VITRIFICATION OF IMMATURE AND MATURE BUFFALO OOCYTES IN GLYCEROL SOLUTION BY A SIMPLE METHOD

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Introduction

The cryopreservation of mammalian embryos has become an integral part of methods to control animal reproduction and development of reproductive technologies (Leibo, et al., 1996). Several cryopreservation methods have been used to preserve embryos and oocytes of many animal species resulting in the birth of live off spring. Vitrification which is is defined as a physical process by which a highly concentrated solutions of cryoprotectants solidifies during cooling, without formation of ice crystals (Niemann, 1991). It offers several advantages over conventional equilibrium methods (Dhali et al., 2000). Vitrification has been successfully applied for cryopreservation of bovine oocytes and embryos at various developmental stages (Le Gal and Massip, 1999). There is however, few report available on the cryopreservation of buffalo oocytes by vitrification. The present study, aimed to investigate the effect of glycerol on the morphological appearance, viability and developmental competence of immature and mature buffalo oocytes in vitro.

Materials and methods

Collection of oocytes: Buffalo ovaries were collected from Cairo abattoir and transported within 2 h to the laboratory in normal saline at 28-37°C. Follicular ovarian oocytes (3-8 mm in diameter) were recovered with M-PBS (modified phosphate buffer saline) supplemented with 10% FCS (Fetal calf serum. Sigma, USA) and 1% antibiotic-antimycotic. All the aspirated cumulus–oocyte complexes (CoCs) with homogenous cytoplasm were used in the study. The oocytes were washed 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).

Experimental Design: Buffalo oocytes were vitrified either immediately after collection (Immature group) or after 24 h of maturation in TCM-199 (mature group). Non vitrified oocytes undergoing the same maturation protocols were used as controls.

In Vitro maturation: The recovered oocytes were cultured in microdroplet of TCM-199(contain 10% FCS and 1% antibiotic–antimycotic),then covered with sterilized mineral oil (Sigma, USA) for 24 h in a Co2 incubator .Maturation was assessed either by staining of

oocytes with 1% acetoorcin for the presence of 1st polar body (Hurtt et al. 2000) or roughly by expansion of cumulus cell mass (Schellander et al. 1989).

Vitrification of oocytes: The vitrification solution (VS) consisted of different concentration of glycerol (3.3%; 6.6% and 10%) dissolved in M-PBS enriched with 10% FCS . The oocytes were suspended in the glycerol solutions(3.3%; 6.6% and 10%) for 5, 5 and 10 minutes intervals respectively at room temperature. During equilibration for 10 minute in the final solution (10% glycerol), about 10-15 oocytes were loadded in 0.25ml- plastic straws as previously described by Curtis (1991).The straws were thawed in a water bath at 37°C for 30s and the oocytes were allowed a 5 minutes equilibration in 10% sucrose solution in M-PBS for one step dilution.The frozen-thawed oocytes (Immature) were further incubated for 24h in TCM-199 under conditioned environment.

Survival of oocytes after vitrification-warming: Oocytes was evaluated as mentioned previously (Dhali, et al., 2000) by their post-thaw morphological appearance under stereomicroscope.

Sperm capacitation and in vitro fertilization of frozen-thawed buffalo oocytes:

Fertilization was performed with frozen-thawed semen capacitated in BO medium (Brackett and Oliphant, 1975), containing sodium caffeine (Sigma, Chemical Co. USA) and heparin (Sigma, USA), at a concentration of 5 to 8 x 106 cells /ml (Niawa et al. 1991). The spermatozoa and oocytes were cocultured for 5h under the same culture condition in Co2 incubator. After insemination, the oocytes were cultured in TCM-199 for 5-6 days in Co2 incubator. The frequency of morula and / or blastocyst was recorded.

Statistical Analysis: The data were analyzed by Chi-square analysis.

Results and Discussion:

The high survival rates of vitrified –thawed buffalo oocytes observed in the present study (70.0%, 72.23% for immutre and mature groups, respectively) compares favorably with other reports in which buffalo oocytes vitrified in 4.5 M ethylene glycol and 3.4 M dimethyl sulfoxide (Dhali, et al. 2000). However, Abdallah (2003) revealed that the percentage of post thawing morphologically normal vitrified mature buffalo oocytes were significantly higher ($p<0.01$) than that of vitrified immature one in 10% glycerol.The maturation rate of immature buffalo oocyte after vitrification-warming was, however, much lower than that for mature and control groups (30.0% VS. 66.67 and 80.0%, respectively). The developmental ability of oocytes frozen at germinal 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). The debate surrounding the appropriate cell stage for cryopreservation of oocytes revolves, primarily, around cytoskeletal elements at different maturation stages (Hurtt et al., 2000). Matured oocytes are more resistant to cryopreservation than immature one (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 subject to cryodamage (Allworth et al., 1993). Data presented here also indicated that buffalo oocytes vitrified at the mature stage cleaved and developed into morula and blastocyst stage after thawing at higher rates than those vitrified at the immature stage (37.50%; 8.34%; 6.67% VS 20.0%; 3.34% ; 2.0%, respectively). These finding which come in accordance with the previous study in buffalo (Abdallah, 2003). This reduction in developmental ability of vitrified immature oocyte could be due to a possible multifactorial cause, including toxic effect of cryoprotectants, ultrastructural damage to the oocytes, and deleterious effects on chromosomes and other cytoplasmic structures (Dobrinsky, 1996). In addition, the freezability of immature oocyte has been reported to be low, and increases as development proceeds to the blastocyst stage after fertilization (Kasai et al., 1979 and Schroeder et al., 1990).

Table 1 : Incidence of immature and mature buffalo oocytes recovered morphology normal after vitrification-thawing.

Group	Number of oocytes vitrified	Number of oocytes recovered	Number of morphological normal oocytes (%)	Number of damaged oocytes (%)
Immature oocyte	120	100	70 (70.0)	30 (30.0)
Mature oocyte	100	90	65 (72.23)	25 (27.77)

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

Group	Number of oocytes damaged	Types of damage		
		Cracking in zona pellucida (%)	Shrinkage of cytoplasm (%)	Leakage of cellular content (%)
Immature oocyte	30	5(16.67)	15(50.0)	10(33.34)
Mature oocyte	25	4(16.0)	13(52.0)	8(32.0)

Table 3: Maturation rates of buffalo oocytes after vitrification –thawing

Group	Number of oocytes cultured	Number of oocyte matured (%)
Immature oocyte	100	30 (30.0)a
Mature oocyte	90	60 (66.67)b
Control	100	80 (80.0)c

Table 4: Cleavage and development rates of buffalo oocytes vitrified at different stage .

Group	Number of inseminated oocytes	Cleavage rate Number (%)	Morula stage Number (%)	Blastocyst stage Number (%)
Immature oocyte	150	30 (20.0)[b]	5 (3.34)[b]	3 (2.0)[b]
Mature oocyte	120	45 (37.5)[a]	10(8.34)[a]	8 (6.67)[a]
Control	100	60 (60.0)[c]	20 (20.0)[c]	12 (12.0)[c]

[a,b,c] values within column with different superscript differ ($p<0.05$)

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