African Journal of Biotechnology Vol. 10(12), pp. 2334-2339, 21 March, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.994
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Nuclear maturation of immature bovine oocytes after vitrification using open pulled straw and cryotop methods

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Accepted 2 March, 2011

To date, at least two well known methods have been widely used for vitrification of oocytes and embryos at different stages in a variety of species. However, there is no reported data regarding the comparative effectiveness of these two methods for vitrification of immature bovine oocytes. The objective of this study is to compare the nuclear maturation of immature bovine oocytes vitrified using open pulled straw (OPS) and cryotop methods. Two experiments were conducted in this study. In the first experiment, cytotoxicity of vitrification solutions (VS) from both methods was studied. After removal of cryoprotectants, cumulus oocyte complexes (COCs) was cultured *in vitro* and cleavage rate was monitored on Day 2 post-insemination (pi), whereas, morulae and blastocyst yields on Days 5 and 8 pi, respectively. The VS solutions significantly reduced zygotic cleavage rate, morulae and blastocyst rates compared with the control group (P < 0.05). The lowest cleavage rate resulted from prolonged exposure time to OPS-VS solutions (35.1%; P < 0.05). However, the morulae and blastocyst rates were significantly higher (P < 0.05) for embryos derived from oocytes exposed to cryotop solutions (40.5 and 22.4%, respectively). In the second experiment, effectiveness of both vitrification methods was compared for cryopreservation of immature bovine oocytes. After warming, COCs were cultured *in vitro* for 24 h. The polar body (PB+) and metaphase-II (MII) stage rates differed significantly among treatment groups. Oocytes vitrified using cryotop solution and device showed higher percentages of PB+ (36%) and MII (51%) rates. In addition, the lowest percentage of degenerated oocytes resulted from cryotop solution. The highest degenerated oocytes obtained by equilibration in OPS solution and vitrified using OPS device (40%; P < 0.05). In conclusion, our data demonstrated that cryotop solution was less toxic to the immature bovine oocytes and vitrification with the cryotop method resulted in higher survival and nuclear maturation rates.

Key words: Immature oocyte, bovine, vitrification, cryotop, open pulled straw (OPS).

INTRODUCTION

Over the last three decades, there have been increasingly rapid advances in the field of reproductive biotechnologies, especially for the cryopreservation of gametes (Isachenko et al., 2005a; Kuwayama et al., 2005a) and ovarian tissue (Isachenko et al., 2008). Although there has been much interest in the cryopreservation of oocytes due to its wide applications for human and animals, there are still many obstacles in obtaining viable oocytes. Ice crystal formation inside the oocyte, chilling injury and cytotoxicity of cryoprotectants (CPAs) are the main barriers to successful cryopreservation (Liebermann et al., 2002). During vitrification, ice crystal formation is prevented due to high cooling/warming rates and vitrification solutions containing high concentrations of CPAs (Smorag and Gajda, 1994; Vajta and Kuwayama, 2006).

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Vitrification not only prevents the formation of ice crystals, but it quickly passes the temperature zone known to cause chilling injury (15 to -5°C). Porcine and bovine oocytes are particularly sensitive to chilling injury and cooling rates (Wu et al., 1999) and are susceptible to meiotic spindle injuries and cytoskeleton ruptures (Aman and Parks, 1994) upon slow cooling to low temperatures.

Two main cryodevices and their associated methods have been widely used for vitrification of oocytes, namely open pulled straw (OPS) (Vajta et al., 1998) and cryotop (Kuwayama et al., 2005c). The design of the cryodevices affects cooling/warming rates. The cryotop has significantly increased cooling/warming rates (>23,000 and >42,000°C/min, respectively) due to direct contact with liquid nitrogen and minimal volume (~0.1 µl) of vitrification solution around the sample. It has been found that, extremely high warming rates are also important to prevent damage at the time of warming (Isachenko et al., 2005b; Isachenko et al., 2005c; Seki and Mazur, 2008).

