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Effects of vitrification and post-thawing interval on the cytoskeleton and subsequent fertilization rate of *in vitro* derived bovine oocytes

T.L. Nedambale[1,2,3#], F. Du[2], J. Xu[2], X.C. Tian[1] and X. Yang[1]

[1] University of Connecticut, Centre for Regenerative Biology/Department of Animal Science, 1392 Storrs Rd., U-4243, Storrs, CT, 06269-USA

[2] Evergen Biotechnologies, Inc., Storrs, CT, 06269-USA

[3] ARC-LBD: Animal Production Institute, Indigenous Genotype Physiology & Biotechnology Development, Private Bag X2, Irene 0062, South Africa

Abstract

Vitrification may alter the cytoskeleton (microtubule, meiotic spindle, microfilament, etc.) and the subsequent fertilization rate of *in vitro* derived bovine oocytes. This study was conducted to evaluate the effect of vitrification and post-thawing incubation periods on the cytoskeleton and fertilization rate of *in vitro* matured (IVM) bovine oocytes. Following 22 h of IVM, 184 fresh matured oocytes (MO) were immediately fertilized *in vitro* and served as a control. The remaining MO (1009) were then vitrified by the solid surface vitrification method. Immediately after thawing, MO were incubated in maturation medium in 5% CO_2 at 39 °C for 0, 30, 60, 90 and 120 min respectively. Following incubation, half of the MO from each vitrified-thawed treatment group (0, 30, 60, 90, and 120 min) was stained with fluorescein isothiocyanate conjugated (FITC) and propidium iodide (PI) to evaluate the microtubule and DNA or spindle under laser-scanning confocal microscopy. The remaining half from the vitrified-thawed MO treatment groups was washed three times in Brackett and Oliphant's fertilization medium and *in vitro* fertilized. Cleavage and blastocyst rates were recorded 48 h post-fertilization. Results demonstrated that vitrification damaged MO zona pellucida (ZP), microtubule (MT), meiotic spindle (MS), and caused chromosomal fragmentation. Both the cleavage (84%) and blastocyst rates (50%) of the control group were significantly higher compared to the vitrified-thawed treatment groups. However, extending the incubation period of vitrified MO to 120 min after thawing (prior to fertilization) improved cleavage (65%) and blastocyst (13%) rates 48 h post-fertilization. Fertilizing vitrified MO immediately (0 min group) after thawing resulted in the lowest cleavage (42%) and blastocyst (1.9%) rates. In conclusion, vitrification reduces the subsequent fertilization rate of MO, however, a prolonged post-thawing incubation period (120 min) improves survival, cleavage and blastocyst formation rates, and enhances the reorganization of MO's cytoskeleton (MT and MS).

Keywords: Vitrification, cytoskeleton, bovine, oocytes, fertilization, *in vitro* matured

[#] Corresponding author. E-mail: lucky@arc.agric.za, nedambale@hotmail.com

Introduction

In the mid 80's, vitrification entered the mainstream of animal embryo technologies (Rall & Fahy, 1985) as an alternative cryopreservation technique to the traditional slow-freezing method. Since then vitrification of mammalian embryos and oocytes has been the subject of many investigations, and has been achieved simply by plunging the sample directly into liquid nitrogen (Rall & Fahy, 1985; Arav *et al.*, 2002; Nedambale *et al.*, 2006). Furthermore, several attempts to improve the survival rate of oocytes pioneered the establishment of different cryopreservation techniques such as the solid surface vitrification (SSV) method (Dinnyés *et al.*, 2000), open-pulled-straw (OPS, Vajta *et al.*, 1998) and Cryo-loop (Lane & Gardner, 2001). Despite this extensive research over the past 20 years, limited progress has been made. The survival rate of cryopreserved and thawed oocytes in terms of development to the blastocyst stage remains low, and only few calves have been born (Vajta *et al.*, 1998). Perhaps vitrification of oocytes is currently one of the greatest challenges facing female gamete storage and assisted reproductive technologies (ART). Effective means of cryopreservation of bovine oocytes followed by the higher survival rate would be important for improving blastocyst formation rate following IVF. Thus, more research is needed in order to gain a better understanding of the morphological cytoskeleton of bovine oocytes that could be crucial in providing a cryo-biological basis to develop a more suitable vitrification protocol. The present study was carried out to

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evaluate the effects of vitrification and post-thawing intervals on the cytoskeleton and subsequent fertilization rate of *in vitro* derived bovine oocytes.

