Vitrification of Swamp Buffalo Oocytes

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ABSTRACT

The study was conducted to examine the role of various factors (presence/absence of cumulus cells, meiotic stage, oocyte source, pre-equilibration time, warming condition, method of vitrification) to establish the optimum condition for vitrification of buffalo oocytes. Survival was based on the morphological normality post-warming, completion of first meiosis and penetrability post fertilization in vitro.

In Experiment 1, survival of germinal vesicles (GV) stage cumulus-oocyte-complexes (COCs) or denuded oocytes was significantly lower than the control regardless of pre-equilibration time used except when using 10-minute exposure time. The maturation rate of vitrified oocytes was lower compared to the control. In Experiment 2, the survival of vitrified/warmed GV oocytes had no difference with the control regardless of the warming conditions used although the maturation rate was lower. Among treatments, descending trehalose concentration and exposure time appeared better than direct warming and step-wise warming with descending trehalose concentration with same exposure time. In Experiment 3, vitrification of oocytes at GV or metaphase II (M_2) stage showed no difference in terms of survival and fertilizability but use of the cryotop method appeared better than the minimum drop size (MDS) method. In Experiment 4, oocyte source had no effect on the survival and fertilizability of vitrified/warmed M_2 stage oocytes. In Experiment 5, the cleavage and blastocyst formation rate of vitrified/warmed oocytes was lower than the control.

The results showed that buffalo GV and M_2 stage oocytes can survive vitrification either by MDS or cryotop method, complete first meiotic division, be fertilized and produce embryos although to limited extent.

INTRODUCTION

Nowadays, attention is focused on vitrification as a rapid and efficient method for cryopreservation of biological systems. Vitrification is defined as the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling (Kauzmann, 1948). During vitrification, translational molecular motion are significantly arrested, but without any of the changes brought about by freezing. Gametes and/or organs capable of being vitrified need not satisfy classical constraints of optimal cooling and warming rates, but instead can nearly escape both "solution effects" injury and the dangers of intracellular freezing. To achieve good survival following vitrification, gametes and/or embryos must be able to tolerate exposure to and dehydration by highly concentrated solution (Rall *et. al.*, 1987).The use of high concentrations of cryoprotectants and high cooling and warming rates prevent ice crystal formation thereby eliminating ice crystal injury, osmotic and chilling injury, zona and blastomere fracture, and alterations of the

cytoskeleton of both gametes and/or embryos.

Vitrification is basically based on direct contact between the vitrification solution containing the cryoprotectant agents and liquid nitrogen (LN_2). Various protocols described for vitrification are very simple, and they allow cells and tissues to be placed directly into the cryoprotectants and then plunged directly into LN_2. However, most vitrification methods developed use the standard straw for holding gametes during cooling, storage and warming, although these straws limit the maximum cooling rates and warming rate to less than 2000 ° per min. Alternative methods allowing direct contact between the embryo containing medium and LN_2 that increased cooling and warming rates have been reported using the electron microscope (Vajta *et al.*, 1998) or cryoloops (Lane *et al.*, 1999). These containers, however, require very small volumes of embryo suspension (less than 2 µl), thus restricting the number of samples per container to 10-15 (EM) grid and 4-6 for open pulled straw (OPS) and cryoloop. On the other hand, large quantities of immature bovine oocytes (up to 65 cumulus-oocyte complexes (COCs) per container) have been successfully vitrified using nylon mesh (Matsumoto *et al.*, 2001). In all these vitrification procedures success has increased.

In buffalo, *in vitro* embryo production may present the best tool to improve genetic progress due to limitations in multiple ovulation-embryo transfer. The only constraint is represented by the low number of oocytes recoverable (Gasparini, 2002) hence by the scarcity of experimental material in the majority of the countries where buffaloes are bred. Thus, oocyte cryopreservation is fundamental in increasing the availability of female gametes for both research purpose and commercial use.

