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Survivability and 1st Meiotic Completion *In Vitro* of Immature Bovine Oocytes after Vitrification

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Oocyte cryopreservation could be used to assist many reproductive processes and rescue gametes when female die unexpectedly or accidentally. In this study, GV stage bovine oocytes were exposed to a vitrification solution (VS_1) of 7.5% EG + 7.5% DMSO in TCM-199 medium with 10% FBS (BM) for 10 min to equilibrate before transferring to vitrification solution (VS_2) of 15% EG + 15% DMSO + 0.6 M sucrose in BM for 30-45 sec. Thereafter, the oocytes were either placed in the cryoloop and plunged into the LN_2 or directly drop into the LN_2. After a month of storage, the oocytes were warmed in a step-wise manner at 3 min each in warming solution with 0.6 M, 0.3 M and 0.15 M sucrose. Then, the oocytes were washed (3x) using the maturation medium before culturing for another 22 hr, fixed and analyzed for meiotic progression. In the cryoloop method, the survival rate was 92.5% (49/53) with a meiotic resumption rate of 87.8% (43/49). The rate of completion of 1st meiosis was 49.0% (24/49). Using the MDS method, the survival rate was 91.1% (51/56) with a meiotic resumption rate of 74.5% (38/51). The rate of completion of 1st meiosis was 31.4% (16/51). The results showed that both vitrification methods could be used for the vitrification of GV stage bovine oocytes, although the cryoloop method appeared more efficient than the MDS method.

Keywords: vitrification, bovine, immature oocytes, meiosis, maturation

Introduction

Oocyte cryopreservation has the potential to be an important adjunct to assisted reproductive technologies (ARTs) in human and domestic animals as it offers the possibility of establishing an oocyte banking system. It would provide an ethically acceptable alternative to embryo freezing and would be fundamental in increasing the availability of female gametes for both research purposes and commercial use. However, the survival of oocytes after

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cryopreservation is dependent on a number of factors including, stage of maturation, oocyte quality or by biophysical factors resulting from the cryopreservation procedure used (Ludwig, 1999). Moreover, the survival of oocytes after thawing/warming is extremely variable and only few fertilized cryopreserved oocytes have developed to term, even when fertilized by intracytoplasmic sperm injection (Shaw *et al.*, 2000). Apparently, the maturity of the oocyte is an important consideration that affects greatly the outcome of cryopreservation. The use of M-II stage oocytes has been less effective, though with few reported live births. Some reasons include, short fertile life span, exquisitely sensitive to chilling and have little capacity for repairing cryoinjury before fertilization (Cha, 2000). The meiotic spindle on which the chromosomes have become aligned is acutely temperature sensitive. Transient cooling to 20 ℃ can cause irreversible disruption of the spindle apparatus while rapid depolarization occurs when the temperature is lowered to 0 ℃ (Pickering, 1990; Zenzes, 2001). An alternative approach to M-II stage oocyte cryopreservation is the use of GV stage oocytes for their chromatin remains at the diplotene stage of prophase I and therefore do not possess a spindle apparatus. While deleterious effects on the cytoskeleton resulting from chilling may be avoided when using GV oocytes, the difficulties associated with the in vitro maturation and extended culture appear to minimize the potential benefits of cryopreserving GV oocytes. Thus, the use of an appropriate protocol that will optimize the survival, meiotic resumption post warming and the eventual completion of 1st meiosis following exposure to stresses associated with cryopreservation has proved to be a major challenge, hence this study.

Materials and Methods

Reagents

Unless specified, all chemicals, reagents and hormones were purchased from Sigma (St Louis, Mo, USA). The basic media for maturation and vitrification of oocytes was a defined tissue culture medium with Earle's salts and L-glutamine (Krisher *et al.*, 1999) supplemented with 10% fetal bovine serum (BM).

