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Effects of vitrification of immature bovine oocytes on *in vitro* maturation

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Abstract

The aim of this study was to evaluate the effect of ethylene glycol (EG) concentration, equilibration time, and the use of two disaccharides in the vitrification solution used on immature bovine oocytes that were thawed and then matured *in vitro*. Factorial combinations of the following were tested: three equilibration solutions (ES), containing 3, 20 or 40% EG; three equilibration times, 0.5, 5 or 15 min; and two vitrification solutions (VS), containing 40% EG + 1.0 M trehalose or 40% EG + 1.0 M sucrose. The control treatment had fresh, non-vitrified oocytes maturated *in vitro*. The total number of immature oocytes distributed across the 19 treatments was 2103. The combination VS with sucrose, an equilibration time of 5 min, and a ES with 20% of EG had the highest MII (metaphase II) rate (44.5%) that was more than twice the rate of the next best combination of vitrification treatments but less ($P<0.05$) than the non- vitrified control (74.6%). The lowest MII rates in treatments with sucrose were found in combinations of ES with 40% EG and equilibration times of 5 and 15 min (0.0 and 0.9%, respectively). The highest MII rate for treatments with trehalose was 5.3%. High chromatin condensation rates were found in treatments with trehalose. In conclusion, the use of trehalose in the vitrification solution impaired the oocyte maturation. Better results were obtained with sucrose. A high concentration (40%) of EG in addition to a long equilibration time (5 or 15 min) was detrimental to oocyte maturation. Vitrification of immature bovine oocytes using 20% EG in the ES, an equilibration time of 5 min, and a VS containing 40% EG + 1.0 M sucrose, yielded acceptable *in vitro* maturation rates.

Keywords: vitrification, oocyte, bovine.

Introduction

Unfortunately, the success observed in sperm cryopreservation does not occur with oocyte cryopreservation. Cryopreserved bovine semen has been used commercially for decades, and the conception rates obtained with that semen are comparable to those obtained with natural mating (Watson, 2000). However, experimental results from cryopreservation of bovine oocytes are still extremely variable (Vajta, 2000).

The difficulty, in obtaining acceptable rates of survival and functionality for oocytes after cryopreservation, is due to the size of this cell and its unique morphologic characteristics. Vitrification is an alternative that may increase oocyte survival after thawing. Vitrification is the solidification of a solution at low temperatures without ice crystal formation. This phenomenon requires either rapid cooling rates (Rall, 1987) or the use of concentrated cryoprotectant solutions, which depress ice crystal formation and increase viscosity at low temperatures (Vajta, 2000).

To achieve rapid cooling (2500°C/min), a common procedure is to plunge a sealed straw directly into liquid nitrogen (Palasz and Mapletoft, 1996). More recently, a further increase in the cooling rates (more than 20,000°C/min) was achieved using new rapid-cooling techniques (Martino et al., 1996; Vajta et al., 1998; Lane et al., 1999; Dinnyes et al., 2000; Papis et al., 2000; Matsumoto et al., 2001) that are based on direct contact between the cryoprotectant and the liquid nitrogen. Although good results were obtained using these new techniques, the risk of biological contamination (Bielanski et al., 2000; 2003) has led some researchers to replace open containers with sealed straws in vitrification procedures (Eun et al., 2002; Park et al., 2003).

With vitrification procedures, the exposure time of oocytes to cryoprotectant solutions must be short due to the toxic effects of high cryoprotectant concentrations. However, if the exposure is too short, the penetration of the cryoprotectant will be inadequate and intracellular ice could form, even in the absence of extracellular ice (Otoi *et al.,* 1998). The way to circumvent the noxious effects of cryoprotectants in the vitrification process could be through the use of high cryoprotectant concentrations for short periods of time (Vajta *et al.,* 1998) or increasing the equilibration period by using lower cryoprotectant concentrations (Papis *et al.*, 2000).

Several studies demonstrated that ethylene glycol would be the ideal cryoprotectant (Shaw *et al.*, 1997), because it penetrates membranes faster than glycerol (Cha *et al.*, 2000) and is less toxic than other permeable cryoprotectants (Martino *et al.*, 1996; Cha *et al.*, 2000; Dinnyes *et al.*, 2000). Freezing solutions, containing permeable (usually ethylene glycol) and non-

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permeable cryoprotectants, seem to be more advantageous than solutions containing just a permeable cryoprotectant (Shaw *et al*., 2000).

