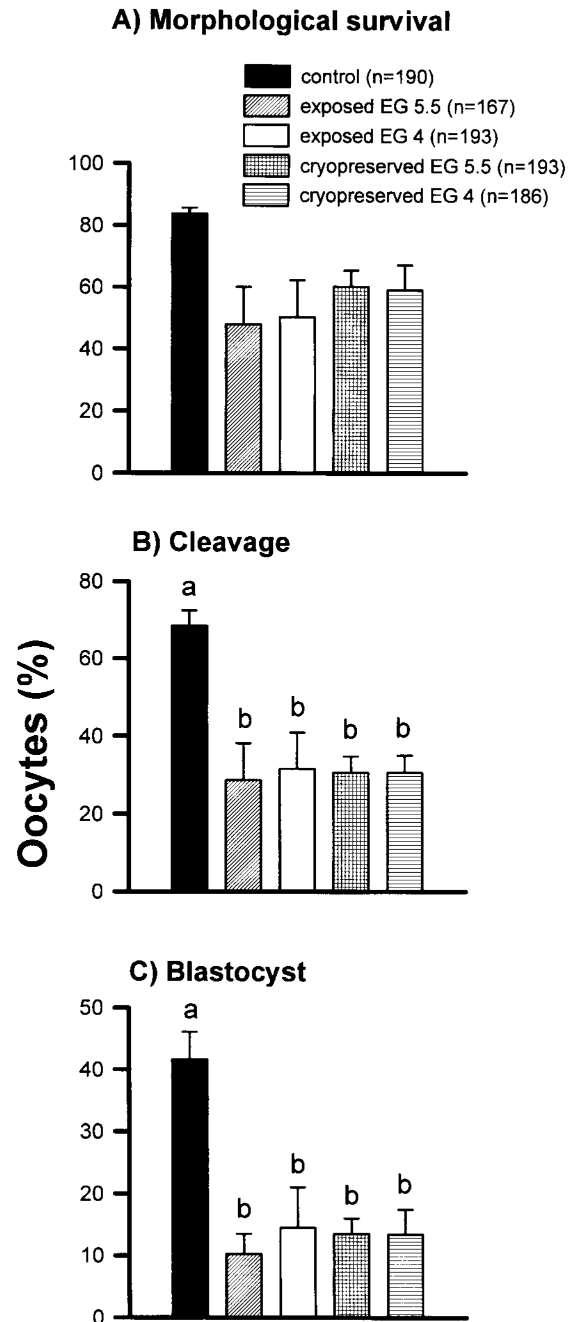

FIG. 5. Survival of oocytes exposed to EG5.5 and EG4 or cryopreserved on grids in EG5.5 and EG4. Percentages were calculated from the total number (n) of oocytes cryopreserved. Different letters (a, b) indicate significant differences between treatments ($p < 0.05$). Data were examined by one-way ANOVA following arcsin transformation. Each bar represents data pooled from four replicates ± SE.

after 22 h in culture, the morphological survival of all treated oocytes ranged from 48% to 60% (Fig. 5A). Although this was lower than the control value (83%), the differences were not significant. On the basis of cleavage (Fig. 5B) or development into blastocysts (Fig. 5C), the differences between the control and treated oocytes increased. For the controls, 68% cleaved and 42% developed into blastocysts. For the treated oocytes, 29–32% cleaved and 10–15% developed into blastocysts, regardless of whether they were only exposed to the CPAs at 35°C or were placed onto grids and plunged into LN_2. The reduction in development to the blastocyst stage was proportionally higher than for cleavage in all the treatments. The normalized figures (calculated as

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a fraction of the development obtained with the controls) were 25–35% for blastocyst development and 42–46% for cleavage.

On Day 8, the hatching rate was significantly lower for blastocysts derived from oocytes exposed to EG4 (11%) and for those derived from oocytes cryopreserved in EG4 (4%) or EG5.5 (4%) compared to the controls (20%). However, as a percentage of the total blastocysts that had formed by Day 8, differences were no longer significant for the total hatching rates on Day 9, 10, or 11. The total hatching rates on Day 11 were 72% and 69% for blastocysts from oocytes cryopreserved in EG4 and EG5.5, respectively. For those only exposed to EG4 and EG5.5, the hatching rates were 50% and 77%, respectively; for the overall controls the hatching rate was 75%.