Oocyte collection and selection for vitrification

Bovine ovaries were collected at a local abattoir and transported to the laboratory within 5 hr in sterile physiological saline solution (0.9 % NaCl, w/v) supplemented with antibiotics (0.1 mg/ml Penicillin and 500 ug/ml

Streptomycin) maintained at a temperature of 35-37 °C. The follicular oocytes were aspirated from 3-5 mm follicles using an 18- gauge needle attached to a 10- ml disposable plastic syringe (Fig. 1). The follicular aspirates were pooled in a test tube maintained at 37 °C in a water bath. After 5 min, part of the follicular fluid was decanted and the sediment searched for cumulus-oocyte complexes (COCs) using a stereomicroscope at low magnification. Oocytes with intact, unexpanded cumulus cell investments and with evenly granulated ooplasm were selected for use in the experiments. Selected COCs were washed twice in Hepes buffered TCM-199 with Earles salt supplemented with 10 % fetal calf serum (FCS). The COCs were then denuded free of cumulus cells by pipetting and used for vitrification (Fig. 2).

Fig. 1. Bovine ovaries collected from the slaughterhouse (A) and the aspiration of follicles (B).

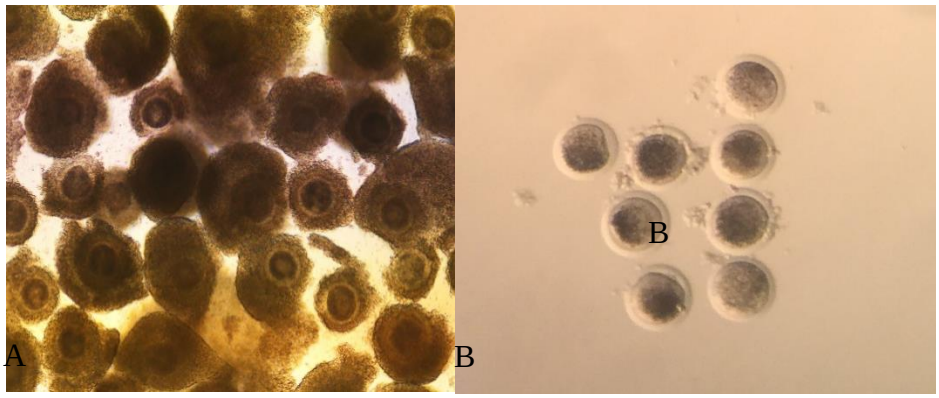

Fig. 2. Bovine COCs (A) and after denudation by pipetting (B).

Vitrification and warming

Two vitrification solutions were prepared in BM. Vitrification solution 1 (VS_1) consist of 7.5% ethylene glycol (EG) + 7.5% dimethyl sulfoxide (DMSO) and vitrification solution 2 (VS_2) with 15.0% EG + 15.0% DMSO + 0.6M sucrose. The oocytes were exposed to VS_1 (5 oocytes/ml) for 10 min before transferring to VS_2 for 30-45 sec at room temperature (21-25 ℃). Thereafter, the oocytes in VS_2 were either loaded in a cryoloop (Fig. 3B; Lane *et al.*, 1999b) or directly dropped into LN_2 in microdrop form (Fig. 3A; Ocampo *et al*., 2014). The cryoloop and droplets/pellets placed in a cryovial were stored in LN_2 tank for at least 1 month before warming. Warming was done in a step-wise dilution pattern in warming solutions (WS) consisting of 0.6 M, 0.3 M and 0.15 M sucrose in BM for 5 min each. Thereafter, the recovered oocytes were washed (at least twice in BM) before transferring to maturation droplets (10 oocytes/50 µl). Survival rate of the oocyte was assessed based on its morphological integrity under an inverted microscope (Ocampo *et al.*, 2014). Oocytes with fragmented zona pellucida and absence of cytoplasmic contents were considered dead oocytes while those that remained morphologically normal were taken for maturation. Freshly collected COCs that were cultured for maturation served as the control.