The disaccharides sucrose and trehalose are the most common non-permeable cryoprotectants used for oocyte cryopreservation. Sucrose acts as a stabilizer, minimizing the effects of high concentrations of ethylene glycol (Fagundes, 2002). Trehalose seems to act directly on lipids and proteins of the membrane, altering their behavior (Holt, 2000), and replacing water molecules on the membrane surface, thus inhibiting denaturation and aggregation of proteins during dehydration (Puhlev *et al*., 2001).

The aim of this study was to evaluate the effects of ethylene glycol concentration, equilibration time, and the use of two disaccharides in the vitrification solution used on immature bovine oocytes that were thawed and then matured *in vitro*.

Materials and Methods

Collection of cumulus-oocyte complexes

The experiment was conducted at the Laboratory of Animal Reproduction, Veterinary Department, Federal University of Viçosa. Ovaries were obtained from cows or heifers at a local slaughterhouse. Immediately after slaughter and evisceration, ovaries were removed and placed in thermo flasks containing physiologic solution at 35 to 38^{0}C and supplemented with streptomycin sulfate (Sigma Chemical Co., St. Louis, MO, USA; Sirard and Bilodeau, 1990). At the end of collection, ovaries were brought to the laboratory in the thermo flasks within two hours of slaughter.

At the laboratory, oocytes were harvested from ovaries by aspirating the follicles (≤ 10 mm) using a 25 x 7 gauge needle attached to a 5 ml syringe. Oocytes were selected and classified morphologically using a stereomicroscope according to Costa et al. (1997a). Only oocytes with at least three layers of compact cumulus cells and a homogeneous ooplasm were used in the experiment.

Experimental design

The experimental design was set up according to a factorial procedure (3 x 3 x 2) based on 3 equilibration solutions (ES), 3 equilibration times, and 2 vitrification solutions (VS) for a total of 18 experimental treatments and one control treatment. Each treatment was replicated five times.

The immature oocytes were distributed across 19 treatments with five replicates for each treatment. Each replicate contained about 20 oocytes per treatment. Three ES's, containing 3, 20, or 40% EG (Sigma), three equilibration times of 0.5, 5 or 15 min, and VS's containing 40% EG + 1.0 M trehalose (Sigma) or 40% EG + 1.0 M sucrose (Sigma) were tested. The control treatment had 110 fresh oocytes that, immediately after selection, were submitted to the *in vitro* maturation (IVM) procedure.

Equilibration and vitrification

The holding medium used for manipulation, equilibration, vitrification, and rehydration solutions was Talp-Hepes (Bavister *et al*., 1983) supplemented with 0.4% of bovine serum albumin (Sigma) and adjusted to 285 mOsm/kg with H_2O. For equilibration, oocytes of each treatment were kept in an ES for 0.5, 5 or 15 min. After the end of the equilibration time, oocytes were transferred to a VS. In the VS, oocytes of all treatments were maintained for one minute. During this time, oocytes were loaded into 0.25 mL straws in the following order: a column of VS, an air bubble, a column of VS containing six to ten oocytes, an air bubble, and a column of VS. The straws were then sealed and plunged directly into liquid nitrogen at the end of one minute. Approximately one-half of the straw was dipped quickly and the remaining half was dipped slowly into the liquid nitrogen.

Thawing and rehydration

The oocytes of all treatments were thawed by exposure of straws to air for 5 sec followed by immersion in a water bath at 37 °C for 30 to 45 sec. After immersion in the water bath, oocytes were gradually rehydrated in trehalose (for treatments 1 to 9) or sucrose solutions (for treatments 10 to 18). Oocytes were expelled into the holding medium with 0.5 M of trehalose or sucrose and held for one minute. Oocytes were then transfered successively into a holding medium with 0.5, 0.33, and 0.17 M of trehalose or sucrose for one min in each solution. At the end of the last rehydration, oocytes were washed successively (three times) in holding medium.

The recovery rate was definied as the number of oocytes counted after the end of rehydration, in relation to the total of oocytes vitrified. Oocytes with fractured zona pellucida and with loss of the cytoplasmic contents were discarded. The remaining ooyctes were submitted to IVM.

In vitro maturation (IVM)

The maturation medium used was TCM 199 (Cultilab, Campinas, SP, Brazil) supplemented with 10% estrous-cow serum and 10 ng/mL of FSH (Calier Lab., Osasco, SP, Brazil; Costa, 1994). *In vitro maturation* was carried out in Petri dishes (35mm diameter) that contained 1.5 mL of maturation medium previously equilibrated for at least 2 h at 38.5 ^{0}C in a humidified atmosphere of 5% CO2 in air. Oocytes were cultured in these conditions for 22 to 24 h. After this period, maturation rates were evaluated.