Materials and Methods

All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO-USA), unless otherwise indicated. Bovine oocytes were aspirated from ovaries collected at an abattoir (Yankton, SD, USA), and then matured in Medium199 plus 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 1% antibiotic/antimycotic (Gibco, Grand Island, NY, USA) and 10 ng/mL epidermal growth factor (EGF) in 5% CO_2 in air at 39 °C for 22 h (Nedambale *et al.*, 2004). Following 22 h of *in vitro* maturation (IVM), 184 matured oocytes (MO; fresh) served as a control and were immediately fertilized. The remaining MO (1009) were randomly aliquoted into five groups: 0 min (n = 199), 30 min (n = 201), 60 min (n = 202), 90 min (n = 204), and 120 min (n = 203). The MO within their respective groups were then vitrified by a solid surface vitrification method (35 % (v/v) EGF + 0.4 M trehalose + 5% (w/v) PVP in M199 + 20% FBS (Dinnyés *et al.*, 2000). Immediately after thawing, MO were incubated in a maturation medium in 5% CO_2 at 39 °C for 0, 30, 60, 90 and 120 min, respectively. Following incubation, half of the MO from each vitrified-thawed treatment groups (0, 30, 60, 90, and 120 min) was stained with fluorescein isothiocyanate conjugated (FITC) and Propidium iodide (PI) to visualize microtubule and DNA or spindle under laser-scanning confocal microscopy (Chang *et al.*, 2004). However, only 20 oocytes (n = 20) per treatment group were examined. The remaining half of the vitrified-thawed MO group was washed three times in Brackett and Oliphant's fertilization medium as described previously (Nedambale *et al.*, 2004), and then transferred into 50 µL drops in a Petri dish covered with mineral oil (Becton Dickinson, Franklin Lakes, NJ, USA). The dishes were incubated at 39 °C in 5% CO_2 in the air. A semen straw (ABS, DeForest, WI, USA) was thawed at 38 °C for 1 min, and washed two times by centrifugation at 453 x g for 8 min. Following the final wash, sperm motility, and concentration were determined. Subsequently, sperm pellets were re-suspended into a volume of 250 µL per treatment group. Forty micro-litres (40 µL) of the sperm were added to each fertilization drop, giving a total concentration of 1 x 10^7 spermatozoa/mL. Oocytes were then incubated with the sperm for 6 h in 5% CO_2 in air at 39 °C. After 6 h of IVF, oocytes were washed six times in TL-HEPES (Bio-Whittaker, Walkersville, MD, USA) and cultured in our previously described sequential KSOM-SOF culture system (Nedambale *et al.*, 2004). The day of fertilization is designated as day zero. Embryo cleavage rate was recorded 48 h post-fertilization and total blastocyst rate on Day 8.

The data was subjected to a one-way analysis of variance (ANOVA) following arc sine transformation (data replicated 4 times). Differences between post-thawing interval groups were determined using Bonferroni's test for pair-wise comparison of means, $P < 0.05$ was considered significant.

Results and Discussion

The results of this study demonstrate that fertilizing vitrified matured oocytes immediately (0 min group) after thawing resulted in the lowest ($P < 0.05$) cleavage (42%) and blastocyst (1.9%) rates. However, extending the incubation period of vitrified MO to 120 min after thawing improved ($P < 0.05$) cleavage (65%) and blastocyst (13%) rates post-fertilization. There were no statistical differences in the cleavage and blastocyst formation rates of vitrified and thawed oocytes, which were derived from 30 (46 and 9%), 60 (45 and 11%), and 90 (50 and 12%) min groups, respectively. As expected, cleavage (84%) and blastocyst rates (50%) of fresh MO (control) were significantly higher compared to the vitrified-thawed groups. Significantly higher survival rates were recorded in the 0 (82%) and 30 (80%) min groups than in the 60 (61%), 90 (66%) and 120 (67%) min groups, respectively (Figure 1).

As shown in Figure 2, in a retrospective comparison among the treatment groups following FITC and PI staining, a significantly higher MO in 0 min group had abnormal spindle (50%) and microtubule (35%), compared to the other groups. However, microtubule (22 and 15%) and spindle (13 and 9%) of vitrified MO reorganized and showed less damages following incubation for longer periods (90, and 120 min, respectively) post-thawing. The highest overall damages (OA) in vitrified-thawed MO were observed in 0 (92%) and 30 (69%) min compared to the other groups.

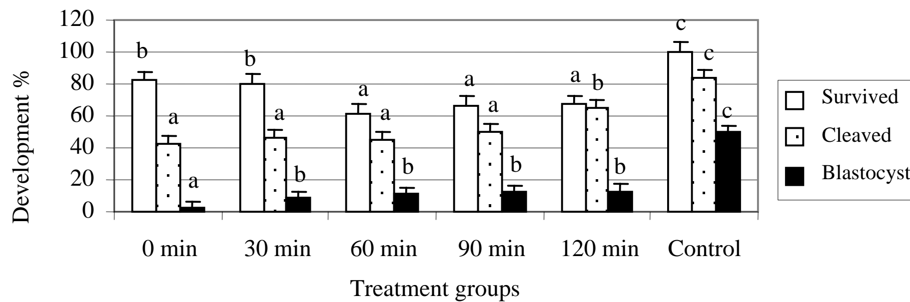

Figure 1 Post-thawing survival rate and embryo development following *in vitro* fertilization of fresh (control) and vitrified bovine oocytes fertilized at different intervals. [a,b,c]Values with different superscripts across the bars are significantly different (P < 0.05)