The OPS and cryotop methods employ different concentrations of CPAs along with their differing cryodevices. High concentrations of CPAs are necessary to achieve a vitrified state. However, CPAs at high concentrations are toxic to cells, lead to osmotic shock and ultimately cell death (Arav et al., 1993). Extremely high cooling rates enable the use of lower concentrations of CPAs and may reduce cellular damage. The OPS method, which uses higher concentrations of CPAs, has resulted in successful cryopreservation of oocytes in variety of species (Berthelot et al., 2001; Vieira et al., 2002) however; the concentration of CPAs in this method might adversely affect the viability of future embryos. The cryotop method uses lower concentration of CPAs and has been successfully used for cryopreservation of human (Kuwayama et al., 2005a), *in vitro* matured bovine (Chian et al., 2004), ovine (Succu et al., 2007), porcine (Liu et al., 2008) and buffalo oocytes (Gasparrini et al., 2007; Attanasio et al., 2009).

Chilling sensitive mature porcine oocytes vitrified by the cryotop method showed higher survivability and embryo development when compared with the OPS method (Liu et al., 2008). Therefore, cryotop method may also be useful for successful cryopreservation of immature bovine oocytes. However, there is a lack of data regarding the vitrification of immature bovine oocytes; the OPS method has been used to vitrify immature bovine oocytes but there is no data regarding the cryotop method for vitrifying of these oocytes. The aim of this study is to compare *in vitro* viability rates of immature bovine oocytes vitrified using OPS and cryotop methods and finally, to propose a superior method for cryopreservation of immature bovine oocytes.

MATERIALS AND METHODS

Reagents

Unless otherwise is indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Oocyte collection

Ovaries were collected from local abattoirs and transported to the laboratory within 3 h at 34 to 36°C in phosphate-buffer saline (PBS; P-4417) containing penicillin-streptomycin (100,000 IU of penicillin and 100 mg of streptomycin per liter). Before recovery of oocytes, each ovary was rolled on a sterile tissue paper to dry its surface. Cumulus-oocyte complexes (COCs) were obtained by slicing method, in washing solution (WS) consisting of tissue culture medium 199 (Medium199, 12340; Gibco, Carlsbad, CA, USA) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Earl's salts, L-glutamine and 2 mg/ml sodium bicarbonate modified by the addition of 4 mg/ml bovine serum albumin (BSA, fraction V, A-3311) and gentamycin 50 µg/ml (G-1264). Visible ovarian follicles (2 to 6 mm) were sliced using a surgical blade in a 90 mm sterile disposable petri dish (Nunc, Denmark) containing 2 ml of WS. Each petri dish was examined under a stereomicroscope (Olympus SZ40, Japan) at 10-40X magnification. COCs surrounded by more than 3 to 4 cumulus cell layers and homogenous ooplasm were selected and gently transferred to another washing petri dish.

Toxicity test, vitrification and warming

Immature oocytes recovered from ovaries were washed three times in WS and subsequently washed twice in holding solution (HS; Hepes-buffered medium 199 + 20% adult bovine serum (Sigma, B-9433)) and incubated for 15 min.

Toxicity test

After 15 min incubation in HS solution, immature oocytes were assigned to one of the four treatments to test the toxicity of solutions previously used for vitrification (Vieira et al., 2002; Kuwayama et al., 2005c; Liu et al., 2008). Treatment groups were: (1) Control group, no exposure to vitrification solutions, (2) cryotop group, exposed to the lower concentration of CPAs in solutions designed for the cryotop method (Kuwayama et al., 2005c) without plunging into liquid nitrogen, (3) and (4) OPS group, exposed to the higher concentrations of CPAs in solutions designed for the OPS method (Vieira et al., 2002). To determine the cytotoxicity of longer exposure to the high CPA concentrations of OPS solutions, oocytes were separated into two groups: (3) short exposure (OPS-A: 30-45 sec for vitrification solutions (VS)1 and 25 sec for VS2); (4) long exposure (OPS-B: 10-12 min for VS1 and 60 sec for VS2). After exposure, CPAs were removed instantly by submerging the oocytes to the serial warming solutions designed for each method. The vitrification solution formulations are described further. After treatments, oocytes were inseminated and cultured *in vitro* for 10 days. The cleavage, morula and blastocyst formation rates were assessed 24 h, 4 and 7 days post insemination, respectively.