Evaluation of maturation status

For evaluation of maturation rates, cumulus cells were removed from oocytes (Costa *et al*., 1997b). Next, oocytes were placed in a hypotonic medium, fixed on glass slides, and stained with 2% of orcein according to Costa *et al*. (1997c). The slides were examined at 1000X magnification under immersion oil to evaluate the stage of nuclear maturation. Oocytes were classified according to the configuration of the chromosomes as metaphase II (MII), anaphase I (AI), metaphase I (MI), prophase I (PI), condensed chromatin (CC), or without chromosomal configuration (WCC). Oocytes in MII were considered mature.

Statistical analysis

The analysis was set up according to the factorial design (3 x 3 x 2) of the experiment. The data collected from the experiment were subjected to an arcsine transformation, and the transformed values for individual treatments were compared by ANOVA using the SAS program (SAS, 1999). When ANOVA revealed a significant (P<0.05) treatment effect, the transformed data were compared by paired t-tests.

Results

The ES, equilibration times, and VS did not influence the recovery rate. The mean recovery rate was 97% of the 1993 oocytes vitrified. In general, the incidences of fractured zona pellucida and loss of cytoplasmic content were low (1.8 and 0.7%, respectively) and did not vary among the treatments.

The highest MII rate in treatments that used trehalose was 5.3%. With most of the trehalose treatments, very few oocytes reached the MII stage (Table 1). Using sucrose (Table 2), an equilibration time of 5 min and a ES with 20% EG (treatment 14) yielded the highest MII rate (44.5%). Table 2 also shows that the lowest MII rates following treatments that used sucrose were found in combinations of ES with 40% EG and an equilibration time of 5 or 15 min (0.0 and 0.9%, respectively). Using the concentration of 40% EG, the equilibration time of 0.5 min was less detrimental (maturation rate of 16.8%).

Table 1. *In vitro* maturation rates of immature bovine oocytes vitrified with solutions containing trehalose

Treatment	ES	ET	N	MII % (SD)	AI % (SD)	MI % (SD)	PI % (SD)	WCC % (SD)	CC % (SD)
Control (0)	-	-	110	74.6a (5.4)	0.0a (0.0)	14.5c (7.5)	0.0a (0.0)	2.7a (3.8)	7.8a (6.2)
1	3%	15 min	110	0.0b (0.0)	0.0a (0.0)	14.9c (4.2)	3.1[a] (4.7)	11.3[a],b (6.8)	70.6b,c,d (12.7)
2	20%	15 min	111	0.0b (0.0)	0.0a (0.0)	2.5[a],b (3.4)	0.0a (0.0)	12.6[a],b (8.8)	84.9d,e (5.7)
3	40%	15 min	108	0.0b (0.0)	0.0a (0.0)	0.0a (0.0)	0.0a (0.0)	13.7b (8.6)	87.1e (7.9)
4	3%	5 min	104	0.0b (0.0)	0.0a (0.0)	9.5b,c (6.9)	0.0a (0.0)	16.7b (7.3)	73.8b,c,d,e (11.3)
5	20%	5 min	105	5.3b (5.1)	0.0a (0.0)	25.8d (12.1)	0.0a (0.0)	9.0a,b (2.3)	59.9b (13.5)
6	40%	5 min	104	0.0b (0.0)	0.0a (0.0)	1.0a,b (2.2)	2.1[a] (4.7)	11.8[a],b (5.5)	85.1d,e (3.6)
7	3%	0.5 min	100	1.2b (2.8)	0.0a (0.0)	9.4b,c (10.2)	1.2[a] (2.8)	8.0a,b (7.3)	80.1c,d,e (20.1)
8	20%	0.5 min	104	3.0b (4.1)	0.0a (0.0)	12.5c (12.6)	2.6a (3.6)	13.7b (13.5)	68.3b,c (15.9)
9	40%	0.5 min	105	1.4b (3.2)	0.0a (0.0)	6.5[a],b,c (5.1)	3.4[a] (4.7)	12.1[a],b (8.0)	79.4c,d,e (11.1)

* values in same column without common letters differ (P<0.05)
ES: equilibration solution (% of ethylene glycol); ET: equilibration time; N: number of oocytes evaluated; MII: metaphase II; AI: anaphase I; MI: metaphase I; PI: prophase I; WCC: without chromosomal configuration; CC: chromatin condensation; SD: standard deviation.