The respective mean numbers of cells ± SE of the hatched blastocysts were 238.8 ± 15.9 (n = 11) and 234.2 ± 17.6 (n = 12) for embryos derived from oocytes cryopreserved in EG4 and in EG5.5; for those only exposed to the CPAs, the respective values for embryos derived from oocytes exposed to EG4 and to EG5.5 were 225.5 ± 22.8 (n = 11) and 215.9 ± 32.8 (n = 12). These figures were not significantly different from the control values (228.1 ± 10.4, n = 26).

Fertilization

Fertilization was evaluated at 18 h postinsemination. At that time, some of the oocytes had degenerated and could not be evaluated because they had been further damaged by the fixation process. Therefore, the fertilization data (Fig. 6) represent only oocytes that were judged from their morphology to have survived fixation: 87% and 72% of the control and cryopreserved oocytes, respectively. These percentages of morphological survival were equivalent to the ones shown in Figures 4A and 5A. In comparison to the controls, the percentage of penetrated oocytes was significantly reduced in frozen oocytes (62% vs. 89%). However, the proportion of normally fertilized zygotes (calculated from the penetrated oocytes) was not statistically different (60% in the frozen group, 66% in the controls). Polyspermy was similar in the two groups (cryopreserved, 20%; control, 23%). There was an increase in the proportion of oocytes abnormally fertilized after cryopreservation, but the differences from the controls were not significant (20% vs. 11%). The main fertilization abnormalities were lack of decondensation of the sperm head and asynchrony in pronucleus formation.

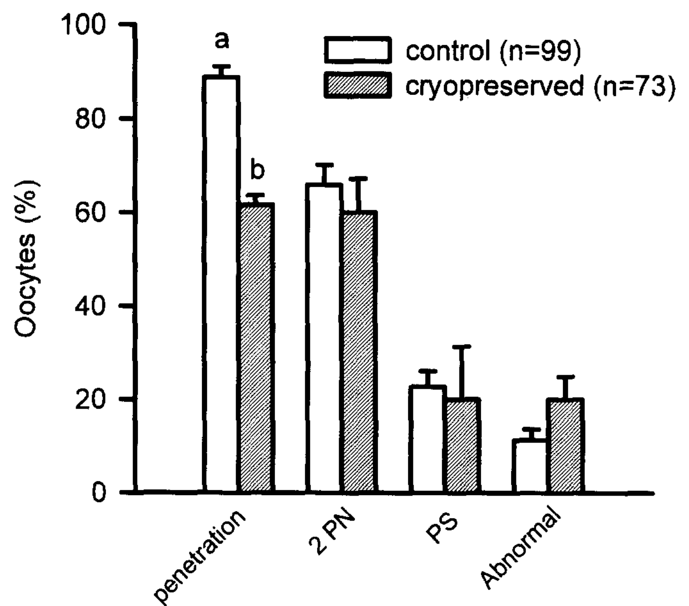

FIG. 6. Fertilization of oocytes cryopreserved on grids in EG5.5, compared to controls. Penetration was calculated as a percentage of the total number (n) of oocytes evaluated; 2 PN, PS, and Abnormal were calculated as percentages of penetrated oocytes. Different letters (a, b) indicate significant differences between treatments ($p < 0.05$). Data were examined by one-way ANOVA following arcsin transformation. Each bar represents data pooled from three replicates ± SE.

DISCUSSION

These data demonstrate that in vitro-matured bovine oocytes can be cryopreserved with very high cooling rates. After being warmed and fertilized, 15% of such oocytes developed into blastocysts, a rate clearly higher than what has been obtained up to now with conventional freezing protocols. Since the first paper on this subject was published less than 10 yr ago [29], several groups have described procedures to freeze bovine oocytes. However, embryonic development from oocytes frozen either by equilibrium or by nonequilibrium cooling has been low, with usually < 3% of the oocytes developing into blastocysts [13–20, 22, 30]. There are only three reports of pregnancies or births after the transfer of embryos derived from frozen bovine oocytes [13–15].

In vitro-matured bovine oocytes are known to be extremely sensitive to chilling; Aman and Parks [31] showed that the metaphase II spindle was damaged in 56% of oocytes cooled to 25°C for only 1 min, and only 10% of oocytes cooled to 4°C for 1 min contained normal spindles. The fertilization rate of oocytes exposed to 0°C for only 2 min [7] and the cleavage of oocytes cooled to 0°C or −7°C [32] are significantly reduced. We have reported recently that the developmental competence of immature oocytes is also impaired after chilling to 10°C for 30 min or to 0°C for only 30 sec [33], and that the chilling damage is time-dependent. Although immature chilled oocytes can undergo meiotic maturation and be fertilized in vitro, their subsequent embryonic development is compromised. The present results show a dramatic effect of cooling on the developmental potential of in vitro-matured oocytes. The reduction in developmental competency is highly correlated with the du-

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ration of exposure to 0°C. Chilling damage develops very rapidly; the developmental potential is halved by exposure of the oocytes to 0°C for only 5 sec (Fig. 2).