Vitrification

Each cryodevice (cryotop and OPS) were tested with the two concentrations of CPAs associated with each device, high and lower concentrations.

Vitrification using cryotop solution: The cryotop protocol was adapted from Kuwayama et al (2005c). Groups of four COCs were incubated in the first vitrification solution (VS1; 7.5% DMSO and 7.5% EG in HS) for 10 to 12 min and then, transferred to the

second vitrification solution (VS2; 15% DMSO, 15% EG and 0.5 M sucrose in HS) for a further 60 s. Instantly, oocytes were loaded using an OPS (group B) or onto a cryotop (group A) and submerged into liquid nitrogen (LN_2) for storage. The time of exposure from VS2 to liquid nitrogen was less than 90 s. All vitrified oocytes were maintained in liquid nitrogen at least for 10 days.

Immediately after removing cryotop from liquid nitrogen, the cryotop device was submerged in 3 ml pre-warmed (39°C) HS plus 1 M sucrose (T1) and oocytes were detached smoothly from cryotop device. Immature oocytes were left in T1 for one minute and then, transferred to HS plus 0.5 and 0.15 M sucrose solution for 3 and 5 min, respectively. Finally, the immature oocytes were washed twice in HS for 5 min each and processed for *in vitro* maturation.

Vitrification using OPS solution

Vitrification using the OPS method was previously described by Vieira et al. (2002). Groups of four COCs were incubated in the first vitrification solution (VS1; 10% DMSO and 10% EG in HS) for 35 to 40 s and then, transferred to the second vitrification solution (VS2; 20% DMSO, 20% EG and 0.5 M sucrose in HS) for a further 25 s. Instantly, oocytes were loaded onto either cryotop (group C) or OPS (group D) cryodevices and plunged into liquid nitrogen (LN_2) for storage. The time of exposure from VS2 to LN_2 was no longer than 30 s. Vitrified samples were maintained in LN_2 for at least 10 days.

Immediately after removing the OPS or cryotop from LN_2, they were submerged in 3 ml pre-warmed (39°C) HS plus 0.25 M sucrose (designated T1) and oocytes smoothly detached from the cryotop or expelled from the OPS. Immature oocytes were left in T1 for five minute and then, transferred to HS plus 0.15 M sucrose solution for 5 min. Finally, the immature oocytes were washed twice in HM for 5 min each and processed for *in vitro* maturation.

In vitro maturation (IVM)

Immature oocytes were washed twice in medium 199 supplemented with 4 mg/ml BSA and washed again in maturation solution containing Hepes-buffered medium 199 supplemented with 10% fetal calf serum, 0.2 mM sodium pyruvate (P-5280), 50 µl/ml Gentamycin (G-1264) and 1 µg/ml oestradiol-17β (E-8875). Approximately, 20 to 30 oocytes were incubated in 400 µl of maturation solution under mineral oil in 4 well plates for 22 to 24 h at 38.5°C under 5% CO_2 atmosphere with high humidity.

Nuclear maturation stage

For assessment of maturation 24 h after IVM, a representative number of oocytes were denuded using 0.1% (w/v) hyaluronidase (Type 1-S) in Hepes-buffered medium 199 by vortexing. The cumulus-free oocytes were fixed in aceto-ethanol mixture (1:3, v/v) at 5°C for 24 h. Once fixed, oocytes in groups of 5 to 10 were mounted on slides. Cover-slip with 4 spots of silicon glued at the corners was gently pressed down until it touched and secured the oocytes. Afterward, aceto-lacmoid stain was passed under the cover-slip and remained there for 5 min for staining. Subsequently, a decolorizing solution (aceto-glycerol) was passed through to remove the stain residuals. Stained oocytes were examined under light microscope (400 x) for nuclear stages determination.