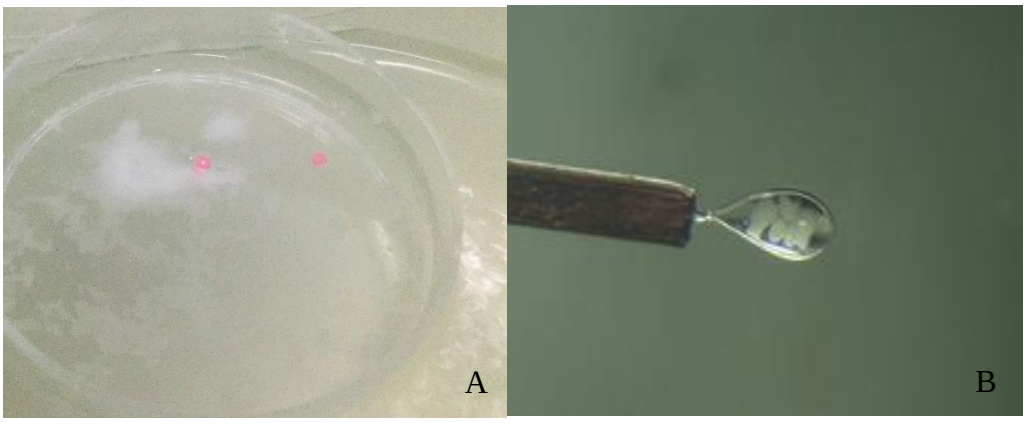

Fig. 3. MDS method showing droplets of VS_2 containing the oocytes (A) and the cryoloop with the oocytes (B) just before plunging into LN_2.

Evaluation for meiotic resumption and completion

At the end of maturation period (22 hr) in BM, the oocytes were mounted on glass slides with cover slip supported by droplets of a paraffin-

vaseline (1:12, v/v) mixture, fixed and cleared with acetic acid:ethanol solution (1:3, v/v) for 24 hr or more. Cleared specimen were stained with 1% orcein dissolved in 40% acetic acid in water and observed under phase contrast optics at 200-400 x magnification. Oocytes that have undergone germinal breakdown stage to telophase 1 stage were considered to have resumed meiosis while those with visible 2nd metaphase plate and a polar body were regarded as matured/completed 1st meiotic division (Fig. 4; Ocampo *et al.*, 2001).

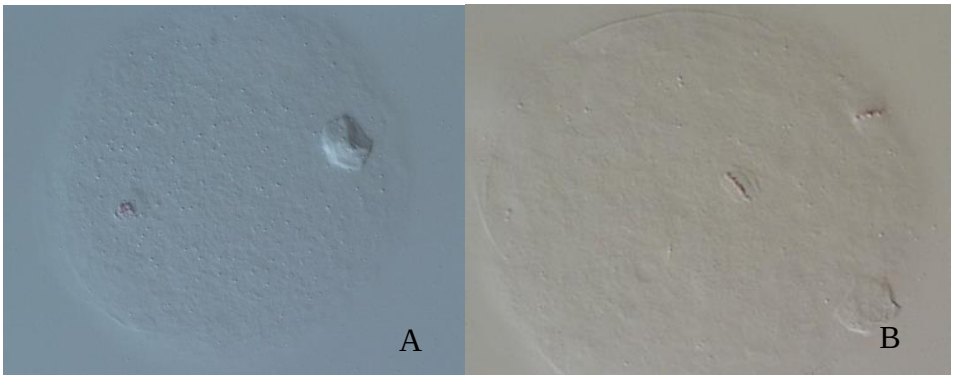

Fig. 4. Post warmed oocytes resuming meiosis (A) and an oocyte completing 1st meiosis (B) as evidenced by having a 2nd metaphase plate and a 1st polar body.

Results and Discussion

In this study, we have demonstrated that GV stage bovine oocytes can be successfully vitrified by both the cryoloop and MDS method using a combination of EG + DMSO as CPAs.

A total of 192 good oocytes were used, of which 60, 71 and 61 oocytes served as the control (non-vitrified) and vitrified groups using the cryoloop and MDS method, respectively. The survival (morphologically normal) rate of both control and vitrified groups showed no significant differences ranging from 91.1 % to 93.3 % (Table 1), although the percentage of intact morphology in vitrified-warmed GV oocytes was lower than the control. The assessment of survival rate based on morphological integrity has been used by several authors as a non-invasive parameter, indicative of cryoinjury (Prentice and Anzar, 2011). A normal oocyte that survived the vitrification process have clear cytoplasm with uniform texture and homogenous granulation, rounded symmetrical shape, intact nuclear and cellular membrane and no sign of lysis or leakage of cellular content. In related studies using the cryoloop method, similarly high survival rate has been reported for cryopreserved immature oocytes (bovine (90.5%), Mavrides and Morroll, 2002; goat (92.0%), Begin

