



Effect of different types of cryoprotectants on developmental capacity of vitrified-thawed immature buffalo oocytes

K.H. El-Shahat^{1,3}, A.M. Hammam²

¹Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University

²Department of Animal Reproduction and Artificial Insemination,
National Research Center, Dokki, Egypt.

Abstract

Studies were conducted to compare viability of immature buffalo oocytes vitrified in different types of cryoprotectants on the post-thaw morphological appearance, the *in vitro* maturation and developmental competence of buffalo oocytes. The vitrification solution (VS) consisted of Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.5 M sucrose, 0.4% bovine serum albumin (BSA) and different types of molar (M) concentrations of the cryoprotectants which were composed of either glycerol (G), ethylene glycol (EG) or dimethyl sulfoxide (DMSO) in order to determine the best type of vitrification cryoprotectants. The concentrations tested were 4 M, 7 M and 7M concentration of G, EG and DMSO, respectively. Cumulus oocyte complexes (COCs) were obtained from slaughterhouse ovaries. Oocytes and their cumulus cells were vitrified immediately after collection. The COCs were pre-equilibrated in 50% of the VS for 3-5 min, then kept in VS for 1 min and loaded in pre-sterilized 0.25 ml straws for 7-10 days of storage in liquid nitrogen. The straws were thawed in warm water at 37°C for 10 s. The COCs were equilibrated in 0.5 M sucrose in modified phosphate buffer (M-PBS) for 5 min and then washed in washing medium (TCM-199 plus 10% FCS). Oocyte-cumulus cells were evaluated for morphological damage. Morphologically normal COCs (Oocyte-cumulus cells) were cultured *in vitro* and evaluated for maturation. Oocytes were fertilized with frozen-thawed semen capacitated in Brackett and Oliphant (BO) medium containing heparin and caffeine and were evaluated for cleavage and embryonic development. The results revealed that the proportion of buffalo oocytes found to be morphologically normal was significantly ($P < 0.05$) higher in EG and DMSO than those obtained in G (85.0 and 83.33 vs. 65.0%, respectively). Among the damaged oocytes, cracking of zona pellucida was the most frequent abnormality observed. A significantly higher ($P < 0.05$) percentage of maturation derived from vitrified -thawed immature oocyte in EG than those obtained in G (47.05, vs. 30.76%, respectively). A significantly higher proportion of oocytes were cleaved in EG and DMSO compared to those obtained in G (28.57 and 25.71 vs.

10.0%; $P < 0.05$, respectively). A similar trend was observed in blastocyst stage produced *in vitro*. However, *in vitro* developmental competence was higher for vitrified-thawed fresh oocytes (control) than those obtained from all groups of cryoprotectants. In conclusion, the 7 M solution of EG or DMSO could be used for vitrification of immature buffalo oocytes for their subsequent utilization in the *in vitro* maturation, fertilization and embryo production.

Keywords: *Bubalus bubalis*, dimethyl sulfoxide, ethylene glycol, fertilization, glycerol, *in vitro* maturation, straw, ultra rapid freezing.

Introduction

The cryopreservation of mammalian embryos has become an integral part of methods to control animal reproduction. Cryopreservation is of practical importance for the development of reproductive technologies such as nuclear transfer, cryobanking of animal species and the routine or commercial application of breed improvement such as embryo transfer (Leibo *et al.*, 1996; Wani, 2002). Several cryopreservation methods such as conventional (slow), equilibrium rapid freezing (vitrification) and ultra rapid freezing have been used to preserve embryos and oocytes of many animal species resulting in the birth of live offspring. Vitrification, which is a relatively recent approach, is defined as a physical process by which a highly concentrated solution of cryoprotectants solidifies during cooling, without formation of ice crystals (Niemann, 1991). Vitrification is a common method for cryopreservation of gametes and embryos. Although successful oocyte vitrification has been achieved in several animal species, subsequent progress is still limited especially in buffalo (Mahmoud *et al.*, 2013). Many compounds act as cryoprotectants and are used for protection of cells against freezing. There are two types of cryoprotectants: (a) membrane permeating which can freely diffuse the membrane such as glycerol (G), ethylene glycol (EG) and dimethyl sulfoxide (DMSO); (b) non membrane permeating which cannot permeate the cell membrane such as sugars (Pedro *et al.*, 2005). The primary problem with cooling or cryopreservation of oocytes is the low percentage of