The MII rate of the treatment that used ES with 20% EG, an equilibration time of 5 min, and a VS with sucrose was lower (P<0.05) than the control treatment (44.5 and 74.6%, respectively). However, the rates of PI, AI, MI, CC and WCC were not different (P>0.05; Table 2). High CC rates were observed when trehalose was used in the VS (Table 1). But, elevated CC rates were also observed when sucrose was added to the VS (Table 2).

Table 2. *In vitro* maturation rates of immature bovine oocytes vitrified with solutions containing sacarose

Treatment	ES	ET	N	MII % (SD)	AI % (SD)	MI % (SD)	PI % (SD)	WCC % (SD)	CC % (SD)
Control (0)	-	-	110	74.6a (5.4)	0.0a (0.0)	14.5c (7.5)	0.0a (0.0)	2.7a (3.8)	7.8a (6.2)
10	3%	15 min	103	19.4c (7.2)	0.00a (0.0)	12.3a (8.9)	4.8b (4.8)	13.3b (8.3)	49.3b (6.7)
11	20%	15 min	105	12.0c (13.1)	0.00a (0.0)	18.0a (10.0)	2.3a,b (3.3)	10.9a,b (4.6)	56.8b (11.8)
12	40%	15 min	102	0.9d (2.1)	1.0a (2.3)	0.00b (0.0)	2.2a,b (3.1)	16.9b (8.4)	78.9d (5.0)
13	3%	5 min	104	18.1c (9.6)	0.00a (0.0)	11.7a (6.9)	2.4a,b (3.3)	11.8a,b (11.0)	55.9b (15.6)
14	20%	5 min	109	44.5b (6.9)	1.0a (2.3)	20.5a (11.1)	1.3a,b (3.0)	12.6a,b (11.9)	19.9a (13.0)
15	40%	5 min	105	0.0d (0.0)	0.00a (0.0)	13.6a (6.5)	5.3b (9.1)	15.3b (11.8)	65.8c (11.7)
16	3%	0.5 min	104	11.0c (5.6)	0.00a (0.0)	14.6a (7.1)	3.7a,b (3.4)	6.2a,b (4.7)	64.4b,c (15.4)
17	20%	0.5 min	108	21.6c (13.7)	0.00a (0.0)	12.4a (9.0)	2.4a,b (3.4)	11.1a,b (9.2)	52.5b (22.3)
18	40%	0.5 min	103	16.8c (9.0)	1.0a (2.3)	19.0a (5.1)	1.0a,b (2.3)	9.1a,b (6.2)	53.0b (10.2)

* values in same column without common letters differ (P<0.05)

ES: equilibration solution (% of ethylene glycol); ET: equilibration time; N: number of oocytes evaluated; MII: metaphase II; AI: anaphase I; MI: metaphase I; PI: prophase I; WCC: without chromosomal configuration; CC: chromatin condensation; SD: standard deviation

Discussion

Although full-term development of embryos derived from vitrified immature bovine oocytes has already been reported (Vieira *et al*., 2002), most results obtained using this technique were considered unsatisfactory because of low blastocyst development rates (less than 10%). With such poor results, we decided to evaluate the *in vitro* maturation rate, which is a much simpler procedure and enabled the testing of a larger number of vitrification protocols. In the present experiment, the highest maturation rate was obtained using the VS with sucrose, an equilibration time of 5 min, and a ES with 20% EG. Using a similar protocol, Kuchenmeister and Kuwayama (1997) vitrified immature oocytes in 0.25 mL straws, and obtained a cleavage rate of 42.0% and a blastocyst development rate of 5.7%. However, in that study, rehydration with sucrose was done in the straw ("one-step" method), different from the conventional method used in the present experiment. Studies that used bovine embryos, frozen using the "one-step" method, indicate that pregnancy rates obtained with this method are usually 5 to 10% less than the conventional method of rehydration (Reichenbach *et al*., 2002). So, it is possible that the rehydration method used here was more efficient. However, the direct comparison of the methods mentioned above is not possible, because Kuchenmeister and Kuwayama (1997) did not evaluate the maturation rate.

From analyzing the results of maturation rate when 40% EG was used for different equilibration times (0.5, 5 or 15 min), it is possible to deduce that high concentration of EG for a long equilibration time harmed the oocytes. This effect was also reported by Valdez *et al*. (1992) who observed a significant reduction in the survival rates of mouse blastocysts exposed to 40% EG for 10 min. When exposing bovine oocytes to 20% EG for 10 min, Ullah *et al*. (1997) showed that these oocytes were even more sensitive to EG due to a drastic reduction in the rate of embryonic development. Im *et al*. (1997) suggested that oocytes, maintained for long periods in cryoprotectant solutions, can have reduced viability due to biochemical (inactivation of enzymes needed for meiotic progression) and/or biophysical events (lipid elution from membranes) instead of osmotic stress.