Successful vitrification of unfertilized oocytes has been reported in some mammalian species (Wood *et al.*, 1993; Kuleshova *et al.*, 1999; Viera *et al.*, 2002).The rate of subsequent fertilization and development are much lower however, than in those obtained using fresh oocytes. An insufficient cooling rate during vitrification is believed to be one of the limiting factors (Vajta, 1997).The simplest way to overcome this is by directly dropping the oocytes into LN_2 as first proposed for mouse embryos (Landa and Tepla, 1990) then successfully used for bovine embryos and oocytes (Riha *et al.*, 1991; Papis *et al.*, 2000) and goat embryos (Ocampo *et al.*, 2001). Attempts of its application in immature buffalo oocytes (Soriano *et al.*, 2003) had encouraging results. However, the optimal condition for its efficient use has not yet been established and the developmental ability of vitrified immature and *in vitro* matured buffalo oocytes has not been examined.

This study aimed to establish the optimum conditions for vitrification of both immature and *in vitro* matured buffalo oocytes by investigating the possible influence of some biological (*e.g.*, meiotic stage, cumulus cells and oocyte source) and related factors (*e.g.*, pre-equilibration exposure time, warming condition) using the minimum drop size (MDS) and cryotop methods. The developmental competence of post-warmed oocytes after *in-vitro* maturation (IVM) and *in vitro* fertilization (IVF) to produce embryos was also examined.

METHODOLOGY

Reagents and Media

Unless otherwise stated, all reagents were purchased from Sigma Chem (USA). *In vitro* maturation medium was TCM-199 medium buffered with 25 mM Na bicarbonate supplemented with 10% FCS, 0.2 mM Na pyruvate, 0.5 µg per ml FSH, 1 µg per ml 17β-estradiol, 50 µM cysteamine and antibiotics (BM). The fertilization medium was modified Brackett and Oliphant (mBO) medium supplemented with 3 mg per ml BSA and 2.5 mM theophylline. The embryo culture medium was modified synthetic oviductal fluid (mSOF) + 5% fetal calf serum (FCS).

Oocyte Collection and Maturation

COCs were recovered from ovaries of slaughtered buffaloes by aspiration of 3-5 mm antral follicles using an 18-gauge needle attached to a 5 ml plastic syringe, or from ovaries of donor buffaloes through transvaginal ultrasound-guided follicular aspiration (TUFA). Only COCs with compact, non-atretic cumulus cells (at least 3 layers) and homogenous ooplasm were selected and allocated in 50 µl drops (10-15 COCs per drop) of maturation medium under mineral oil in a 5% CO_2 incubator at 39ºC for 22-24 hr.

Vitrification and Warming Solution

Vitrification solution 1 (VS1) consist of BM + 20% FCS + 4% ethylene glycol (EG) while vitrification solution 2 consist of (VS2) of BM + 20% FCS + 40% EG + 50 mg per ml PVP + 0.3 M trehalose. The warming solution (WS) was BM + 20% FCS + 0.3 M trehalose. Oocyte survival was based on the integrity of the oocyte membrane and *zona pellucida* together with homogeneity of the ooplasm. Surviving oocytes were transferred into drops of IVM medium for maturation (in case of immature oocytes) or for 1 hr (in case of matured oocytes) before subjecting for fertilization.

***In Vitro* Fertilization and Culture**

Frozen-thawed sperm of fertile bull were used for IVF and processed by the swim-up method. Briefly, the semen suspension was put in a test tube, added with 5 ml of mBO medium and centrifuged (2000 rpm for 5 min) for washing (twice). The sperm pellet was then layered with 2 ml fertilization medium and put in the incubator for 1 hr. Afterwards, about 1.8 ml of the upper portion of the semen suspension was recovered and washed. The sperm pellet was re-suspended in the same medium to give an initial sperm suspension of 2 x 10^6 sperm per ml. A 50 µl aliquot of the sperm suspension was added in droplets of fertilization medium containing pre-washed oocytes to co-incubate for at least 6 hr with a final sperm concentration of 1 x 10^6 sperm per ml. After sperm-oocyte co-incubation, the extra sperms attached to the *zona pellucida* were removed by pipetting, and washed twice before culturing. Embryo developmental stage attained was assessed morphologically every 2 days until the 7^{th} day of culture in mSOF medium + 5% FCS in an incubator set to 39°C with a humidified atmosphere of 5% CO_2 and 90% N_2.