In vitro fertilization (IVF)

Oocytes used in the toxicity test were used for *in vitro* fertilization. The fertilization method was previously described by Parrish et al., (1988) with some modifications. Briefly, matured oocytes were washed three times in tyrode's albumin lactate pyruvate buffered with HEPES (Talp-Hepes) and twice in fertilization medium. Viable spermatozoa were obtained by centrifugation of frozen-thawed spermatozoa on Bovipure (Nidacon International AB, Gothenburg, Sweden) discontinuous density gradient (2 ml of top-layer over 2 ml of bottom-layer) for 20 min at 300 x g at room temperature. Viable spermatozoa collected at the bottom of the tube, were washed with 5 ml of sperm-TALP supplemented by 6 mg/ml BSA (fraction V, A-3311) and centrifuged for 10 min at 300 x g. After centrifugation, 150 µl of the pellet was selected. Spermatozoa were counted in a hemacytometer and diluted in the appropriate volume of IVF-Talp to give a concentration of 1 x 10^6 spermatozoa/ml. IVM-oocytes were transferred in groups of up to 7 into 50 µl of fertilization solution supplemented with 10 µg/ml heparin-sodium salt (Sigma, H-3393). Spermatozoa suspension was added to each fertilization droplet. Oocytes and sperms were incubated for 18 h at 38.5°C in 5% CO_2 in humidified air.

In vitro culture (IVC)

Eighteen hours after IVF, oocytes were freed of cumulus cells by high speed vortexing and washed twice in Talp-Hepes and culture medium. For each well, 10 to 15 oocytes were transferred to 400 µl of synthetic oviductal fluid (SOFaaci) supplemented with 5% adult bovine serum (B-9433) and 1 µg/ml gentamycin under mineral oil. Every two days after transfer to culture medium, 200 µl of culture medium replenished with 200 µl fresh incubated SOF medium. Cleavage and blastocyst rates were recorded at 48 h and 7 days post insemination, respectively.

Experimental design

A total of 535 COCs were recovered and used in two experiments: For the first experiment (toxicity test), 391 COCs were exposed to equilibration and vitrification solutions without cooling. After removal of cryoprotectants and exposure to warming solution, they were matured, fertilized and cultured *in vitro* for 9 days.

For the second experiment (vitrification/warming), 144 immature oocytes were firstly vitrified using OPS and cryotop solutions and cryodevices, using the same CPA exposure times as in the toxicity test. After warming, oocytes were assigned to IVM for a period of 24 h after which nuclear maturation, polar body extrusion and degeneration rates were evaluated. Oocytes were monitored for the presence of the first polar body. Afterward, all of the oocytes were fixed and stained except the degenerated oocytes. Nuclear maturation stages for each group were determined under the light microscope.

Statistical analysis

Significant differences among treatments were revealed by one-way analysis of variance followed by Duncan's multiple range test for mean comparisons ($p < 0.05$) using the Statistical Analysis System (SAS) software (version 9.1 for Windows; SAS Inst., Cary, NC).

RESULTS AND DISCUSSION

Experiment 1

Toxicity testing

Results in Table 1 clearly indicate that exposure to equi-

Table 1. Toxicity testing of solutions on immature bovine oocytes subsequent development.

Solution	No. of Oocyte	Cleavage (%)	Morulae (%)	Blastocyst (%)
Control	99	77.56 + 2.54[a]	53.98 + 1.68[a]	31.20 + 1.96[a]
cryotop	94	58.46 + 3.40[b]	40.53 + 3.59[b]	22.49 + 3.35[b]
OPS-A	105	55.82 + 3.20[b]	33.18 + 3.48[c]	14.20 + 2.44[c]
OPS-B	93	35.13 + 1.47[c]	19.81 + 2.68[d]	8.66 + 2.30[d]

Data were pooled from 5 replicates. [a,b,c] Values with different superscripts in the same column are significantly different ($P < 0.05$).
OPS-A, timing type A (shorter exposure to CPAs); OPS-B, timing type B (longer exposure to CPAs).

Table 2: Polar body rate of vitrified/warmed immature oocytes after maturation in different groups.