³Corresponding author: attiakh@yahoo.com
Phone: +20(2)6468-8386; Fax: +20(2)3572-5240
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oocytes retaining the ability to undergo normal maturation and fertilization. This may be attributed to the exposure to the cryoprotectant and the cooling process (Le Gal, 1996). To improve the effectiveness of vitrification of buffalo oocytes, the present study evaluated the comparative effect of cryoprotectants (glycerol, dimethyl sulfoxide and ethylene glycerol) on the developmental capacity of vitrified-thawed immature buffalo oocytes.

Materials and Methods

Collection of oocytes

Buffalo ovaries were collected from an abattoir and were transported within 2 h to the laboratory in 0.9% normal saline supplemented with antibiotics (100 ug/ml streptomycin and 100 iu/ml penicillin) at 37°C. Follicular oocytes (3-8 mm in diameter) were aspirated using 20-gauge needle attached to a 5 ml syringe. The aspiration medium consisted of M-PBS (modified phosphate buffer saline) supplemented with 10% FCS (Fetal calf serum, Sigma, USA). All the aspirated cumulus-oocyte complexes (COCs) with homogenous cytoplasm were used in the study. The oocytes were washed three times in Tissue Culture medium (TCM-199, HEPES modification with Earle's salt and L-glutamine, Sigma, USA) plus 10% FCS and 1% antibiotic - antimycotic (Gibco, Switzerland).

Vitrification of oocytes

Freshly collected oocytes and their cumulus cells were cryopreserved by ultra rapid cooling as described previously by Das (1997) with some modifications. The vitrification solution (VS) comprised of Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.5 M sucrose, 0.4% bovine serum albumin (BSA) and different types of molar (M) concentrations of the cryoprotectants which are either glycerol (G), ethylene glycol (EG) or dimethyl sulfoxide (DMSO) in order to determine the best type of vitrification cryoprotectants. The concentrations tested were 4 M, 7 M and 7M concentration for G, EG and DMSO, respectively (Yadav *et al.*, 2008). The oocytes were pre-equilibrated in 50% of the vitrification solution (prepared by dilution of VS in DPBS) for 3-5 min and then kept in VS for 1 min and loaded (4-5 oocytes per straw) in pre-sterilized 0.25 ml straws (IMV, France). The straws were heat-sealed and pre-cooled by keeping the straws over liquid nitrogen level (LN₂) vapour for 2 min at the height of about 5 cm from LN₂. The straws were then plunged in LN₂ and stored for 7-10 days.

Thawing and evaluation

The straws were warmed rapidly by transferring them to a water bath at 37°C for 10 s. The

content of the vitrified straw was emptied in a 35 mm Petri dish and the oocytes were allowed a 5 min equilibration in warm 10% sucrose solution in M-PBS for one-step dilution. The oocytes were washed 3 times in TCM-199 plus 10% FCS.

Oocyte-cumulus cells were evaluated as mentioned previously (Dhali *et al.*, 2000) by their post-thaw morphological appearance under stereo microscope. Normal oocytes have spherical and symmetrical shape with no signs of lysis, membrane damage, swelling, degeneration or leakage of the cellular content; but abnormal oocytes show ruptured zona pellucida or fragmented cytoplasm with signs of degeneration. The survival percentage was calculated as the proportion of normal oocytes against the total number vitrified. Only the oocytes with complete compact dense cumulus oophorus after vitrification-thawing were used for IVM. The cryoprotectant was removed by placing oocytes in 50% VS and then transferred to DPBS. The morphologically normal oocytes were matured and fertilized *in vitro*. Freshly collected oocytes were simultaneously matured *in vitro* and kept as control.