Most attempts to freeze bovine oocytes have involved conventional procedures similar to those developed to freeze embryos. These protocols include slow or direct cooling of the oocytes to the seeding temperature of about −7°C, holding the oocytes at this temperature for a few minutes before and/or after the seeding, and finally cooling them at < 1°C/min to an intermediate subzero temperature of ∼ −30°C before plunging them into LN_2. Therefore, oocytes must be exposed for extended times to temperatures below +10°C, temperatures now shown to be damaging. Considering the temperature range of +10°C to −10°C, we can calculate that oocytes frozen according to the various slow cooling procedures described in the literature must have been exposed to those temperatures for periods between 10 and 40 min [13, 16–21, 30]. Chilling sensitivity alone, therefore, can explain the low rate of development obtained after freezing and thawing of bovine oocytes. There is no evidence to suggest that cryoprotectants can prevent chilling damage in that temperature range. Niemann et al. [32] reported that the developmental potential of bovine oocytes cooled to 0°C or −7°C was compromised even though the oocytes had been equilibrated in 2 M DMSO or propylene glycol.

Even the first two reports of bovine oocyte cryopreservation documented this chilling damage in immature [29] and in in vitro-matured oocytes [30]. Examining the effect of every step of the freezing protocol on subsequent oocyte development, Glass and Voelkel [30] concluded that cooling oocytes to −7°C, as well as seeding and holding them at that temperature, was responsible for the loss in viability observed after oocyte freezing. However, little attention has since been directed to solving this problem. Recent studies have attempted modifications of conventional protocols, including the use of different CPAs, cooling rates, and dilution procedures, but the overall development after freezing-thawing is still very low [18–20]. In a very comprehensive study in which more than 3000 oocytes were frozen, Schellander et al. [20] concluded that changing conventional freezing variables would probably not improve the cryopreservation of cattle oocytes.

The question is whether even rapid cooling or vitrification will produce cooling rates fast enough to circumvent the extreme chilling sensitivity of bovine oocytes. The fastest cooling rate that can be obtained by plunging a plastic insemination straw directly in LN_2 is ∼2500°C/min [11]. Therefore, rapid freezing exposes oocytes to harmful temperatures (assumed to lie between +15° and −15°C) for ∼1 sec during cooling to −196°C and again during warming to +35°C. Even this short exposure might be enough to compromise the viability of some of the oocytes. The lower chilling sensitivity of mouse oocytes in comparison to cattle oocytes [9, 31] would explain why the former can be frozen through use of slight modifications of conventional embryo freezing protocols, either by slow cooling [34, 35] or by rapid cooling/vitrification [36, 37]. Bovine embryos before the morula stage, and porcine oocytes and embryos up to the hatched blastocyst stage, are chilling-sensitive and cannot be cryopreserved successfully using conventional protocols [38–41].

The clearest example of chilling-sensitive embryos that have been successfully frozen is *Drosophila* embryos. This has only been possible with use of extremely high cooling rates to circumvent chilling damage [23–25]. This is the approach that we have followed in the present work. Having demonstrated that chilling injury of bovine oocytes is time-dependent, we reasoned that cooling the oocytes at very high rates might enable them to pass through the damaging temperature zone in the fluid state fast enough to circumvent the chilling damage, until the entire system solidified below −130°C. To achieve very high cooling rates, we have developed a cryopreservation method that uses electron microscope grids as a physical support on which to plunge oocytes directly into LN_2, similar to the method described by Steponkus et al. [23] for *Drosophila* embryos. We believe that the significant improvement in survival, compared to that of oocytes cryopreserved in straws, is due to the very high cooling rates achieved with the grids. The cooling rate estimated for *Drosophila* embryos cooled on grids that were plunged directly into LN_2 was 3000°C/min, a rate only about threefold higher than that obtained with straws [23]. The small diameter of bovine oocytes (0.15 mm) compared to the size of *Drosophila* embryos (1 × 1.5 mm), and the small diameter (7 μm) of the filaments of the grid we used (see Fig. 1), ought to have increased the cooling rates in our experiments. The observation that some oocytes cryopreserved in straws (but none on grids) exhibited the yellowish appearance of chilled oocytes is consistent with the argument that cooling rates achieved with grids were very much higher than with straws.