Group	A (n=47)	B (n=44)	C (n=46)	D (n=47)
Solution	Cryotop		OPS	
Cryodevice	Cryotop	OPS	Cryotop	OPS
Polar body +	36.2% (17/47)[a]	29.5% (13/44)[a]	19.6% (9/46)[b]	17.0% (8/47)[bc]
Polar body -	46.8% (22/47)[a]	50.0% (22/44)[a]	45.7% (21/46)[a]	42.6% (20/47)[ab]
Degenerated	17.0% (8/47)[a]	20.5% (9/44)[a]	34.8% (16/46)[b]	40.4% (19/47)[b]

Data were pooled from 4 replicates. [a,b,c] Values with different superscripts in the same row are significantly different ($P < 0.05$).
Group A, cryotop solution + cryotop device; Group B, cryotop Solution + OPS device; Group C, OPS solution + cryotop device; Group D, OPS solution timing type A + OPS device.

libration solution (ES) and VS solutions negatively affected immature oocytes viability and their subsequent developments. There was no significant difference between cryotop (58.4%) and OPS-A (55.8%) solutions in terms of cleavage rate although they were significantly lower than those recorded in the control group (77.5%). Seemingly, prolonged exposure to OPS solutions (OPS-B group) radically decreased the cleavage rate (35.1%; $P < 0.05$). The type of cryoprotectant solution significantly decreased morulae and blastocyst rates ($P < 0.05$). A higher percentage of embryos reached the morulae and blastocyst stage after exposure to cryotop solutions when compared with OPS solutions (40.5 and 22.4%, respectively).

Experiment 2

Table 2 shows the percentage of oocytes with (PB+) or without (PB-) first polar body after vitrification in different treatments. The percentage of PB+ oocytes obtained from groups A and B was higher ($P < 0.05$) than those obtained from other groups. The lowest percentage of PB+ oocytes resulted from groups D and C ($P < 0.05$). Interestingly, by applying both OPS solutions and OPS cryodevice (Group D) the highest percentage of degenerated oocytes were obtained. On the other hand, cryotop solutions used with the cryotop device exhibited the lowest percentage of degeneration (Table 2; $P<0.05$).

Furthermore, the percentage of oocytes in MII stage decreased with increasing cryoprotectant concentration ($P < 0.05$; Table 3). Therefore, the cryotop method (Group A) showed the highest percentage of oocytes that reached MII stage. Almost half of the remaining viable oocytes in group D (40%) ceased nuclear maturation at the germinal vesicle (GV) stage which was significantly higher than the cryotop solution group (groups A and B).

In this study cryotop and OPS methods were compared to find a superior method for cryopreservation of imamture bovine oocytes to produce higher survivability and nuclear maturation after warming and *in vitro* culture. Immature oocytes were equilibrated in OPS and cryotop vitrification solutions (Vieira et al., 2002) with a fixed equilibration time (Kuwayama et al., 2005a). Results obtained from the toxicity test, confirmed the higher potential of immature bovine oocytes vitrified using the cryotop method.

Vitrification methods with higher cooling rates require lower concentrations of cryoprotectants, while still achieving a vitrified state. Furthermore, finding the proper mixture of cryoprotectants diminishes the osmotic shock, toxicity (Vajta and Nagy, 2006; Morato et al., 2008) and specific toxicity of each cryoprotectant (Morato et al., 2008). Due to the differences in concentrations of cryoprotectant solutions and timing of equilibration among laboratories and also between species (Fujihira et al., 2005; Bogliolo et al., 2007; Muenthaisong et al., 2007), we designed a toxicity test to find the least toxic solutions by varying equilibration times for the solutions used. Based on the data obtained, we found that cryotop solution is less toxic to immature bovine oocytes and long-term equilibration in higher concentration of cryo-

Table 3. Nuclear maturation of vitrified immature oocytes after staining in different groups.