In vitro maturation (IVM)

The morphologically normal oocytes were cultured in 50 µl of TCM-199 + 10% FCS and 1% antibiotic - antimycotic covered with mineral oil (Sigma, USA) in four-well culture plate (10 to 15 oocytes per droplet) for 24 h in a Co₂ incubator (5% Co₂ and 95% relative humidity) at 39°C. In addition, a number of COCs was run as a control group in each *in vitro* maturation trial. Maturation was assessed by expansion of cumulus cell mass (Schellander *et al.*, 1989).

Sperm capacitation and in vitro fertilization of frozen-thawed buffalo oocytes (IVF)

Fertilization was performed with frozen-thawed semen which was capacitated in BO medium (Brackett and Oliphant, 1975) containing 3.88 ml sodium caffeine benzoate (Sigma, Chemical Co. USA) and 0.02 mg/ml heparin (Sigma, USA). The spermatozoa were washed twice by centrifugation at 1850 rpm for 5 min. The sperm suspension was then diluted 1:1 with BO medium containing 20 mg/ml BSA (bovine serum, albumin, fraction V. Fluka, Switzerland). The concentration of sperm for fertilization was 5 to 8 x 10⁶ cells /ml of BO medium (Niawa *et al.*, 1991). After 24 h, matured oocytes were washed 3 times in BSA-containing BO medium, and were co-cultured for 5 h under the same condition in Co₂ incubator. After insemination the oocytes were washed in TCM-199+10% FCS and 1% antibiotic-antimycotic 3 times and incubated for 5-6 days in Co₂ incubator. The frequency of morula and/or blastocyst was recorded.



Statistical analysis

The experiment was replicated 5 times. The data were analyzed using Chi-square analysis according to Snedecor and Cochran (1976).

Results

Survival and morphological evaluations of vitrified-thawed oocytes:

Table 1 illustrates the morphological evaluations immediately after warming of vitrified buffalo oocytes using straws in different cryoprotectants. The proportion

of buffalo oocytes found to be morphologically normal was significantly ($P < 0.05$) higher in EG and DMSO than those obtained in G (85.0 and 83.33 vs. 65.0%, respectively) after vitrification-thawing. Furthermore, the proportion of damaged oocytes was significantly ($P < 0.05$) higher in G (35.0%) than those in EG (15.0%) and DMSO (16.66%). Various morphological abnormalities observed in oocytes after vitrification-thawing included cracking of zona pellucida, leakage of cellular content and shrinkage of cytoplasm but without significant difference among various cryoprotectants of buffalo oocytes (Table 2). Among the damaged oocytes, cracking of zona pellucida was the most frequent abnormality observed.

Table 1. Incidence of immature buffalo oocytes recovered with normal morphology after vitrification-thawing among different cryoprotectants.

Types of cryoprotectant	Number of oocytes vitrified	Number of morphological normal oocyte-cumulus cells (%)	Number of damaged oocytes (%)
EG	100	85 (85.00) ^a	15 (15.00) ^a
DMSO	90	75 (83.33) ^a	15 (16.66) ^a
G	100	65 (65.00) ^b	35 (35.00) ^b

Within the same column values with different superscripts (^{a,b}) are significantly different ($P < 0.05$).

Table 2. Types of damage observed in buffalo oocytes after vitrification-thawing.

