In vitro production of buffalo embryos from stepwise vitrified immature oocytes

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Summary

This study was conducted to produce buffalo embryos *in vitro* from stepwise vitrified immature oocytes. Cumulus oocyte complexes (COCs) were obtained from the ovaries of slaughtered buffalo and were collected from the local abattoir. Selected COCs were exposed to a vitrification solution consisting of 40% ethylene glycol (EG) plus 0.3 M trehalose and 20% polyvinyl pyrrolidone (PVP) for 1 min and loaded in 0.25 ml plastic mini-straws containing 100 µl of 10% sucrose. The loaded cryostraws were cryopreserved by stepwise vitrification and were stored in liquid nitrogen for 4 to 6 months. Data analysis revealed a high percentage of post-thawing morphologically normal immature oocytes (80.7%) with a low percentage of damaged oocytes. There were no significant differences in the maturation (82.1%), cleavage (47.6%) and buffalo embryo development (15.4%) produced by the stepwise vitrified immature oocytes in comparison to the three observations in fresh oocytes (88.3%, 50.4% and 19.4%, respectively, $p<0.05$).

Keywords

Buffalo, Egypt, Embryo, Oocyte, Vitrification.

Produzione *in vitro* di embrioni di bufalo da ovociti immaturi vitrificati in stepwise

Riassunto

Questo studio è stato condotto per produrre embrioni di bufalo in vitro *da ovociti immaturi vitrificati in stepwise. I complessi cumulo-ovocita (COC) sono stati prelevati dalle ovaie di esemplari macellati presso il mattatoio locale. I COC selezionati sono stati esposti a una soluzione di vitrificazione contenente etilenglicole (EG) al 40% più trealosio 0,3 M e polivinilpirrolidone (PVP) al 20% per 1 min., quindi sono stati caricati in microstraw di plastica da 0,25 ml contenenti 100 µl di saccarosio al 10%. I cryostraw caricati sono stati sottoposti a crioconservazione mediante vitrificazione in stepwise e conservati in azoto liquido per 4-6 mesi. L'analisi dei dati ha evidenziato post-congelamento un'elevata percentuale (80,7%) di ovociti immaturi morfologicamente normali e una bassa percentuale di ovociti danneggiati. Non sono emerse differenze significative in termini di maturazione (82,1%), divisione cellulare (47,6%) e sviluppo embrionale (15,4%) per quanto riguarda gli ovociti immaturi vitrificati in stepwise rispetto alle tre osservazioni su ovociti freschi (rispettivamente 88,3%, 50,4% e 19,4%,* p<0,05*).*

Parole chiave

Bufalo, Egitto, Embrione, Ovocita, Vitrificazione.

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Introduction

Recent research investigating the *in vitro* culture of immature oocytes to the morula and/or blastocyst stage of development has been primarily focused on the improvement of cryopreservation methods of immature oocytes to overcome cryoinjury-induced blocks to development.

A practical freezing method is a key factor in commercial embryo transfer and production technology and offers the opportunity of implementing novel animal breeding and production programmes.

Although, many studies on conventional vitrification methods (one-step methods) have been undertaken successfully in cattle and other domestic species, the results obtained in buffalo appear to be limited (1, 6).

Several cryopreservation methods, such as slow freeze and vitrification, have been used to preserve embryos and oocytes of many animal species, resulting in the birth of live offspring. Vitrification is defined as a physical process by which a highly concentrated solution of cryoprotectants solidifies during cooling, without the formation of ice crystals (8). This offers several advantages over conventional equilibrium methods, e.g. faster and simplified freezing and thawing procedures, high percentages of oocyte/embryo survival and no requirements for a freezing machine (5). Vitrification was reported to be suitable for cryopreservation of bovine oocytes (7). However, there were no reports available on the cryopreservation of buffalo oocytes by stepwise vitrification. Therefore, our study was conducted to produce buffalo embryos *in vitro* from stepwise vitrified immature oocytes.

Materials and methods

All materials were purchased from the Sigma Chemical Company, in St Louis (Missouri) unless otherwise indicated.

Collection of oocytes

Cumulus oocyte complexes (COCs) were obtained by aspiration of 2-6 mm from abattoir ovaries as described by Totey *et al.* (10). The age of the female animals ranged from 5 to 15 years. The COCs were washed three times with tissue culture medium (TCM-199), supplemented with 10% foetal calf serum (FCS) and 50 μg/ml gentamycin sulphate.

Experimental design

Immature buffalo oocytes were cryopreserved after collection using the stepwise vitrification technique. Non-vitrified oocytes subjected to the same *in vitro* fertilisation technique were used as controls.