Groups nuclear stage	A (n=39)	B (n=35)	C (n=30)	D (n=28)
Solution	Cryotop		OPS	
Cryodevice	Cryotop	OPS	Cryotop	OPS
GV	17.9% (7/39)[b]	22.9% (8/35)[b]	33.3% (10/30)[ab]	32.1% (9/28)[ab]
GVBD-MI	23.1% (9/39)	20.0% (7/35)	16.7% (5/30)	21.4% (6/28)
MII	51.3% (20/39)[a]	48.6% (17/35)[a]	43.3% (13/30)[b]	39.3% (11/28)[b]
Unclassified	7.7% (3/39)	8.6% (3/35)	6.7% (2/30)	7.1% (2/28)

Data were pooled from 4 replicates. [a,b,c] Values with different superscripts in the same row are significantly different ($P < 0.05$). Group A, Cryotop solution + cryotop device; Group B, cryotop solution + OPS device, Group C, OPS solution + cryotop device; Group D, OPS solution timing type A + OPS device.

protectants (OPS-B) significantly decreases the viability and consequent embryo developments of immature bovine oocytes.

In the present study, we have found that survivability (Table 2, represented by the number of degenerate oocytes) of immature bovine oocytes after 24 h culture in maturation medium was higher in group A where lower concentration of cryoprotectants and the cryotop cryodevice was used. These higher survival rates can be attributed to lower toxicity of cryotop solution, a lower volume of vitrification solution surrounding the oocyte and ultra rapid cooling rates in the cryotop method (Kuwayama et al., 2005a). In addition, it has been shown that small volumes and direct contact to liquid nitrogen results in a lower percentage of cracked zonas and plasma membrane lysis (Vajta, 1997). The survival rate post warming from group A (cryotop method) was similar to those reported for matured bovine (Kuwayama et al., 2005b) and buffalo oocytes (Gautam et al., 2008; Attanasio et al., 2009) but was higher than those reported for immature oocytes vitrified by OPS method (Hurtt et al., 1999; Luna et al., 2001).

Polar body extrusion and MII stage in group A was significantly higher compared to the other groups. The proportion of oocytes that reached the MII stage in this study is comparably higher than those of Cetin and Bastan (2006). The cryotop method has been designed by Kuwayama et al. (2005a) to provide ultra high cooling/warming rates employing lower concentrations of cryoprotectant mixtures. Upon mounting of oocytes on thin strip of cryotop, almost all of the vitrification solution around the oocyte is removed (~0.1 µl) and a thin layer of vitrifi-cation solution remains around the oocyte. Furthermore, the LN2 exposed surface / volume ratio is higher than OPS method (Liu et al., 2008). Thus, direct contact with liquid nitrogen results in a higher cooling rate preventing chilling injury and internal ice crystal formation. The most important parameter is higher warming rates achieved using cryotop method. Isachenko et al (2005c) proved that faster warming rates are more important than cooling rates to prevent devitrification during warming. As mentioned earlier, cryoprotectant solutions used with the cryotop method were also lRHess toxic to immature bovine oocytes when compared with OPS solutions. The reason for this is the reduced concentration of permeable cryoprotectants, which are potentially toxic to the oocytes (Kuwayama et al., 2005a; Morato et al., 2008).

In conclusion, our data demonstrated that cryotop solution was less toxic to the immature bovine oocytes and vitrification with the novel cryotop method resulted in high survival and developmental rates. Further studies are needed to monitor the gene expression and ultrastructure changes of vitrified/warmed immature bovine oocytes.

ACKNOWLEDGEMENT

This research was supported by a research grant from Agro-Biotechnology Institute, Ministry of Science, Technology and Innovation Malaysia (5488200) to Dr. Abas Mazni Othman.

Abbreviations

CPAs, Cryoprotectants; **OPS,** open pulled straw; **WS,** washing solution; **PBS,** phosphate-buffer saline; **COCs,** cumulus-oocyte complexes; **BSA,** bovine serum albumin; **HS,** holding solution; **IVM,** *in vitro* maturation; **IVF,** *in vitro* fertilization; **IVC,** *in vitro* culture; **SOF,** synthetic oviductal fluid; **ES,** equilibration solution; **VS,** vitrification solution.

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