2003). Apparently, the oocytes held in cryoloop for vitrification achieved a faster cooling and warming rate than other vitrification methods (eg., straw/open pulled straw method and traditional slow freezing techniques), thus the disruption in cryoloop method seems to occur less than the disruption of microtubules in conventional methods. In this method, the oocytes are carried within a film of VS which makes it possible to directly warm and immediately dilute them thus reducing their exposure to unsuitable temperatures and concentrated cryoprotectants (Saki *et al.*, 2005). In here, a thin nylon loop is used to suspend a film of CPA with the oocytes to directly submerge them in LN_2. This small volume of VS allows the oocyte to pass through the damaging temperature zone of +10 °to -10 ℃ at a very high rate (Luster, 2004). Whereas, the MDS method allows keeping the oocytes without damage due to dessication through the use of a minimal amount of VS (Ocampo *et al.*, 2014). The VS volume is about 0.07 – 0.1 µl which is almost 50% lower than other vitrification methods. The benefit in using MDS method than conventional vitrification methods is it maximizes the cooling rate up to 130,000 ℃/min, allowing up to 50% reduction in CPA concentration. It also increases the warming rate and decreases the likelihood of ice crystal formation (Zhang *et al.*, 2011). In related studies, the use of MDS method in the vitrification of swamp buffalo and bovine GV stage oocyte has resulted to <80.0% survival rate (Kim *et al.*, 2005; Ocampo *et al.*, 2014).

Table 1. Survival rate of vitrified-warmed GV stage bovine oocytes.

Vitrification	**No. of oocytes (%)**		
method	**vitrified**	**recovered**	**survived**
Control*		60 (100.0)[a]	56 (93.3)
Cryoloop*	71	53 (74.6)[b]	49 (92.5)
MDS**	61	56 (91.8)[a]	51 (91.1)

*'**Data from 5 and 3 replicates. [a,b]Values differ significantly (P<0.05).

In the control, 92.9% resumed meiosis after culture with 85.7% completing 1st meiosis. In cryoloop vitrified group, 87.8% resumed meiosis following culture post-warming with 49.0% completing 1st meiosis. Also, in MDS vitrified group, 74.5% resumed meiosis with 31.4% completing 1st meiosis. Significant differences (P<0.05) was noticeable between the non-vitrified and vitrified groups (Table 2). The number of oocytes that showed signs of degeneration after the maturation period was higher in the vitrified group. Nonetheless, the results obtained was comparable to earlier studies in buffalo with maturation rate of 21-47.0% (Ocampo *et al.*, 2014), but lower in

bovine and goat (<90.0%) when using the cryoloop method (Begin, 2003; Kim *et al.*, 2005). From these findings, morphologically normal oocytes post-warming could be incompetent of meiosis indicating that morphological assessment alone is a limiting factor in predicting developmental potential of vitrified-warmed GV oocytes. In other species, improved developmental competence of vitrified-warmed GV stage oocytes were reported after using either cryoloop or MDS method (Arav *et al.*, 1993; Lane *et al.*, 1999a). In conclusion, GV stage bovine oocyte vitrification still requires much developmental work to improve the competence for meiotic resumption and its subsequent completion. The potential benefits, convenience and lower cost of using both the cryoloop and MDS methods which could be translated to successful programs of GV oocyte vitrification will stimulate efforts in the improvement of protocols for application in other ARTs.

Table 2. Meiotic resumption and completion of GV stage bovine oocytes after vitrification.

Vitrification	**No. of oocytes (%)**		
method	**cultured**	**resumed meiosis**	**matured**
Control*	56	52 (92.9)[a]	48 (85.7)[a]
Cryoloop*	49	43 (87.8)[a,b]	24 (49.0)[b]
MDS**	51	38 (74.5)[b]	16 (31.4)[b]

*:**Data from 5 and 3 replicates. [a,b] Values differ significantly (P<0.05).

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