For the concentration of 40% EG, the time of 0.5 min seemed less detrimental than other equilibration times tested. In fact, in a toxicity test, Wani *et al*. (2004) immersed immature buffalo oocytes in solution containing 7.0 M (38.9%) EG for 45 sec and obtained maturation rates similar to the control group. Concerning the use of 3 and 20% EG for 0.5 min, it is possible that 3% EG was too low given the reduced amount of time. Even the use of 20% EG was not capable of yielding good maturation rates. Possibly, with a reduced equilibration time, combining EG with other cryoprotectants may be beneficial. Using an equilibration time of 25 to 30 sec, Luna *et al*. (2001)

and Brandão (2002) combined 10% DMSO with 10% EG in the ES and obtained maturation rates of 38.8 and 8.3% in immature bovine oocytes, respectively. With the same vitrification protocol, Vieira *et al*. (2002) obtained cleavage rates of 46.4 to 49.0% and blastocyst formation rates of 3.5 to 6.1%, also having used immature bovine oocytes.

In the present study, the use of 3% EG with equilibration for 15 min and a VS with trehalose or sucrose did not produce satisfactory maturation rates (0.0 and 19.4%, respectively). Using ES's (3 and 4% EG, respectively) and equilibration times (10 to 15 min) similar to those used in the present study, Papis *et al*. (2000) and Dinnyes *et al*. (2000) found high cleavage rates (75.0 and 62.0%, respectively). Probably, the mature oocytes and the rapid freezing method (vitrification in droplets and solid surface vitrification, respectively) that they used were responsible for the high cleavage rates. It is likely necessary to adapt the freezing protocol according to the type of oocyte (mature or immature) because the protocol used in the studies referenced above attained high maturation and cleavage rates using mature oocytes but not with immature oocytes.

The maturation rates obtained using trehalose was poor when compared to the use of sucrose. The trehalose molarity used in the present experiment may not have been optimal. Arav *et al*. (1993) compared the osmotic and cytotoxic effect of different concentrations (0.25, 0.5 or 1.0 M) of sucrose and trehalose on immature bovine oocytes, and observed that higher fertilization rates (70%) were achieved with the exposure to 0.25 M trehalose. In addition, using the same concentration of trehalose, Rayos *et al*. (1994) did not observe differences in fertilization rates between the use of sucrose or trehalose with EG.

The increased cooling rate used in the vitrification procedure decreases some of the chilling injury such as plasma membrane and cytoskeleton rupture, microtubule depolymerization, chromosome dispersion, and premature release of the cortical granules (Aman and Parks, 1994; Fuku *et al*., 1995; Schmidt *et al*., 1995; Wu *et al*., 1999; Saunders and Parks, 1999). However, some cryo-damage still occurs which likely resulted in the majority of the vitrified oocytes in the present experiment that did not reach the MII stage.

In fact, the majority of the vitrified oocytes in the present study were arrested in the MI stage or classified as WCC or CC. The absence of chromosomal configuration (WCC) is commonly considered as a sign of degeneration (Fagundes, 2002). The CC can indicate that maturation has begun or that degeneration has occurred. According to Stojkovic *et al*. (1999), CC is an essential biochemical mechanism in the cell cycle that occurs in the pre-metaphase I phase. In the case of oocytes from the present experiment, cryopreservation might have inhibited the activity of the meiotic regulators that inhibited the maturation process (Wu *et al*., 1999). The CC also characterizes one of the first events of the process of cellular death, apoptosis (Betts and King, 2001; Farin *et al*., 2001); therefore, oocytes of the present experiment might have also entered the process of degeneration. It is difficult to establish what really occurred because the oocytes were evaluated only after 24 h of culture.

Nevertheless, the use of trehalose in the vitrification solution clearly impaired oocyte maturation. Better maturation rates were obtained with the use of sucrose. In addition, the high concentration of ethylene glycol (40%) combined to a long equilibration time (5 or 15 min) were detrimental to oocyte maturation. Acceptable *in vitro* maturation rates were observed only when immature bovine oocytes were vitrified using 20% EG in the ES, an equilibration time of 5 min, and a VS containing 40% EG + 1.0 M sucrose.

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