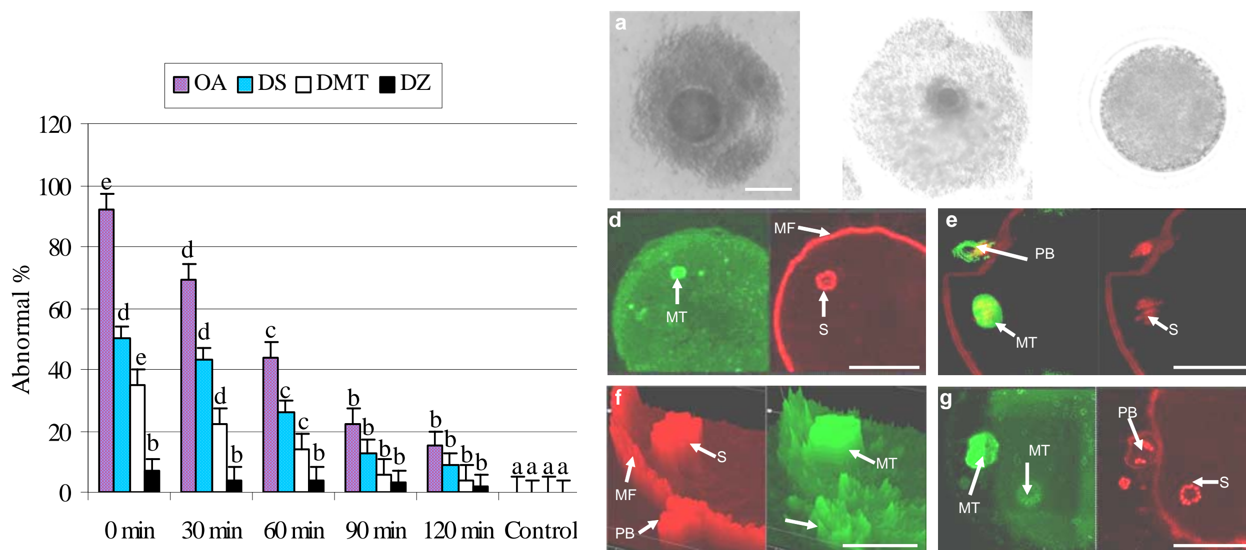

Figure 2 Morphological evaluation of cytoskeleton structure (Microtubule (MT), microfilament (MF) and meiotic spindle (S) of vitrified-thawed bovine oocytes. The histogram illustrates all the abnormalities observed at different groups. [a-e]Values with different superscripts across bars are significantly different (P < 0.05). The OA = overall abnormalities, DS = damaged spindle, DMT = damaged microtubule, DZ = damaged zona. (**a**) Normal immature bovine oocyte after ovary aspiration. (**b**) Matured oocyte with well-expanded cumulus cells selected for vitrification 22 h after IVM. (**c**) Denuded matured oocyte following removal of cumulus cells from control group (not vitrified). **(d)** Normal MT attached to S and MF circling the cytoplasm of the oocyte, control group (not vitrified). **(e)** A normal MT attached to abnormal or damaged S, and abnormal MT attached to damaged small polar body (PB), derived from 0 min group. **(f)** Three dimension (3-D) structure taken from different level positions that combined to form a well-defined 3-D structure of a normal spindle, MF, and PB. Subsequently, few or depolymerised MT attached to PB and normal MT attached to PB, from the 120 min group. **(g)** Normal MT attached to normal PB, and abnormal or small MT attached to normal spindle of matured oocyte 120 min post-thawing. Bar =100 µm

This study demonstrated that vitrification adversely affects subsequent *in vitro* fertilization and survival rates and the ability to reorganize the damaged meiotic spindle and microtubule of MO (Figure 2). However, these adverse effects tended to improve significantly by post-thawing incubation prior to *in vitro* fertilization. This seems to allow reorganization of the MO's cytoskeleton and to be time dependent. These results are consistent with the findings of Pickering *et al.* (1990), who reported that exposure of human or mouse oocytes to cryopreservation procedures and low temperature disrupts its cytoskeleton. Zona pellucida

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has also been found to be damaged and hardened due to cryopreservation resulting in decreased fertilization rates (Fuku *et al.*, 1995).

Conclusion

In conclusion, vitrification reduced the subsequent survival and fertilization rates of *in vitro* matured oocytes. However, extended post-thawing incubation periods (120 min) improved cleavage and blastocyst formation rates post-fertilisation. Microtubule and spindle reorganization of vitrified-thawed oocytes incubated for 120 min prior to fertilization showed better reorganization and less damage than all other groups of vitrified oocytes considered in this study

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