To test whether the use of even faster cooling rates would improve survival, we cryopreserved some oocytes by plunging the grids directly in N_2 slush. According to Steponkus et al. [23], the use of N_2 slush ought to increase the cooling rate of a 20-μl sample over the range of 0° to −60°C from 3000°C/min to 24 000°C/min. In a recent experimental study of cooling very small samples, Han et al. [42] confirmed that quenching samples in N_2 slush rather than in LN_2 substantially increases cooling rates. Furthermore, they showed that the size of the sample also greatly influences cooling rate; the respective rates of 2-mm and 0.2-mm samples were 30 000°C/min and 180 000°C/min. Perhaps the latter rate would apply to bovine oocytes that have a 0.15-mm diameter and are contained in < 1 μl of fluid. The fact that development into blastocysts of oocytes only exposed to CPAs was not different from development of oocytes cryo-

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preserved on grids suggests that the damage observed after cryopreservation can be attributed to the effects of the CPA, rather than to the cryopreservation procedure per se. Thus, the development observed after CPA exposure (10–15% blastocyst formation) may be the maximum achievable with any cooling rate with use of these particular CPAs.

Bovine oocytes seem to be very sensitive to exposure to CPA solutions. The exposure of in vitro-matured oocytes to CPA solutions of low concentrations, such as 1.5–2 M propylene glycol or DMSO, for 10–20 min has no effect on subsequent cleavage [30, 43]. However, oocytes are more likely to be damaged when exposed to high solute concentrations present in vitrification solutions [22, 44]. No differences were observed after exposure of the oocytes to either of the two CPA solutions tested in the present study. The lower concentration of 4 M EG ought to have reduced the likelihood of osmotic damage during CPA dilution.

The decreased survival observed after CPA exposure can be explained either by a direct toxic effect of the CPA itself or by osmotic effects. The higher sensitivity of immature oocytes as compared to in vitro-matured bovine oocytes [44] might reflect a toxic effect of the CPAs. However, osmotic stress produced by exposure to solutions of very high osmolality also has deleterious effects on oocyte survival, analogous to those described for mouse zygotes [45]. The hydraulic conductivity of immature bovine oocytes is only half that of in vitro-matured oocytes [46], a fact that would render the former more likely to suffer osmotic shock during dilution of a CPA.

Our observations are consistent with the interpretation that the oocytes were damaged by osmotic changes rather than a direct toxic effect of the CPA. Direct dilution of the CPA in which the oocytes were suspended yielded lower survival than the 3-step dilution that we have used (data not shown). Our microscopic observations of oocytes exposed to EG4 and EG5.5 (data not shown) revealed that the response of the oocytes was dramatic, with a very rapid contraction of the vitellus itself. This contraction was usually uneven, the shrinking oocyte adopting an irregular nonspherical shape and collapsing inwards. The oocytes used in our experiments had one or two layers of corona cells. The persistence, after IVM, of microfilaments in the foot processes extending from corona cells through the zona onto the surface of the oocyte [47] could explain the irregular shrinkage observed in bovine oocytes exposed to CPAs. We believe that this folding or compaction of the cytoplasm might have deleterious "mechanical" effects on its integrity.

One possibility often suggested as a way of avoiding the damaging effects of CPA solutions is to expose oocytes at lower temperatures, as was done by Rall and Fahy in the original vitrification procedure [11]. The fertilization rates of human oocytes decrease after exposure to DMSO at 37°C, but not at 0°C [48]. However, the extreme chilling sensitivity of bovine oocytes precludes this approach. Another possibility would be to use lower CPA concentrations. We have obtained similar results using either 4 M EG + 0.5 M sucrose or 5.5 M EG + 1 M sucrose, suggesting that with very high cooling rates, the CPA concentration is not a critical factor. This is consistent with observations obtained for rapidly cooled 8-cell mouse embryos; survival of embryos cryopreserved in 2 M EG + polyvinylpyrrolidone + galactose was the same as that of embryos cryopreserved in 6 M EG + polyvinylpyrrolidone + galactose [49]. We do not know the intracellular EG concentration within the oocytes at the time when they were plunged into LN_2, but it is our view that oocyte dehydration is probably more important for successful cryopreservation than the absolute amount of CPA present in the oocyte. As was suggested many years ago for mouse embryos [50], a minimum amount of CPA in the oocyte is almost certainly necessary to protect it against cryoinjury, since embryos cannot be cryopreserved with sucrose alone [51, 52]. Although the solution of 4 M EG + sucrose does not vitrify when cooled in straws, the very small volume (< 1 μl) present on the surface of the grids probably does vitrify. Regardless of that, vitrification of the extracellular solution is not necessary to obtain high survival of rapidly cooled embryos [49, 53]. What is undoubtedly more important is that the high intracellular concentration of solutes and macromolecules achieved as a consequence of dehydration of the oocytes exposed to the CPA solution contributes to intracellular vitrification, as discussed by Rall [11]. This is especially so with very high cooling rates and very small volumes, even with low CPA concentrations [49, 52].