Types of cryoprotectant	Number of oocytes damaged	Types of damage		
		Cracking of zona pellucida (%)	Shrinkage of cytoplasm (%)	Leakage of cellular content (%)
EG	15	8 (53.34)	3 (20.0)	4 (26.67)
DMSO	15	9 (60.00)	2 (13.34)	4 (26.67)
G	35	20 (57.14)	5 (14.28)	10 (28.57)

In vitro maturation and fertilization of vitrified-thawed oocytes

The maturation rates of buffalo oocytes after thawing and removal of cryoprotectant are illustrated in Table 3. A significantly higher ($P < 0.05$) proportion of oocytes were matured in the non-vitrified control buffalo oocytes compared to vitrified immature oocytes in EG, DMSO and G. Internal group comparison revealed that vitrified-thawed oocytes in EG and DMSO had higher maturation rates compared to G groups (47.05 and 46.67 vs. 30.76%; respectively). However,

only significant differences ($P < 0.05$) existed between EG and G groups for the maturation rate of oocytes. As shown in Table 4, a significantly higher proportion of oocytes were cleaved in EG and DMSO compared to G (28.57 and 25.71 vs 10.0%; $P < 0.05$, respectively). A similar trend was observed in blastocyst produced *in vitro* while the percentage of morula stage did not significantly vary among the different groups. On the other hand, the *in vitro* cleavage and blastocyst stage were significantly ($P < 0.05$) lower in oocytes cryopreserved in EG, DMSO and G as compared to control.

Table 3. Maturation rates of buffalo oocytes vitrified in different cryoprotectants.

Types of cryoprotectant	Number of oocytes cultured	Number of oocyte matured (%)
EG	85	40 (47.05) ^b
DMSO	75	35 (46.67) ^{bc}
G	65	20 (30.76) ^c
control	100	65 (65.00) ^a

Within the same column values with different superscripts (^{a,b,c}) are significantly different ($P < 0.05$).



Table 4. Cleavage and development rates of buffalo oocytes vitrified in different cryoprotectants.

Types of cryoprotectant	Number of fertilized oocytes	Cleavage rate Number (%)	Morula stage Number (%)	Blastocyst stage Number (%)
EG	40	10 (28.57) ^b	2 (20.00) ^a	1 (10.00) ^b
DMSO	35	9 (25.71) ^b	2 (22.23) ^a	1 (11.12) ^b
G	20	5 (10.00) ^c	1 (20.00) ^a	0 (0.00) ^c
control	65	30 (46.15) ^a	8 (26.67) ^a	8 (26.67) ^a

Within the same column values with different superscripts (^{a,b,c}) are significantly different ($P < 0.05$).

Discussion

In the present study the rate of morphologically normal immature buffalo oocytes following vitrification/thawing was higher in EG and DMSO than those obtained in G. These findings compare favorably with previous reports of Dhali *et al.* (2000), Wani *et al.* (2004) and Yadav *et al.* (2008) in buffaloes. Furthermore, Mahmoud *et al.* (2013) reported that the rate of morphologically intact oocytes following vitrification/warming was high, ranging from 87.7% in straws to 90.8% in Cryotops using a mixture of 3 M DMSO + 3 M EG. It has been observed that the immature germinal vesicle stage oocytes tolerate the cryopreservation damage more efficiently compared to oocytes at metaphase-II and cumulus compact oocytes are less vulnerable to cryo-injuries compared to their denuded counterpart. The vitrification treatment does not affect the proportion of oocytes with intact morphology after warming, although it has been reported that mammalian oocytes are very sensitive to high concentration of cryoprotectant (Purohit *et al.*, 2012). The cumulus cell removal prior to *in vitro* maturation or vitrification has shown a detrimental effect on oocyte morphology for both immature and mature vitrified equine (Tharasanit *et al.*, 2009), and bovine oocytes (Modina *et al.*, 2004). The damaged oocytes recorded herein following vitrification/thawing may be due to the large lipid-like material found in oocytes of many species, since lipid removal or lipid polarization reduces chill- and cryo-injury (Otoi *et al.*, 1997).