COCs were equilibrated at room temperature in 10% ethylene glycol (EG) in modified Dulbecco phosphate-buffered saline (mPBS) for 5 min (PBS supplemented with 10% FCS and 0.6 % bovine serum albumin [BSA]) and were then equilibrated again for 5 min in 10% EG and 0.3 M trehalose present in mPBS. The embryos were vitrified for 1 min in a pre-cooled (on ice) vitrification solution consisting of 40% EG and 0.3 M trehalose and 20% polyvinyl pyrrolidone (PVP) (2).

Groups of 5-10 immature oocytes were rapidly loaded into 0.25 ml straws (Bicef, L'Aigle) in accordance with the double column Curtis method (4). In this respect, a column of 9-10 mm of vitrification solution containing immature oocytes was loaded between two columns of 9-10 mm layers of a 10% sucrose solution and separated from the oocytes by a 5-7 mm layer of air bubbles. Once the straw had been loaded, the unloaded end was sealed using a heat sealer.

Stepwise vitrification technique

The loaded cryostraws were immediately and carefully touched by the forceps that had previously been maintained in a liquid nitrogen insulating cup (–196°C) at the straw wall surrounding the fluid segment containing the immature oocytes for approximately 3-7 sec. The cryostraws were then placed on the surface of the liquid nitrogen present in the tank for approximately 20-30 sec. The loaded cryostraws were plunged into a labelled goblet containing liquid nitrogen (–196°C) and stored for 4-6 months.

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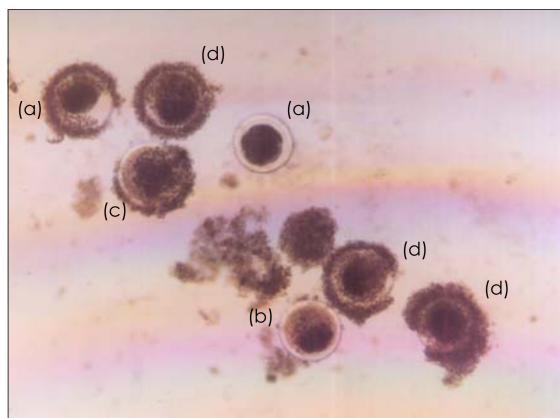
Thawing procedure of cryostraws

Bielanski *et al.* (3) described the thawing procedure that was used in our study. Briefly, the straws were immersed for 10 sec in a water bath at 30°C. The contents of each straw were emptied into a Petri dish containing 2 ml of mPBS and mixed by slight agitation. Immature oocytes were washed 2-3 times in a culture medium (TCM-199 + 10% FCS) and co-cultured in 50 µl drops of this medium at 38°C in a humidified atmosphere of 5% CO_2.

Assessment of survival of oocytes

The post-thawing survival of embryos was observed under a stereomicroscope (M6C-9, N9116734, Russia). Oocytes were judged morphologically as survivors (Fig. 1) when the spherical and symmetrical shape had no signs of lysis, membrane damage, swelling, degeneration or leakage of the cellular content; oocytes were considered abnormal (Fig. 1) when a ruptured zona pellucida or a fragmented cytoplasm with signs of degeneration were present (5).



(a) Shrinkage of cytoplasm
(b) Leakage of cellular content
(c) Rupture of cell membrane
(d) Non-shrinkage cytoplasm (morphological normal oocytes)

Figure 1
Morphological abnormalities in post-thawed stepwise vitrified immature buffalo oocytes

In vitro fertilisation of stepwise vitrified thawed buffalo oocytes

Morphologically normal post-thawed COCs were washed twice with TCM-199, supplemented with 10% FCS and 50 µg/ml gentamycin sulphate.

Maturation was performed in 50 µl drops (10 COCs/drop) of the same medium used for washing and was supplemented with 10 µl/ml pregnant mare serum gonadotropin (PMSG) (Folligon, Intervet, Cairo), 10 µl/ml human chorionic gonadotropin (hCG, Pregnyl, Nile Company for Pharmaceuticals and Chemical Industries, Cairo). The reaction with the post-thawed COCs was conducted with mineral oil, for an incubation time that lasted between 22 h and 24 h at 38.5°C and in an atmosphere of 5% CO_2.

The contents of two 0.25 ml straws were thawed in a water bath at 37°C for 30 sec, layered beneath 1.0 ml of Bracket-Oliphant (BO) medium supplemented with 10 mM caffeine, 10 µg/ml heparin and 20 mg/ml BSA (fraction V). The thawed material was centrifuged at 700 × g units for 8 min at room temperature. The sperm pellet of frozen thawed semen was resuspended in the same medium to give a final concentration of 10-16 × 10^6 sperm cells/ml.

Matured oocytes were washed three times in a sperm suspension medium, kept in a 50 µl droplet (10 oocytes/drop) for the same medium then covered with warm sterile mineral oil and incubated for 1 h at 38.5°C in a humidified atmosphere of 5% CO_2. Thereafter, oocytes were inseminated with a sperm suspension (50 µl/droplet) and incubated for 24 h at 38.5°C in a humidified atmosphere of 5% CO_2 for fertilisation.