If dehydration is one of the most important factors determining the effectiveness of the CPA solution under our conditions, another way to decrease potential toxic effects of CPAs would be to shorten the exposure time. Preliminary observations indicate that oocytes exposed to 5.5 M EG or 4 M EG require very few seconds to contract to their minimum volume, although a precise study of the volume changes during the CPA exposure would be necessary to fix the optimum time of exposure. Nakagata et al. [10] obtained good survival of mouse oocytes after vitrification using a very short exposure (5–15 sec) to a solution containing 2 M DMSO, 1 M acetamide, and 3 M propylene glycol. Hamano et al. [14] used a 10-sec exposure to the same solution to vitrify bovine oocytes, with 9 of 100 oocytes developing into blastocysts after fertilization and culture.

We observed a decrease in the penetration rate of sperm into cryopreserved oocytes. The same observation has been previously described for bovine oocytes after slow cooling or vitrification [13, 18]. With mouse oocytes, cooling alone as well as CPA exposure can decrease fertilization rates as a consequence of zona hardening mediated through the premature release of the cortical granules (reviewed in [8, 9]). The zonae of dead immature bovine oocytes (from frozen ovaries) are penetrated at lower rates than those of in vitro-matured oocytes [54], but it is difficult to conclude

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from these results whether or not this is a consequence of the direct effect on the zona. Although we have not examined the effect of CPA exposure on the fertilization rate in the present study, our previous observations indicate that fertilization itself is not affected by chilling to 0°C for 1 min or less (Martino and Leibo, in preparation). Moreover, the fact that similar development rates were obtained with oocytes cryopreserved on grids and with those only exposed to the CPA also suggests that it is the CPA alone, not the chilling, that was probably responsible for the lower fertilization rates. Apart from the decrease in the total fertilization rate, oocytes that survived cryopreservation and were penetrated by a spermatozoon had a cytoplasm able to support formation of morphologically normal pronuclei. In addition, the proportion of CPA-exposed or cryopreserved oocytes that cleaved was higher, compared to the control, than the proportion developing to the blastocyst stage. Thus, the parameters estimated within shorter times after warming (morphological survival, fertilization, cleavage) are less affected than those evaluated at later times (blastocyst formation).

Although formation of blastocysts derived from cryopreserved or CPA-exposed oocytes was completed by Day 8, as in the control, some of them showed a slightly delayed development. We observed a lower degree of expansion at Day 7 or 8 in some of the blastocysts from cryopreserved oocytes, and found a lower hatching rate on Day 8. However, cumulative hatching rates, as well as the number of cells in blastocysts, were not different from the control values, indicating that the quality of these embryos was similar to that of normal, in vitro-produced embryos. Efforts are in progress to determine the ultimate viability of those embryos by their transfer into recipient cows.

In conclusion, we have developed a method to cryopreserve bovine oocytes using very high cooling rates to circumvent the extreme chilling sensitivity of this type of cell. The decrease in survival that we observed after warming cryopreserved oocytes can be largely explained by the injury produced by the CPAs. The derivation of different CPA solutions or modification of the exposure/removal procedures may yield improved survival. Although this method is more difficult to perform than standard procedures using straws, its application may be justified in special cases. For example, when there is great concern about possible chromosomal damage caused by cooling or freezing, this method may permit effective cryopreservation of oocytes from female domestic animals of high genetic value or of human oocytes.

ACKNOWLEDGMENTS

We thank Mr. Pedro Puig for his assistance and advice with the statistical analyses of the data. We also thank Mr. Thomas Kroetsch and United Breeders Inc., Guelph, Ontario, for providing the cryopreserved samples of bull sperm used in this study; Professor K.J. Betteridge for his advice and very useful review of this manuscript; and Dr. H.D. Goff and Ms. Sandy Smith for their generous help and for the use of their high capacity vacuum equipment to prepare N_2 slush.

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