Evaluation of Oocyte Maturation and Fertilization

Both non-vitrified and vitrified/ warmed oocytes were washed (2x) at the end of maturation period before fixation for about 48 hr in aceto-methanol (1:3, v/v) solution at room temperature, stained with 1% orcein in 45% (v/v) acetic acid in water and examined for evidence of nuclear maturation (Experiments 1 and 2), distinguished by the presence of 1st polar body or presence of penetrating sperm head and/or male pronucleus with visible sperm tail (Experiments 3 and 4) using phase contrast microscopy (200 – 400 x magnification).

Experimental Group

In Experiment 1, factorial studies (2 x 4) were designed to investigate the effect of cumulus cells and pre-equilibration time in VS1 on the survival of GV stage oocytes using the MDS method. Oocytes were divided into 2 groups (with or without cumulus cells) corresponding to 4 pre-equilibration time (3, 5, 10 and 15 min) used, then exposed to VS2 for 45 sec before directly placing in LN_2. After 1 wk of storage the oocyte pellets (5 COCs) with the oocytes were recovered and warmed by directly placing in wells of WS (300 µl) for 5 min, washed twice and evaluated.

In Experiment 2, different steps of warming were investigated on the survival of GV stage oocytes. The control represented non-vitrified oocytes. Treatment 1 (T_1) represented a direct step warming condition as described above. In treatment 2 (T_2), vitrified

oocytes in pellet form were directly placed in wells of WS for 5 min each with descending trehalose concentration (0.3, 0.15 and 0.075 M) before washing and evaluation. In treatment 3 (T_3), vitrified COCs in pellet form were directly placed in wells of WS with descending trehalose concentration of 0.3 M, 0.15 M and 0.075 M for 2 minutes, 1 minutes and 1 minutes exposure time, respectively before washing and evaluation.

In Experiment 3, the effect of meiotic stage (GV and metaphase 2 stage (M_2) and vitrification methods (MDS versus Cryotop method) were evaluated on the survival of buffalo oocytes before using for IVF. The oocytes were exposed to VS1 and VS2 for 10 min and 45 sec, respectively before directly placing in LN_2. Step-wise warming condition of descending trehalose concentration and exposure time was used before evaluating and selecting survived oocytes for IVF.

In Experiment 4, the effect of oocyte source (*in vitro* derived versus *in vivo* derived through TUFA) on the survival and fertilizability of M_2 stage oocytes were examined. Selected good quality COCs were matured *in vitro* before vitrification (using cryotop method as described above) and fertilization.

In Experiment 5, the developmental competence of *in vitro* matured oocytes was evaluated following fertilization by culturing in mSOF medium + 5% FCS. The control group represented oocytes collected from the local abattoir while the treatment groups represented vitrified/warmed oocytes using the cryotop method and step-wise warming condition of descending concentration and exposure time.

Statistical Analysis

The General Linear Models procedure and Duncan's Multiple Range Test of Statistical Analysis were used to determine differences among treatment groups in each experiment. Value differences of $p<0.05$ were considered statistically different.

