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## Vitrification of Swamp Buffalo Oocytes

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**Abstract** In this study, various factors (eg., presence/absence of cumulus cells, meiotic stage, oocyte source, pre-equilibration time, warming condition, method of vitrification) were examined in trying to establish the optimum condition for vitrification of buffalo oocytes. Survivability was based on the morphological normality post-warming, completion of 1<sup>st</sup> meiosis and penetrability post fertilization in vitro. In Expt. 1, data gathered showed that survivability of GV stage COCs or denuded oocytes was significantly lower than the control regardless of pre-equilibration time used except when using 10 min exposure time. The maturation rate of vitrified oocytes was lower against the control. In Expt. 2, the survivability of vitrified/warmed GV oocytes had no difference with the control regardless of the warming conditions used but 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 but same exposure time. In Expt. 3, vitrification of oocytes at GV or M<sub>2</sub> stage showed no difference in terms of survival and fertilizability but the use of cryotop method appeared better than the MDS method. In Expt. 4, oocyte source had no effect on the survival and fertilizability of vitrified/warmed M<sub>2</sub> stage oocytes. In Expt. 5, The cleavage and blastocyst formation rate of vitrified/warmed oocytes was lower against the control. The results showed that buffalo GV and M<sub>2</sub> stage oocytes can survive vitrification either by MDS or cryotop method, complete 1<sup>st</sup> meiotic division, be fertilized and produce embryos although at limited extent.

**Keywords:** Oocytes, vitrification, survival, maturation, fertilization, embryos

### Introduction

Nowadays, attention has focused on vitrification as a rapid and efficient method for cryopreservation of biological systems. Apparently, vitrification can be and has been defined in different ways (Kauzmann, 1948), but essentially is the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling. During vitrification, the solution is to become a glass, translational molecular motion are significantly arrested,

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making the effective end of biological time but without any of the changes brought about by freezing. So that, gametes and/or organs capable of being vitrified need no longer 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 a good survival following vitrification, gametes and/or embryos must be able to tolerate exposure and dehydration to 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, also osmotic and chilling injury, zona and blastomere fracture, 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 the liquid nitrogen (LN<sub>2</sub>). The various protocols describe for vitrification are very simple, and they allow cells and tissue to be placed directly into the cryoprotectants and then plunged directly into LN<sub>2</sub>. However, most vitrification methods developed used 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 C/min. Alternative methods allowing direct contact between the embryos containing medium and LN<sub>2</sub> that increased cooling and warming rates have been reported using the electron microscope grids (Martino *et al.*, 1996), in thin-walled open pulled straw (Vajta *et al.*, 1998) or on cryoloops (Lane *et al.*, 1999). These containers however, require very small volume of an embryo suspension (less than 2 µl), thus restricting the number of samples per container to 10-15 (EM) grid and 4-6 for OPS and cryoloop. On the other hand, large quantities of immature bovine oocytes (up to 65 COCs/container) have been successfully vitrified in using a nylon mesh (Matsumoto *et al.*, 2001). In all these vitrification procedures success has been increased. In buffalo, in vitro embryo production may present the best tool to improve genetic progress due to limitation with multiple ovulation-embryo transfer programs. The only constraint is represented by the low number of oocytes recoverable (14) and 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 purposes 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 vitification 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<sub>2</sub> 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 was not examined. This study aimed to establish the optimum conditions for the vitrification of both immature and in vitro matured buffalo oocytes by investigating the possible influence of some biological (eg., meiotic stage, cumulus cells and oocyte source) and related factors (eg., pre-equilibration exposure time, warming condition) using the minimum drop size (MDS) and cryotop methods. The developmental competence of post-warmed oocytes after IVM/IVF to produce embryos was also examined.

## **Materials and methods**

### ***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/ml FSH, 1 µg/ml 17β-estradiol, 50 µM cysteamine and antibiotics (BM). The fertilization medium was modified Brackett and Oliphant (mBO) medium supplemented with 3 mg/ml BSA and 2.5 mM theophylline. The embryo culture medium was modified synthetic oviductal fluid (mSOF) + 5% FCS.

### ***Oocyte collection and maturation***

Cumulus-oocyte complexes (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 a homogenous ooplasm were selected and allocated in 50 µl drops (10-15 COCs/drop) of maturation medium under mineral oil in a 5% CO<sub>2</sub> incubator at 39°C for 22-24 hr.

### ***Vitrification and warming solution***

The vitrification solution 1 (VS1) consist of BM + 20% FCS + 4% ethylene glycol (EG) and vitrification solution 2 (VS2) of BM + 20% FCS + 40% EG + 50 mg/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 the zonapellucida together with the homogeneity of theoplasm. 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 semen suspension was recovered and washed. The sperm pellet was re-suspended in the same medium to given an initial sperm suspension of  $2 \times 10^6$  sperm/ml. Then, a 50  $\mu$ l 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  $1 \times 10^6$  sperm/ml. After sperm-oocyte co-incubation, the extra sperms attached to the zonapellucida were removed by pipetting, washed twice before culturing. Embryo developmental stage attained was assessed morphologically every 2 days until the 7<sup>th</sup> day of culture in mSOF medium + 5% FCS in an incubator set to 39°C in a humidified atmosphere of 5% CO<sub>2</sub> and 90% N<sub>2</sub>.

