International Journal of Agricultural Technology 2017 Vol. 13(7.1):1147-1154
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Meiotic Resumption and Completion *In Vitro* of Immature Buffalo Oocytes after Vitrification

Renz Carlo A. Cajucom[1], Elfren F. Celestino Jr[1], Lerma C. Ocampo[2,3], Virgilio D. Viernes[1] and Marlon B. Ocampo[1,2*]

[1]College of Veterinary Science and Medicine, Central Luzon State University, [2]Reproductive Biotechnology and Physiology Unit, Philippine Carabao Center and [3]Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, 3120 Philippines.

Renz Carlo A. Cajucom, Elfren F. Celestino Jr, Lerma C. Ocampo, Virgilio D. Viernes and Marlon B. Ocampo. (2017). Meiotic Resumption and Completion *In Vitro* of Immature Buffalo Oocytes after Vitrification. International Journal of Agricultural Technology 13(7.1):1147-1154.

Cryopreservation of buffalo oocytes can be done in using various methods of vitrification with different success rate. In this study, germinal vesicle (GV) stage buffalo oocytes were exposed to VS_1 (7.5% EG + 7.5% DMSO in BM) for equilibration (10 min) before transferring to VS_2 (15% EG + 15% DMSO + 0.6 M sucrose in BM) for 45 sec loaded in cryoloop and plunged directly into LN_2 or directly plunging into LN_2 in microdrops. After a few weeks of storage, the oocytes were warmed in step-wise dilution pattern for 3 min each in 0.6 M, 0.3 M and 0.15 M sucrose in BM, transferred to a washing solution (3x) before culturing in maturation droplets. In using the cryoloop method, the survival rate recorded was 86.5% (64/78) with a meiotic resumption rate of 85.9% (55/64). The maturation rate was 34.4% (22/64). In using the MDS method, the survival rate was 88.5% (54/75) with a meiotic resumption rate of 88.9% (48/54). The maturation rate was 42.6% (23/54). These findings indicate that both vitrification methods can be used for cryopreservation of GV stage buffalo oocytes. Future studies should be directed on attaining a higher maturation rate post warming, including its fertilizability, capacity for embryo development and eventual production of live birth.

Key words: Vitrification, buffalo, immature oocytes, meiotic resumption, maturation

Introduction

Vitrification has become the most practical and efficient method of choice for the cryopreservation of mammalian oocytes (Ambrosini *et al.*, 2006; Prentice and Anzar, 2011). However, a universal protocol that could be used effectively for all species have yet to be determined. The cryotop/cryotech method described for vitrification of human oocytes has reported a 100% survival rate (Kuwayama, 2005). Others have used different vitrification methods with varying results as in whale (Fukui and Kuwayama, 2004), cat (Merlo *et al.*,2008; Cocchia *et al.*, 2010), cattle (Massip and Donnay, 2003),

* **Corresponding author:** Marlon B. Ocampo; **Email:** ocampomarlon29@yahoo.com

goat (Le Gal, 1996; Purohit *et al*., 2012), pig (Isachenko *et al.*, 2001), mouse (Lane and Gardner, 1999) and sheep (Ebrahimi *et al.*, 2010).

In water buffalo, attempts have been made in the vitrification of GV stage oocytes (Dhali *et al.,* 2000b; Wani *et al.,* 2004a,2004b; Yadav *et al.*, 2008; Ocampo *et al.*, 2014). The use of GV stage oocytes has the advantage of being more resistant to cold induced damage once the chromatin is still protected by nuclear envelope and no spindle apparatus is present (Luciano *et al.*, 2009). However, the low surface-to-volume ratio of the oocytes that makes it difficult for water and cryoprotectants to move across the cell plasma membrane rendered it extremely sensitive to chilling (Pereira and Marques, 2008). During the process of cryopreservation, the oocyte suffer considerable morphological and functional damage, although the extent of cryoinjuries depends on a number of factors including the species, origin of the oocytes, method of cryopreservation, type of CPAs and others. The mechanisms for cryoinjuries is yet to be fully understood and until more insight is gained, improvement of oocyte cryopreservation will remain difficult. That's exactly the reason why several vitrification devices have been developed and tested, each with a specific method of minimizing the volume of the vitrification solution. The aim is to increase the cooling velocity and heat transfer and prevent ice crystal formation (Arav *et al.,* 2002). So far, the cryotop method appeared to be the most efficient method for vitrification of oocytes and embryos (Kuwayama, 2007). The only constraint on the choice of its use is the possibility of microbial disease transmission and viral contamination (AbdelHafez *et al.,* 2011) and in developing countries is the cost. Therefore, development and/or selection of other methods which is cost effective and efficient in supporting the viability of GV stage buffalo oocytes becomes necessary. One particular method of interest is the use of MDS method (Ocampo *et al*., 2014). The technique is an open type method that allows the samples to come into direct contact with LN_2. Its use in goat embryos has resulted to live births (Ocampo *et al*., 2002) and showed potential applications in buffalo oocyte cryopreservation. Another is the cryoloop method (Lane and Gardner, 1999), which consists of a tiny nylon loop (20 µm wide, 0.5-0.7 mm in diameter) mounted on a small stainless tube inserted into the lid of a cryovial (Hampton Res., Laguna Niguel, CA, USA). A metal insert on the lid enables the use of a stainless steel handling rod with a small magnet (Crystal Wand with Tab; Hampton Res) for manipulation of the loop at low temperature. Similarly, its initial use in the vitrification of some mammalian oocytes showed remarkable results. In this study, we compare the efficacy of both the cryoloop and MDS methods for vitrification of GV stage buffalo oocytes by determining their survival, meiotic resumption and subsequent completion of 1st meiosis post- warming.