RESULTS AND DISCUSSION

Table 1 shows the survival and maturation rates of oocytes vitrified with or without cumulus cells after exposure

Table 1. Survival of GV stage buffalo oocytes after exposure to VS1 at different time in the presence or absence of cumulus cells

Oocyte Condition	Exposure time (min)	No. of oocytes (%)			
		Exposed	Recovered	Survived	Matured
With CCs					
Control		50	50 (100.0)	47 (94.0)[a]	39 (83.0)[a]
	3	50	48 (96.0)	21 (43.7)[b]	3 (14.3)[b]
	5	52	47 (90.4)	36 (76.6)[c,e]	8 (22.2)[b,c]
	10	50	47 (94.0)	40 (85.1)[a,c]	4 (35.0)[d]
	15	50	48 (96.0)	38 (79.1)[c,e]	11 (28.9)[c,d]
Denuded					
Control		50	50 (100.0)	45 (90.0)[a]	36 (80.0)[a]
	3	50	47 (94.0)	19 (40.4)[b]	2 (10.5)[b]
	5	50	50 (100.0)	32 (64.0)[d]	8 (25.0)[c]
	10	55	50 (90.9)	40 (80.0)[a,c,e]	9 (22.5)[c]
	15	52	50 (96.2)	36 (72.0)[c,e]	8 (22.2)[c]

Means with different superscript within same column differ (P<0.05). Data taken from 5 replicates

to different pre-equilibration time. The percentage recovery rates in all treatments following 1 wk of storage in pellet form in cryotubes were more than 90%. Survival rate of oocytes was higher at 5-15 min than 3 min pre-equilibration time. Similarly, maturation rate after 24 hr of culture was higher in oocytes pre-equilibrated for 5–15 min than those exposed for 3 min. However, survival and maturation rates obtained in the treatment groups were significantly lower ($p<0.05$) than the control.

In Table 2, presents survival and maturation rates of oocytes following different warming condition. Survival rates of oocytes showed no significant difference between the non-vitrified and vitrified groups based on morphological appearance. However, significant differences in maturation rate between the control and the treatment groups were observed. Also, difference in maturation rate between T_3 (52.3 %) and T_1 (35.0 %) was observed.

Recovery and survival rates of oocytes post warming using either MDS or cryotop method showed no difference (Table 3). The fertilizability of vitrified/warmed GV stage oocytes after culturing for maturation similarly showed no difference. Oocytes vitrified at M_2 stage had a higher fertilizability rate than those vitrified at GV stage though not significant when using the MDS method.

M_2 stage oocytes after IVM of COCs derived from local abattoir or through TUFA were vitrified by the cryotop method (Table 4). The recovery and survival rate of vitrified groups showed no difference from the control. The fertilization rate of vitrified group regardless of the source of the oocytes (*in vitro* derived; 39.4% and *in vivo* derived; 43.9%) had no difference but were significantly lower than the control (76.0%).

Table 5 shows the cleavage and blastocyst formation rate of non-vitrified (control) and vitrified M_2 stage oocytes. Both cleavage (72.0%) and blastocyst formation rate (25.0%) of non-vitrified oocytes were higher

Table 2. Survival of GV stage buffalo oocytes after exposure to different warming conditions

Warming Condition	No. of oocytes (%)			
	Vitrified	Recovered	Survived	Matured
Control		50 (100.0)	47 (94.0)	39 (82.9)[a]
T_1	50	47 (94.0)	40 (85.1)	14 (35.0)[b]
T_2	55	53 (96.4)	46 (86.8)	21 (45.6)[b,c]
T_3	50	50 (100.0)	44 (88.0)	23 (52.3)[c]

Means with different superscript within same column differ ($p<0.05$). Data taken from 5 replicates

Table 3. Survivability and fertilizability of buffalo oocytes vitrified at different meiotic stages using two vitrification methods

Meiotic Stage	Vitrification Method	No. of oocytes (%)				
		Vitrified	Recovered	Survived	Inseminated	Fertilized
GV	MDS	52	50 (96.2)	43 (86.0)	21 (48.8)	6 (28.6)[a]
	Cryotop	52	48 (92.3)	43 (89.6)	22 (51.2)	7 (31.8)[a]
M_2	MDS	50	50 (100.0)	45 (90.0)	45 (100.0)	17 (37.8)[a,b]
	Cryotop	60	60 (100.0)	56 (93.3)	56 (100.0)	25 (44.6)[b]

Means with different superscript within same column differ ($P<0.05$).Data taken from 5 replicates.