In our study the survival rate of *in vitro* matured, vitrified-thawed buffalo oocytes in G was 30.76 % as assessed by expansion of cumulus cell mass. This result is in accordance with Hammam and El Shahat (2005) in buffalo, but still lower than the results of El-Shahat and Hammam (2012) who reported a 63.63% maturation rate in sheep depending on the same previous assessment of maturation. This difference may be due to different concentration of G and species differences. In spite of the present high recovery rate of morphologically normal oocytes in all types of cryoprotectant, the maturation rate was lower in vitrified-thawed oocytes than those in control. The lower maturation rate may be the consequence of several factors including the one-step dilution procedure employed after warming, as well as the ultra-rapid temperature changes during vitrification and warming,

which may have caused extensive damage to membranes and structures in the ooplasm (Yoon *et al.*, 2000; Mahmoud *et al.*, 2010a, b). The freeze-thaw process is known to induce an alteration in the physicochemical properties of intracellular lipids (Kim *et al.*, 2001) and such damages may render the oocyte incapable of retaining its developmental competence. Despite the protective effects of cryoprotectants during freezing they may impose concentration-, time- and temperature-dependant toxicity (Fahy *et al.*, 1984). The maturation rate recorded herein was highest in EG and DMSO followed by the G group. Mahmoud *et al.* (2013) observed that EG and DMSO were used for the cryopreservation of buffalo oocytes with the straw method. This is due to their low molecular weight; these compounds can easily permeate through cell membranes rapidly (25-30 s) to achieve concentration equilibrium across the cell membrane. Moreover, EG, because of its high permeability and low cytotoxicity, has been found to be convenient for vitrification of bovine and equine oocytes (Hurt *et al.*, 2000; Cetin and Bastan, 2006). In our investigation, the developmental capacity of the oocytes vitrified in glycerol was lower compared to those in EG or DMSO. These findings were in accordance with the previous study in buffaloes (Yadav *et al.*, 2008). Glycerol induces osmotic damage to the cytoplasm owing to its low membrane permeability (Szell *et al.*, 1989). Osmotic stress produced by the cryoprotectants has deleterious effect on survival of mature bovine oocytes (Martino *et al.*, 1996). The low permeability of the cells to glycerol may increase the risk of osmotic stress during thawing and dilution as the water enters the cells more quickly than the glycerol is lost. This could explain the poor survival rate and thus maturation rates with subsequent embryonic development of oocytes vitrified in glycerol. On other hand, the developmental capacity of the vitrified-thawed immature buffalo oocytes was significantly lower compared to control in the present study. This could be due to the use of immature rather than mature oocytes for vitrification, since the developmental ability of oocytes frozen at germinal vesicle vesicle-stage (immature) has been reported to be much lower than that of *in vivo* or *in vitro* matured oocytes in mice (Schroeder *et al.*, 1990) and cattle (Lim *et al.*, 1992). Matured oocytes are more resistant to cryopreservation than immature ones (Isachenko *et al.*, 1998). The cytoskeleton of the first meiotic division in immature oocytes is particularly susceptible to damage. Matured



oocytes display a more flexible cytoskeleton, which may be one reason that they are less subjected to cryodamage (Allworth and Albertini, 1993). In addition, the French straw is a popular cryodevice (packaging system) as it is inexpensive, and cells and tissues remain sterile. However, vitrification of bovine oocytes in 0.25-ml straws causes a delay in heat loss from the solutions, possibly leading to devitrification, i.e. intracellular recrystallization during warming (Morató *et al.*, 2008). Considerable progress has been made in increasing cooling and warming rates by developing alternative packaging systems (Succu *et al.*, 2007). Cryodevices such as OPS (Vajta *et al.*, 1998) and Cryotops (Kuwayama *et al.*, 2007) have drastically improved the cooling rate by reducing the surface to volume ratio and thus exposing the minuscule vitrification drop directly to LN₂ (Liebermann *et al.*, 2002).

In conclusion, 7M solution of EG or DMSO can be used for vitrification of immature buffalo oocytes for their utilization in the *in vitro* maturation and fertilization as well as embryonic production.

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