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

Buffalo 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 35-37º C. The follicular oocytes were aspirated from 3-5 mm follicles using an 18- gauge needle attached to a 10- ml disposable plastic syringe. The follicular aspirates were pooled in a test tube maintained at 37º 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 (Fig. 1). 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.

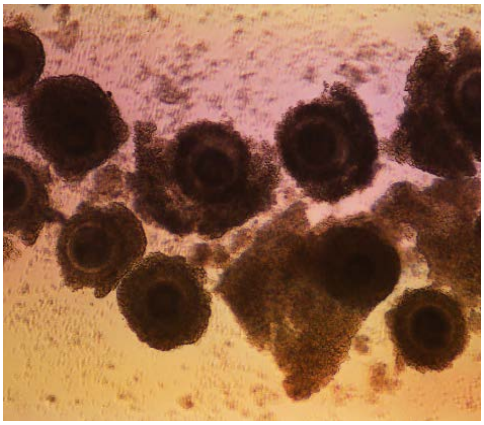

Fig. 1. GV stage buffalo oocytes.

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°C). Thereafter, the oocytes in VS_2 were either loaded in a cryoloop (Fig. 2A; Lane and Gardner, 2001) or directly dropped into LN_2 in microdrop form (Fig. 2B; Ocampo *et al.*, 2014). The cryoloop and droplets/pellets placed in a cryovial were stored in LN_2 tank for at least 3 wks 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 3 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 (Fig. 3 A; 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.

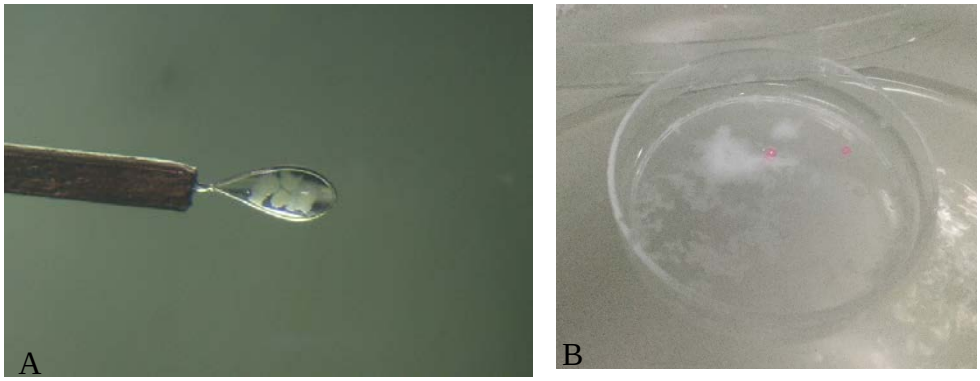

Fig. 2. Cryoloop vitrification (A) and MDS vitrification (B) of GV stage oocytes.