Table 4. Effect of oocyte source on the survival and fertilizability of buffalo oocytes

Oocyte Source	No. of oocytes (%)				
	Vitrified	Recovered	Survived	Inseminated	Fertilized
Control		52	50 (96.2)	50	38 (76.0)[a]
In vitro	115	112 (97.4)	104 (92.8)	104	41 (39.4)[b]
In vivo	96	90 (93.7)	82 (91.1)	82	36 (43.9)[b]

Means with different superscript within same column differ (P<0.05). Data taken from 5-8 replicates

Table 5. Development of buffalo oocytes after vitrification and fertilization

Treatment	No. of oocytes (%)		
	Inseminated	Cleaved	Blastocyst
Control	68	49 (72.0)[a]	17 (25.0)[a]
Vitrified	55	22 (40.0)[b]	2 (3.6)[b]

Means with different superscript within same column differ (P<0.05). Data taken from 5-8 replicates

(P<0.05) than vitrified oocytes (40.0% cleavage and 3.6% blastocyst formation rate).

Live births have been reported from successful cryopreservation of mammalian oocytes through slow cooling (Chen, 1986; Al-Hasani, 1987) and vitrification methods (Kuleshova *et al.*, 1999; Vajta *et al.*, 1998). Despite these successes however, oocyte freezing has remained slow to be adopted due to extreme variability in the survivality post thawing/ warming and low percentage of development to term either after conventional IVF or intracytoplasmic sperm injection. Apparently, the species-specificity requirements of oocytes/ embryos are constraints to the successful application of a particular cryopreservation technique.

In this study, a number of factors were investigated in an effort to optimize the needed conditions for cryopreservation of buffalo oocytes. The choice of using the MDS method of vitrification in Experiments 1 and 2 was based on successful application in goat embryos with resulting live births (Ocampo *et al.*, 2001). The method allowed direct contact of oocyte-containing VS2 solution to LN_2, eliminating possible insulation effect of container wall used in other vitrification methods.

The presence or absence of cumulus cells prior to vitrification may have a direct impact on oocyte survival post warming. Although not proven, cumulus cells was suggested to offer some protection against sudden changes and stresses induced by rapid influx of CPA during equilibration and CPA removal during warming (Fabri, 2001). Our results showed that both survival and maturation rates of COCs or denuded oocytes had no significant difference in relation to the pre-equilibration time used, although the values obtained were numerically higher in COCs and in 10 min exposure time. In contrast, earlier work in bovine oocytes using MDS method reported higher survival and maturation rate when using 3 min pre-equilibration time (VS2) and 1 min exposure time to VS1 (Kim *et al.*, 2007). The observed difference could however be attributed on the CPA used and oocyte permeability. On the other hand, the maturation rate of vitrified oocytes was lower than the fresh oocytes despite no significant difference on the percentage recovery post warming and survival rate at 10 min exposure time. Possible damage on intercellular contact between the cumulus

cells and oocytes via gap junction brought about by cryopreservation (Diaz, *et al.*, 2005; Fuku *et al.*, 1995) could have resulted to low maturation rate. It is known that intercellular contact via gap junction plays an important role in metabolic cooperation between the oocytes and cumulus cells during the growth phase and final maturation of oocytes (Rojas, 2004).

Also, oocyte survival is dependent on both cooling and warming rates as influenced by the type of CPA used and its concentration. Warming may restore the normal spindle morphology of the oocytes, hence appropriate removal of CPA from vitrified oocytes significantly affects its survival and efficiency of the method used (Attanasio *et al.*, 2007). In this study, survival rates obtained in different warming methods examined had no difference based on morphology but maturation rate was highest in oocytes warmed in T_3 compared to T_1 and T_2, indicating better removal of CPA from the oocytes when using T_3. Overall maturation rate however, remained significantly lower versus the control suggesting that the oocytes were compromised even with normal morphology post warming. Such responses could be attributed to biological responses of the oocytes to warming condition, eg., abnormal spindle fiber formation post warming (Aman and Parks, 1994; Saunders and Parks, 1999).