### ***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 (eg., Expt. 1 and 2), distinguished by the presence of 1<sup>st</sup> polar body or presence of penetrating sperm head and/or male pronucleus with visible sperm tail (eg., Expt. 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 survivability of GV stage oocytes using the MDS method. COCs were divided into 2 groups (with or without cumulus cells) corresponding to the 4 pre-equilibration time (eg., 3, 5, 10 and 15 min) used, then exposed to VS2 for 45 sec before directly placing to the LN<sub>2</sub>. After 1 wk of storage, the 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 survivability of GV stage oocytes. The control represent the non-vitrified oocytes. Treatment 1 (T<sub>1</sub>) represent a direct step warming condition as described above. In treatment 2 (T<sub>2</sub>), vitrified oocytes in pellet form were directly placed in wells of WS for 5 min each with descending trehalose concentration (eg., 0.3, 0.15 and 0.075 M) before washing and evaluation. In treatment 3 (T<sub>3</sub>), 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 min, 1 min and 1 min exposure time, respectively before washing and evaluation.

In Experiment 3, the effect of meiotic stage (eg., GV and metaphase 2 stage (M<sub>2</sub>)) and vitrification methods (eg., MDS versus Cryotop method) were evaluated on the survivability 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 into the LN<sub>2</sub>. 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 (eg., in vitro derived versus in vivo derived through TUFAs) on the survivability and fertilizability of M<sub>2</sub> stage oocytes were examined. Selected good quality COCs were in vitro matured before vitrification (using Cryotop method as described above) and fertilization.

In Experiment 5, the developmental competence of in vitro matured oocytes were evaluated following fertilization by culturing in mSOF medium + 5% FCS. The control group represent the oocytes collected from the local abattoir while the treatment group represent the 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 System were used to determine the differences among treatment groups in each experiment. Values difference of  $P < 0.05$  were considered statistically different.

### Results

Table 1 shows the survival and maturation rates of oocytes vitrified with or without cumulus cells after exposure to different pre-equilibration time. The percentage recovery rate in all treatments following 1 wk of storage in pellet form in cryotubes were more than 90%. The survival rate of oocytes with or without cumulus cells after pre-equilibration time of 5 – 15 min was higher than 3 min exposure time. Similarly, the maturation rate after 24 hr of culture was higher in oocytes pre-equilibrated for 5 – 15 min than those exposed for 3 min. However, all figures obtained in the treatment groups survival and maturation rates were significantly lower versus the control.

**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
<b>With CCs</b>					
Control	50	50 (100.0)		47 (94.0) <sup>a</sup>	39 (83.0) <sup>a</sup>
3	50	48 (96.0)		21 (43.7) <sup>b</sup>	3 (14.3) <sup>b</sup>
5	52	47 (90.4)		36 (76.6) <sup>c,e</sup>	8 (22.2) <sup>b,c</sup>
10	50	47 (94.0)		40 (85.1) <sup>a,c</sup>	4 (35.0) <sup>d</sup>
15	50	48 (96.0)		38 (79.1) <sup>c,e</sup>	11 (28.9) <sup>c,d</sup>
<b>Denuded</b>					
Control	50	50 (100.0)		45 (90.0) <sup>a</sup>	36 (80.0) <sup>a</sup>
3	50	47 (94.0)		19 (40.4) <sup>b</sup>	2 (10.5) <sup>b</sup>
5	50	50 (100.0)		32 (64.0) <sup>d</sup>	8 (25.0) <sup>c</sup>
10	55	50 (90.9)		40 (80.0) <sup>a,c,e</sup>	9 (22.5) <sup>c</sup>
15	52	50 (96.2)		36 (72.0) <sup>c,e</sup>	8 (22.2) <sup>c</sup>

<sup>a,b,c,d,e</sup> Values with different superscript within same column differ ( $P < 0.05$ ). Data taken from 5 replicates.

In Table 2, the survival and maturation rates of oocytes following different warming conditions are presented. No difference was observed on the survival rates of oocytes between the non-vitrified and vitrified groups based on its morphological appearance. However, significant differences on the

maturation rate between the control versus the treatment groups was observed. Also, difference on maturation rate between T<sub>3</sub> (52.3 %) and T<sub>1</sub> (35.0 %) was noticed.

**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	47 (94.0)		39 (82.9) <sup>a</sup>
T <sub>1</sub>	50	47 (94.0)	40 (85.1)	14 (35.0) <sup>b</sup>
T <sub>2</sub>	55	53 (96.4)	46 (86.8)	21 (45.6) <sup>b,c</sup>
T <sub>3</sub>	50	50 (100.0)	44 (88.0)	23 (52.3) <sup>c</sup>

<sup>a,b,c</sup>Values with different superscript within same column differ (P<0.05). Data taken from 5 replicates

The 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<sub>2</sub> stage had a higher fertilizability rate than those vitrified at GV stage though not significant when using the MDS method.