Putative zygotes were cultured, under oil, in groups of 10 in 50 µl droplets of TCM-199 supplemented with 10% FCS and 50 µg gentamycin sulphate.

Cultured dishes were incubated for 5-7 days at 38.5°C in a humidified atmosphere of 5% CO_2 and the culture medium was changed every 48 h. The day of fertilisation was considered as day 0 and morula and/or blastocysts for stepwise vitrified and fresh immature oocytes was calculated from the number of embryos which cleaved at least once and were collected on day 6-8 post insemination.

Statistical analysis

The experiment was replicated 10 times and the data were analysed by chi-square analysis (9).

Results

The results of the present study (Table I) revealed a percentage of post thawing morphologically normal immature oocytes frozen by stepwise vitrification technique.

Our statistical analysis (Table II) showed an insignificant (p>0.05 or p>0.01) in the maturation and cleavage rates in stepwise vitrified immature oocytes in comparison to those of fresh oocytes (82.1%, 47.6% vs 88.3 %, 50.4 %, respectively).

The differences between the percentages of developed buffalo embryos resulting from the stepwise vitrified (Fig. 2) and fresh oocyte procedure were non-significant (p>0.05 or p>0.01), although the value of the fresh oocytes was higher (15.4% vs 19.4%, respectively).

Discussion

The high survival rates of vitrified-thawed immature buffalo oocytes observed in the present study compare favourably with other reports of buffalo oocytes cryopreserved using the one-step vitrification method (1).

The high rate of morphologically normal post-thawed immature oocytes in comparison to a previous report (1) can be attributed to the direct contact of the forceps (–196°C) to the wall of the straw, which lead to ultra rapid freezing of oocytes and a decrease in surface tension, thereby reducing ultra structural damage of the oocytes, in addition to stabilising the straw wall during freezing and avoiding the mixing of different segments within the straw and avoiding the explosion of the straw during the thawing process, thereby decreasing the loss and damage of oocytes.

Table I
Frequency of different observations in the stepwise vitrified thawed immature buffalo oocytes

Criteria		Number of stepwise vitrified immature oocytes (%)
Number of stepwise vitrified immature oocytes		520
Number of morphological normal oocytes (%)		420 (80.7%)
Number of damaged oocytes (%)		100 (19.3%)
Types of cryoinjury	Ruptured zona pellucida (%)	35 (35%)
	Shrinkage of cytoplasm (%)	65 (65%)
	Leakage of cell content (%)	10 (10%)

Table II
Developmental competence of stepwise vitrified thawed immature and fresh oocytes

Criteria	Number (%)	
	Stepwise vitrified immature oocytes	Fresh immature oocytes
Number of cultured oocytes	420	283
Matured oocytes (%)	345 (82.1%)[a]	250 (88.3 %)[a]
Cleaved oocytes (%)	200 (47.6 %)[b]	126 (50.4 %)[b]
Buffalo embryos (%)	65 (15.4 %)[c]	55 (19.4 %)[c]

Within the same row, values with the same superscript are insignificantly different from each other (p>0.05)

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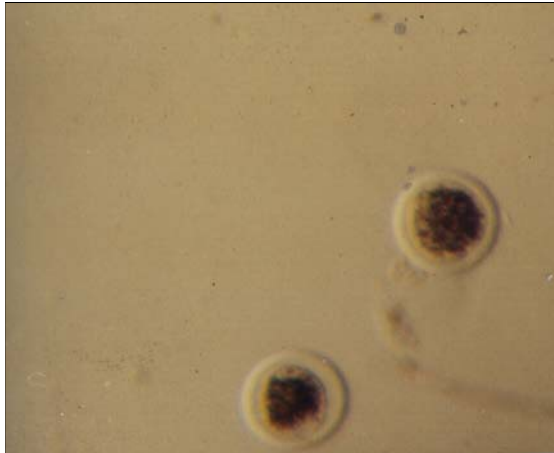

Figure 2
Buffalo embryos produced from stepwise vitrified immature oocytes

The differences between the percentages of developed buffalo embryos obtained by the stepwise vitrified (Fig. 2) and fresh oocyte procedures were non-significant, although the value of the fresh oocytes was higher (15.4% vs 19.4%, respectively). This may be attributed to the ultra rapid freezing and influence of stepwise vitrified method against surface tension, as well as the high permeation ability and low toxicity of 40% EG, in addition the influence of 0.3 M trehalose and 20% PVP against osmotic stress which make this method more convenient for buffalo oocytes.

Conclusion

It is concluded that the stepwise vitrification method used in this study is suitable for the freezing of immature buffalo oocytes on account of the high performance obtained.

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