In Experiment 3, the cryotop method of vitrification appeared better than MDS method in terms of oocyte fertilizability post warming regardless of the meiotic stage prior to vitrification. Apparently, the observation that droplets of VS2 containing the oocytes floating for a few seconds on the surface of LN_2 before sinking when using MDS method could have slowed down the necessary cooling rate for complete vitrification in contrast to the cryotop method wherein the carrier is submerged directly into LN_2. It must be recalled that oocyte survival is strongly dependent on cooling rate though it is influenced by the type and concentration of CPA used. Also, M_2 stage oocytes vitrified by cryotop method was observed to have a higher fertilizability rate than GV stage oocytes. While damage on the cytoskeleton resulting from chilling may be avoided when using GV stage oocytes, the difficulties associated with IVM and extended culture appear to counter the potential benefits of vitrifying GV stage oocytes. Also, chilling reduces the developmental capability of GV oocytes, apart from damage to the meiotic spindle (Martino *et al.*, 1996). In contrast, GV stage oocytes were said to be less susceptible to cryoinjury because they are slightly smaller than M_2 stage oocytes, lack zona pellucida and cortical granules are still in quiescent stage of development. Immature oocytes have a longer period to recover from cryoinjury because they have to mature *in vitro* prior to insemination or other manipulations (Shaw, 2000).

In Experiment 4, oocyte source was found to have no influence on the survival and fertilizability of vitrified oocytes. In the *in vitro* embryo production system reported in buffalo, it was suggested that the heterogenous quality of oocytes recovered from follicles of different stages of growth and atresia from the ovaries of abattoir derived animals and possible cellular damage due to autolytic processes after residing for a prolonged period in excised ovaries contributed to the lower blastocyst yield compared to *in vivo* derived oocytes (Neglia *et al.*, 2003; Manjunatha, 2008). In contrast, *in vivo* derived oocytes are mostly of homogenous quality due to the nature of collection (2x a week) resetting follicular population of homogenously sized follicles resulting to a higher blastocyst yield (Gasparini, 2002). On the contrary, report in cattle have not shown a difference on the developmental competence when two sources of oocytes were compared (Galli *et al.*, 2001). In this study, vitrification of buffalo oocytes regardless of the meiotic stages and source offers no advantage over the other suggesting that the reduced ability of vitrified/ warmed oocytes to be fertilized was due to the detrimental effects occurring during the phase of vitrification.

The developmental competence of vitrified/

warmed oocytes post fertilization to produce blastocyst stage embryos was significantly reduced compared to the control. Apparently, the oocyte's subcellular structure and its component's sensitivity to low temperature, osmotic pressure and ionic strength negatively affected the oocytes developmental competence (Kim *et al.*, 2007; Park *et al.*, 2005; Al-Hasani, 2007; Rall *et al.*, 1987; Papis *et al.*, 2000; Fabri, 2001; Fuku *et al.*, 1995). Our results had a blastocyst formation rate of 3.6%, similar to vitrification of immature bovine oocytes with 2.3% blastocyst formation rate (Kim *et al.*, 2007). The cleavage rate obtained was comparable to the reported immature bovine oocyte vitrification by MDS (Kim *et al.*, 2007) and OPS (Viera *et al.*, 2002) method but better than the rates when using EM grid (Martino *et al.*, 1996) and nylon mesh (Matsumoto *et al.*, 2001). These findings indicate some technical advancement on the vitrification of buffalo oocytes.

CONCLUSION

Swamp buffalo germinal vesicle (GV) and metaphase II (M2) stage oocytes can survive vitrification either by MDS or cryotop method, could complete 1st meiotic division, and be fertilized and produce embryos although to a limited extent. However, more studies should be done to improve the rates of blastocyst formation *in vitro* of vitrified/warmed buffalo oocytes.

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