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

Meiotic Stage	method	Vitrification No. of oocytes (%)				
		Vitrified	Recovered	Survived	Inseminated	Fertilized
GV	MDS	52	50 (96.2)	43 (86.0)	21 (48.8)	6 (28.6) <sup>a</sup>
	Cryotop	52	48 (92.3)	43 (89.6)	22 (51.2)	7 (31.8) <sup>a</sup>
M <sub>2</sub>	MDS	50	50 (100.0)	45 (90.0)	45 (100.0)	17 (37.8) <sup>a,b</sup>
	Cryotop	60	60 (100.0)	56 (93.3)	56 (100.0)	25 (44.6) <sup>b</sup>

<sup>a,b</sup>Values with different superscript within same column differ (P<0.05).Data taken from 5 replicates.

M<sub>2</sub> 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 with the control (non-vitrified group). 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 was lower significantly with the control (76.0%).

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

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

<sup>a,b</sup>Values with different superscript within same column differ (P<0.05). Data taken from 5-8 replicates.

Table 5 shows the cleavage and blastocyst formation rate of non-vitrified (control) and vitrified M<sub>2</sub> stage oocytes. After 48 hr of culture post fertilization, cleaved embryos at 2 – 4 cell stages were transferred to culture medium and evaluated for development up to the 7<sup>th</sup> day. Both the cleavage (72.0%) and blastocyst formation rate (25.0%) of non-vitrified oocytes were higher (P<0.05) than vitrified oocytes (40.0% cleavage and 3.6% blastocyst formation rate).

**Table 5.** Development of buffalo oocytes after vitrification and fertilization

Treatments	No. of oocytes (%)		
	Inseminated	Cleaved	Blastocyst
Control	68	49 (72.0) <sup>a</sup>	17 (25.0) <sup>a</sup>
Vitrified	55	22 (40.0) <sup>b</sup>	2 (3.6) <sup>b</sup>

<sup>a,b</sup>Values with different superscript within same column differ (P<0.05). Data taken from 5 replicates

## Discussion

Live births have been reported from successful cryopreservation of mammalian oocytes through slow cooling (Park *et al.*, 2005; Chen, 1986) and vitrification methods (Kuleshova *et al.*, 1999; Vajta, 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 serve as a constraint on 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 Expt. 1 and 2 was based on our successful application in goat embryos with resulting live births (Ocampo *et al.*, 2001). The method allowed direct contact of oocyte-containing VS2 solution to the LN<sub>2</sub>, eliminating the 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, the cumulus cells was suggested to offer some protection against sudden changes and stresses induced by the rapid influx of CPA during the procedures of equilibration and CPA removal during warming (Fabbri, 2001). Our results showed that both survival and maturation rate of COCs or denuded oocytes have no significant difference in relation to the pre-equilibration time used, although the values obtained were 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.*, 2005). The observed difference could nonetheless 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 the oocytes (Rojas *et al.*, 2004).

Also, oocyte survival is dependent on both cooling and warming rate 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 affect its survival and the efficiency of the method used (Attanasio *et al.*, 2007). In this study, the survival rates obtained in different warming methods examined had no difference based on its morphology but the maturation rate was highest in oocytes warmed in T<sub>3</sub> versus T<sub>1</sub> and T<sub>2</sub>, indicating a better removal of CPA from the oocytes when using T<sub>3</sub>. Overall maturation rate however, remained lower significantly versus the control suggesting that the oocytes were compromised even with normal morphology post warming. Such responses could be attributed on the biological responses of the oocytes to warming condition, eg., abnormal spindle fiber formation post warming (Aman and Parks, 1994; Saunder and Parks, 1999).

In Expt3, 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<sub>2</sub> before sinking when using MDS method could have slow down the necessary cooling rate for complete vitrification to achieved in contrast to the cryotop method

wherein the carrier is submerged directly into the LN<sub>2</sub>. It must be recalled that oocyte survival is strongly dependent on cooling rate though is influenced by the type and concentration of CPA used. Also, M<sub>2</sub> 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 counteract 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<sub>2</sub> stage oocytes, lack zonapellucida 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 Expt. 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 heterogeneous quality of oocytes recovered from the ovaries of abattoir derived animals from follicles of different stages of growth and atresia 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 *et al.*, 2008). In contrast, in vivo derived oocytes are mostly of homogeneous quality due to the nature of collection (2x a week) resetting follicular population of homogeneously sized follicles resulting to a higher blastocyst yield (Gasparini, 2002). On the contrary, reports 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 each other suggesting that the reduced ability of vitrified/warmed oocytes to be fertilized was due to the detrimental effect 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 oocytes subcellular structure and its components 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, 1987; Rall *et al.*, 1987, Papis *et al.*, 2000; Fabbri, 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. However, more studies has to be done to improve the rates of blastocyst formation in vitro of vitrified/warmed buffalo oocytes.

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