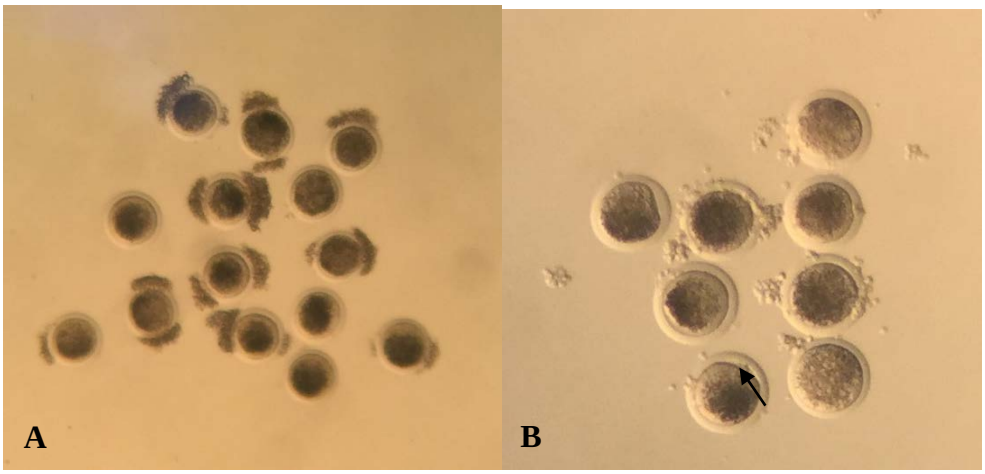

Fig. 3. Survived vitrified-warmed GV oocytes (A) and matured oocytes with 1st polar body (arrow) post culture (B).

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. 3B; Ocampo *et al.*, 2001).

Results and Discussion

In this study, we have demonstrated that GV stage buffalo oocytes can be successfully cryopreserved by both the cryoloop and MDS method using a combination of EG + DMSO as CPAs. A total of 203 good oocytes were used, of which 50, 78 and 75 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 86.5 % to 96.0 % (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 (Tsagias *et al.*, 2006; Prentice and Anzar, 2011). In related studies, a survival rate of 83-89.0% was reported using the straw method of vitrification and EG or DMSO alone or in combination as CPAs, but decreased to 65.0% when using glycerol alone though is dependent on molar concentration. Increased molar concentration to 10 M significantly reduced the survival rate of GV oocytes (Yadav *et al.*, 2008; El-Shahat and Hammam, 2014). When using 1.2-propanediol as CPA through slow freezing method, only 23.0 % survival rate was obtained though with live birth following ICSI (Tucker *et al.*, 1998).

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

Vitrification	No. of oocytes (%)		
method	vitrified	recovered	survived
Control		50 (100.0)	48 (96.0)
Cryoloop	78	74 (94.9)	64 (86.5)
MDS	75	61 (81.3)	54 (88.5)

*Data from 4 replicates.

In the control, 94.0% resumed meiosis after culture with 89.4% completing 1st meiosis. In cryoloop vitrified group, 85.9% resumed meiosis following culture post-warming with 34.4% completing 1st meiosis. Also, in MDS vitrified group, 88.9% resumed meiosis with 42.6% 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% (Dhali *et al.*, 2000b; Yadav *et al.*, 2008; El-Shahat and Hammam, 2014; Ocampo *et al.*, 2014). 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 buffalo 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 buffalo oocytes after vitrification**.

Vitrification	No. of oocytes (%)		
method	cultured	resumed meiosis	matured
Control	50	47 (94.0)	42 (89.4)[a]
Cryoloop	64	55 (85.9)	22 (34.4)[b]
MDS	54	48 (88.9)	23 (42.6)[b]

**Data from 4 replicates. [a,b] Values differ significantly (P<0.05).

References

AbdelHafez F, Xu J, Goldberg J and Desai N. (2011). Vitrification in open and closed carriers at different cell stages: assessment of embryo survival, development, DNA integrity and stability during vapor phase storage for transport. BMC Biotechnol., 11:29.

Ambrosini G, Andrisani A, Porcu E, Rebellato E, Revelli A, Caserta D, Cosmi E, Marci R and Moscarini M. (2006). Oocytes cryopreservation: state of the art. Reprod Toxicol., 22: 250-262.

Arav A, Shebu D and Mattioli M. (1993). Osmotic and cytotoxic study of vitrification in immature bovine oocytes. J Reprod Fertil., 99:353-358.

Arav A, Yavin S, Zeron Y, Natan D, Dekel I and Gacitua H. (2002). New trends in gamete cryopreservation. Mol Cell Endocrinol., 187:77-81.

Cocchia N, Ciani F, Russo M, El Rass R, Rosapane I, Avallone L, Tortora G and Lorizio R. (2010). Immature cat oocyte vitrification in open pulled straws using a cryoprotectant mixture. Cryobiology, 60: 229-234.

Dhali A, Manik RS, Das SK, Singla SK and Palta P. (2000a). Vitrification of buffalo oocytes. Theriogenology, 53:1295-1303.

Dhali A, Manik RS, Das SK, Singla SK and Palta P. (2000b). Post vitrification survival and IVM rate of buffalo oocytes: Effects of EG concentration and exposure time. Anim Reprod Sci., 63:159-165.

El-Shahat KH and Hammam AM. (2014). Effect of different types of cryoprotectants on developmental capacity of vitrified-thawed immature buffalo oocytes. Anim Reprod., 11:543-548.

Ebrahimi B, Valojerdi MR, Eftekhari-Yazdi P, Baharvand H and Farrokhi A. (2010). IVM and gene expression of sheep COCs following different methods of vitrification. Reprod BioMed Online, 20:26-34.

Fukui Y and Kuwayama M. (2004). Effect of cryodevice type and donors sexual maturity on vitrification of minke whale oocytes at GV stage. Zygote, 12:333-338.

Isachenko V, Soler C, Isachenko E, Perez-Sanchez F and Grishchenko V. (2001). Vitrification of immature porcine oocytes: effects of lipid droplets, temperature, cytoskeleton and addition and removal of cryoprotectants. Cryo Lett., 22;157-162.

Krisher RL, Lane M and Bavister BD. (1999). Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. Biol Reprod., 60:1345-1352.
Kuwayama M. (2005). Highly efficient vitrification method for cryopreservation of human oocytes. Reprod BioMed Online, 11:300-308.
Kuwayama M. (2007). Highly efficient vitrification for cryopreservation of human oocytes and embryos: The CryoTop method. Theriogenology, 67:73-80.
Lane M, Bavister BD and Lyons EA. (1999a): Containerless vitrification of mammalian oocytes and embryos. Nature Biotech., 17:1234-1236.
Lane M and Gardner DK. (2001). Vitrification of mouse oocytes using a nylon loop. Mol Reprod Dev., 58: 342-347.
Le Gal F. (1996). In vitro maturation and fertilization of goat oocytes frozen at GV stage. Theriogenology, 45:1177-1185.
Massip A and Donnay I. (2003). Cryopreservation of bovine oocytes: current status and recent developments. Reprod Nutri Dev., 43: 325-330.
Merlo B, Iacono E, Regazzini M and Zambelli D. (2008). Cat blastocyst produced in vitro from oocytes vitrified using the cryoloop technique. Theriogenology, 70: 126-130.
Ocampo LC, Ocampo MB, Mamuad FV, Venturina EV, Mori T and Shimizu H. (2001). Timing of sequential changes on chromosome configurations during the 1st meiosis, 2nd meiosis and cytoplasmic events of swamp buffalo oocytes matured and fertilized in vitro. Phil J Vet Anim Sci., 27:1-11.
Ocampo MB, Ocampo LC, Reyes RO, Silvestre JQ and Villar E. (2002). Viability of frozen goat embryos using a modified minimum drop sized technique of vitrification. Phil J VetAnim Sci., 28(1):46-55.
Ocampo MB, Ocampo LC and Soriano RP. (2014). Vitrification of swamp buffalo oocytes. Intl J Agric Tech., 10 (1):67-78.
Pereira RM and Marques CC. (2008). Animal oocyte and embryo cryopreservation. (Cell Tissue Bank, 9:267-277.
Prentice JR and Anzar M. (2011). Cryopreservation of mammalian oocytes for conservation of animal genetics. Vet Med Int., 21:1-11.
Purohit GN, Meena H and Solanki K. (2012). Effect of vitrification on immature and in vitro matured, denuded and cumulus compact goat oocytes and their subsequent fertilization. J Reprod Fertil., 13:53-59.
Tucker MJ. (1998). Birth after cryopreservation of GV oocytes with subsequent in vitro maturation. Fertil Steril., 70:578-579.
Wani NA, Misra AK and Maurya SN. (2004a): Maturation rates of vitrified-thawed immature buffalo oocytes: Effect of different types of cryoprotectants. Anim Reprod Sci., 84:327-335.
Wani NA, Maurya SN, Misra AK, Saxena VB and Lakhchaura BD. (2004b). Effect of cryoprotectants and their concentration on in vitro development of vitrified-warmed immature buffalo oocytes. Theriogenology, 61:831-842.
Yadav RC, Sharma A, Garg N and Purohit GN. (2008). Survival of vitrified water buffalo oocytes COCs and their subsequent development in vitro. Bulg J Vet Med., 11 (1):55-64.

(Received: 23 October 2017; accepted: 25 November 2017)